INHIBITING P90 RIBOSOMAL S6 KINASE (RSK)/Y-BOX BINDING PROTEIN-1 (YB-1) SIGNALING IS A NOVEL TARGETED THERAPEUTIC STRATEGY WITH THE ABILITY TO OVERCOME DRUG RESISTANCE IN TRIPLE-NEGATIVE BREAST CANCER

by

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ABSTRACT

Despite advances in treating breast cancer, disease recurrence rates remain high and secondary tumors are often refractory to chemotherapy. Currently, the treatment for triple-negative breast cancer (TNBC) relies upon conventional chemotherapeutics as no targeted therapies are available. Although these tumors initially respond well, they paradoxically have the highest relapse rates. Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor abundantly expressed in TNBC (~70% of patients) and associated with disease relapse. It is activated predominantly by phosphorylation via p90 ribosomal S6 kinase (RSK). Once activated YB-1 up-regulates the tumor-initiating cell (TIC) marker, CD44 and promotes drug resistance. These data suggest that blocking YB-1’s activation via RSK inhibition may suppress growth and attenuate the development of chemoresistance in TNBC.

Through an unbiased, functional viability screen comparing breast cancer subtypes, we identified RSK2 as a novel target for TNBC. Pharmacological or siRNA inhibition of RSK2 blocks activation of YB-1, which subsequently decreases growth in TNBC cell lines and delays tumor initiation in immunocompromised mice. Contrary to most conventional chemotherapies, inhibiting RSK/YB-1 signaling eliminates the CD44+/CD24- cell fraction rather than enriching for it. In an effort to identify novel RSK inhibitors, we screened “off-patent” compounds and identified the flavonoid, luteolin, as a RSK inhibitor. We validated that luteolin inhibits RSK in cell-free assays and further demonstrated it blocks the RSK/YB-1/Notch4 signaling pathway. Luteolin phenotypically mirrored the effects of established RSK inhibitor, BI-D1870, and suppressed growth in TNBC (including CD44+/CD24- sorted cells) providing further support for the use of RSK inhibitors to treat this subtype. Finally, we demonstrate that cells that survive standard-of-care chemotherapeutics (paclitaxel and epirubicin) exhibit elevated RSK/YB-1 signaling. Inhibiting this pathway sensitizes TNBC to chemotherapy and reduces the residual cell burden. Importantly, RSK inhibition also demonstrates efficacy against a multidrug resistant cell line and primary, drug-refractory TNBC. When taken together, our data identify RSK as a promising target for the treatment of TNBC. RSK inhibition has the unique ability to
eliminate CD44⁺/CD24⁻ cells and overcome broad-spectrum chemoresistance by blocking activation of YB-1 and as such holds potential to reduce relapse in this aggressive subtype.
PREFACE

Chapter 2: Targeting p90 ribosomal S6 kinase (RSK) eliminates tumor-initiating cells by inactivating Y-box binding protein-1 (YB-1) in triple-negative breast cancers

Kristen Reipas contributed 50% to all aspects pertaining to this manuscript including; experimental conception and design, specifically involving all in vivo and ex vivo cell line studies (Figure 2.1A-D, Figure 2.6A,C), growth assays and immunofluorescence investigating changes in P-YB-1S102 and P-histone H3S10 after treatment with BI-D1870 (Figure 2.3A and Figure 2.4A-C) and assays utilizing CD44+/CD24- TIC-enriched populations (Figure 2.5A-F). Kristen also performed experimentation relating to Figure S2.1, S2.2, S2.3B, S2.5E-F, S2.7B and D, S2.10D and Table S2.3. She contributed 50% to assembly and analysis of data as well as manuscript writing.

Contribution of co-authors:

• Anna Stratford, PhD, performed 50% of experiments. This includes growth assays (Figure 2.2A, C-F), assessment of CD44 expression (Figure 2.3B-D) and mammosphere formation after RSK inhibition (Figure 2.4D-E) as well as knockdown of RSK2 prior to in vivo xenotransplantation (Figure 2.6B). Dr. Stratford also performed: Figure S2.5A-D, S2.6, S2.7C, S2.9A-C, S2.10B-C and Table S2.1.

• Kaiji Hu, PhD, provided technical assistance with Cellomics screening and performed combination treatments with paclitaxel and BI-D1870 (Figure 2.3E) as well as Figure S2.7A.

• Abbas Fotovati, PhD, performed immunohistochemical staining of breast tumor tissue (Figure 2.7B-C and Table S2.4).

• Rachel Brough, PhD, performed the RNAi screening in breast tumor cell line models (Figure 2.2B and Figure S2.4).

• Jessica Frankum, PhD, performed the RNAi screening in breast tumor cell line models (Figure 2.2B and Figure S2.4).

• Mandeep Takhar, assisted with immunoblot experiment (Figure S2.10A).

• Christopher J Lord, PhD, provided the RNAi screening data (Figure 2.2B and Figure S2.4).

• Peter Watson, PhD, provided tumor microarray samples (Figure 2.7B-C).

• Alan Ashworth, PhD, provided the RNAi screening data (Figure 2.2B and Figure S2.4).
• Annette Lasham, PhD, performed the RSK2 survival and subtype analysis (Figure 2.7A and Figure S2.11).

• Cristin G Print, PhD, provided the RSK2 survival and subtype analysis data (Figure 2.7A and Figure S2.11).

• Sandra E. Dunn, PhD, supervised the project and assisted with manuscript editing and writing.

Animal work was performed under approval by the UBC Animal Care Committee (certificate A11-0082).


*both authors contributed equally to this work
Chapter 3: Luteolin is a novel p90 ribosomal S6 kinase (RSK) inhibitor that suppresses Notch4 signaling by blocking the activation of Y-box binding protein-1 (YB-1)

Kristen Reipas contributed 75% in all aspects pertaining to this manuscript including; experimental conception and design, specifically involving growth assays with the short listed compounds in TNBC and CD44+/CD24- populations (Figure 3.2A,D-E), immunoblotting and combination treatments with luteolin and paclitaxel (Figure 3.3A-D), all growth experimentation with the x43 cells (Figure 3.4A-D) and immunoblotting for Notch4 (Figure 3.5D, Figure 3.6A-B immunoblots). Kristen also performed experiments related to Figure S3.6, S3.8. She contributed 75% to assembly and interpretation of data as well as manuscript writing.

Contribution of co-authors:

- Jennifer H. Law, performed immunofluorescence imaging, immunoblotting, non-adherent assays and quantification of CD44 by qRT-PCR of the short-listed compounds (Figure 3.1A-D, Figure 3.2B-C). Jennifer also performed experiments related to Figure S3.1, S3.2, S3.3A-D, S3.4, S3.5.

- Nicole Couto, performed the qRT-PCR experiments assessing Notch4 levels (Figure 3.5C, Figure 3.6A-D and Figure S3.10).

- Sumaiya Islam, performed NanoString gene expression profiling of the x43 cell line (Figure S3.7).

- Yvonne Li, PhD, performed the bioinformatic screen of the Prestwick library to RSK1 as well as the molecular docking of luteolin to known drug targets (Table S3.1 and Table S3.4).

- Huifang Li, PhD, performed bioinformatic docking of the Prestwick library to RSK1 (Table 3.1 and Table S3.2).

- Artem Cherkasov, PhD, supervised the bioinformatic docking of the Prestwick library to RSK1 (Table 3.1 and Table S3.2).

- Karen Jung, MSc, performed the qRT-PCR experiments assessing Notch1-4 after YB-1 knockdown and over-expression (Figure 3.5A-B and Figure S3.9).

- Amarpal S. Cheema, assisted with growth assays testing kaempferol, luteolin and apigenin in TNBC.

- Steven J. M. Jones, PhD, supported the bioinformatics analysis.

- John A. Hassell, PhD, provided the primary x43 cell line as well as intellectual input.
• Sandra E. Dunn, PhD, supervised the project and assisted with manuscript editing and writing.

Chapter 4: p90 ribosomal S6 kinase (RSK)/Y-box binding protein-1 (YB-1) signaling is elevated in residual cells after chemotherapy and inhibiting this pathway can overcome broad spectrum chemoresistance

Kristen Reipas contributed 95% to all aspects pertaining to this manuscript including; experimental conception and design, assembly of data, data analysis/interpretation and manuscript writing.

- Arezoo Astanehe, PhD, performed qRPPA analysis (Figure 4.1).
- Kaiji Hu, PhD, provided technical assistance with Cellomics screening and performed YB-1 knockdown in TNBC cell lines (Figure S4.7).
- Sandra E. Dunn, PhD, supervised the project and assisted with manuscript editing and writing.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AI</td>
<td>aromatase inhibitor</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
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<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
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<td>AML</td>
<td>acute-myeloid leukemia</td>
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<td>AMPK</td>
<td>AMP-dependent protein kinase</td>
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<td>APE1</td>
<td>AP endonuclease 1</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>Bcl</td>
<td>B-cell CLL/lymphoma</td>
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<tr>
<td>BI-D1870</td>
<td>dihydropteridinone 1870</td>
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<tr>
<td>BL</td>
<td>basal-like</td>
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<tr>
<td>BLBC</td>
<td>basal-like breast cancer</td>
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<td>BRCA</td>
<td>breast cancer gene</td>
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<td>CAMK</td>
<td>calcium/calmodulin-dependent protein kinase</td>
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<td>cluster of differentiation</td>
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<td>CDK2</td>
<td>cyclin dependent kinase 2</td>
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<td>chromatin immunoprecipitation</td>
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<td>checkpoint kinase 1</td>
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<td>cAMP response element binding protein</td>
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<td>EGFR</td>
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<td>EpCam</td>
<td>epithelial cell adhesion molecule</td>
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<td>ERCC1</td>
<td>excision-repair cross-complimenting protein</td>
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<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<td>ESA</td>
<td>epithelial-specific antigen</td>
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<td>EV</td>
<td>empty vector</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
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<td>FITC</td>
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<td>GSI</td>
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<td>IKL</td>
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<td>IRES</td>
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<td>LAR</td>
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<td>MAPK</td>
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<td>mTOR-raptor complex 1</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin-1</td>
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<tr>
<td>N4ICD</td>
<td>Notch4 intracellular domain</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
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<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NOD/SCID</td>
<td>nonobese diabetic/severe combined immunodeficiency</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD/SCID lacking interleukin-2 receptor gamma chain</td>
</tr>
<tr>
<td>NTKD</td>
<td>N-terminal kinase domain</td>
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<tr>
<td>OA</td>
<td>ovarian ablation</td>
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<tr>
<td>OS</td>
<td>overall survival</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>OvS</td>
<td>ovarian suppression</td>
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<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase 1/2</td>
</tr>
<tr>
<td>pCR</td>
<td>pathological complete response</td>
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<tr>
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<td>protein data bank</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>phycoerythrin</td>
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<td>paclitaxel</td>
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<td>quantitative real-time polymerase chain reaction</td>
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<td>receptor for hyaluronan-mediated motility</td>
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<tr>
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<td>RSK-like protein kinase</td>
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<tr>
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<td>robust multi-array average</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>ribonucleoprotein</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RSK</td>
<td>p90 ribosomal S6 kinase</td>
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<td>receptor tyrosine kinase</td>
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<tr>
<td>RTKI</td>
<td>receptor tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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<td>smooth muscle actin</td>
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<td>side population</td>
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<td>single-strand break repair</td>
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<tr>
<td>TIC</td>
<td>tumor-initiating cell</td>
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<tr>
<td>TLDU</td>
<td>terminal ductal lobular units</td>
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<tr>
<td>TMA</td>
<td>tumor microarray</td>
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<tr>
<td>TNBC</td>
<td>triple-negative breast cancer</td>
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<tr>
<td>TNM</td>
<td>tumor node metastasis</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>XIAP</td>
<td>X chromosome-linked inhibitor of apoptosis</td>
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<td>Y-box binding protein-1</td>
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<tr>
<td>YRE</td>
<td>YB-1 responsive element</td>
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DEDICATION

To My Family. In particular, Aunt Cathy
CHAPTER 1. INTRODUCTION

1.1 Cancer

Cancer is the quintessential disease of pathological hyperplasia. Through the accumulation of genomic alterations cells acquire novel, aberrant, proliferative capabilities, a phenomenon medically termed neoplasia and often resulting in a tumor or neoplasm – derived from the Greek word neo for “new” and plasma for “formation”. Cancer afflicts nearly every tissue/organ in the body presenting as a diverse range of diseases with the most prevalent being; breast, prostate, lung, colorectal, stomach and cervical cancer. It can further progress from the site of origin to invade other tissues in a process termed metastasis (WHO, 2008). Despite this apparent diversity, all cancers share one defining attribute – uncontrolled cell division. In the pursuit of un-inhibited division, cells acquire six biological capabilities as part of the multi-step process of neoplastic development. Drs. Hanahan and Weinberg coined these attributes “the hallmarks of cancer” and they include 1) sustaining proliferative signaling 2) evading growth suppressors 3) resisting cell death 4) enabling replicative immortality 5) inducing angiogenesis and 6) activating invasion and metastasis (Hanahan and Weinberg, 2000). Since the original publication in 2000, two additional conceptual hallmarks - reprogramming of energy metabolism and evading immune destruction – have begun to emerge. Additionally, the “tumor microenvironment” – normal cells surrounding tumors – have been recognized as active participants in mediating tumorigenesis (Hanahan and Weinberg, 2011).

Currently, cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 (WHO, 2008). Despite major advances in treatment and decreases in mortality, cancer remains a significant burden with adaptive resistance presenting a major challenge in curing this disease. The transition from a “one-size-fits-all” cytotoxic therapeutic strategy to mechanism-based, targeted therapies has revolutionized the way we treat cancer. Our continued progress in developing improved treatments is contingent upon furthering our understanding of the processes that govern carcinogenesis and adaptive resistance then utilizing this knowledge to inform therapeutic development. This review will discuss the
key concepts related to the work in the body of this thesis as well as discuss breast cancer in terms of heterogeneity, treatment and signaling pathways.

1.2 Breast Cancer
Breast cancer is the most prevalent form of cancer in women with approximately 1 in 8 women developing cancer of the breast during their lifetime. It is estimated that ~40,000 women will die of breast cancer in the United States alone in 2012 (SEER, 2012).

1.2.1 Mammary gland architecture
The mammary gland is composed of a branching network of ducts and lobuloalveolar structures. It is one of the few organs that undergoes extensive growth and expansion during adulthood due to hormonal changes (Navarrete et al., 2005). It is comprised of two epithelial cell lineages, luminal and myoepithelial, which rest on a basement membrane to form mammary ductal structures (Figure 1.1). Luminal epithelial cells line the lumen of the duct and produce milk. They are distinguished by CK8, CK18, CD24$^+$ and MUC1$^+$, estrogen receptor (ER$^{-}$) and progesterone receptor (PR$^{-}$) marker expression. Myoepithelial cells generate the contractile force required to transport milk through the ducts. They are identified based on ER$^-$, PR$^-$, CK14$^+$, CK5/6, CD10$^+$ and smooth muscle actin (SMA) marker expression (Polyak, 2007). Human mammary ducts are organized into branching networks that end in clusters of small ducts that constitute the terminal ductal lobular units (TDLUs), with the vast majority of breast cancers arising in the TDLUs (Visvader, 2009). Attachment of epithelial cells to the basement membrane is critical for maintaining epithelial cell polarity and function while disruption of this well-organized architecture occurs during carcinogenesis (Bissell and Radisky, 2001). Most human cancers arise from epithelial cells and are termed carcinomas. Breast cancers of the mesenchyme, sarcomas, also occur but account for less than 1% of all malignancies (McGowan et al., 2000). Breast carcinomas can be further characterized based on pathological characteristics, genetic profiles and molecular features, which provide information on patient prognosis and response to therapy.
Figure 1.1 Mammary gland architecture.

A) Schematic depiction of mammary gland architecture including distinguishing markers of luminal and myoepithelial cells (grey boxes). B) A visual representation of a longitudinal section of a terminal ductal unit from a mouse mammary gland stained via haematoxylin/eosin. C) Cross section of a mouse mammary duct stained for SMA (red) to distinguish myoepithelial cells from luminal cells.
1.2.2 Pathological classification

Breast cancer can be broadly divided into carcinoma in situ (CIS) or invasive carcinomas. CIS can be further subdivided into lobular carcinoma in situ (LCIS) or ductal carcinoma in situ (DCIS). LCIS itself is not a premalignant lesion, but identifies women at increased risk of developing subsequent invasive breast cancer, generally of ductal classification (NCI, 2013). As such, careful observation and regular mammographies are recommended to help achieve early detection. LCIS usually presents as multicentric and bilateral. There is no evidence that re-excision is required, however, the use of tamoxifen has been shown to reduce the risk of developing invasive breast cancer and is considered the standard-of-care (Fisher et al., 1998). In contrast, DCIS can progress to become invasive. They are a heterogeneous group of histopathologic lesions that account for approximately 18% of all newly diagnosed invasive and non-invasive breast cancers (NCI, 2013). It presents as multicentric disease in 40% of cases, with a 25%-50% incidence of recurrence and 50% of recurrences being invasive carcinoma when treated with breast-conserving surgery alone (Lagios et al., 1982). As such, treatment has previously consisted of mastectomy. However, recent studies indicate that breast-conserving surgery combined with radiation with or without tamoxifen decreases the risk of both non-invasive and invasive recurrence (Bijker et al., 2006; Fisher et al., 1999).

Malignant breast cancer develops over many years, progressing from premalignant proliferative lesions, such as atypical hyperplasia and CIS eventually acquiring invasive properties to become metastatic disease (Allred et al., 2001). A staging system for invasive carcinomas called, the tumor node metastasis (TNM) system, was developed by the American Joint Committee on Cancer (AJCC) in order to group patients with respect to prognosis. This system incorporates characteristics of the primary tumor (T) such as invasiveness and size, along with lymph node status (N) and the presence of local or distant metastasis (M) (NCI, 2013). A stage I tumor is less than 2 cm in diameter with no axillary node involvement. Stage II is either a tumor < 5 cm in diameter with involvement of the ipsilateral axillary node or any tumor > 5 cm in diameter without node involvement. Stage III is characterized by extensive ipsilateral axillary node positivity or ipsilateral supraclavicular lymph node involvement and tumor extension into the chest wall or skin in
the form of ulceration. Stage III also includes inflammatory carcinoma. Stage IV is any disease with distant metastasis.

In addition to the TNM system, breast cancer can be classified based on histological grade, which also gives an indication of prognosis. This system considers; cell morphology, the similarity of cancer cells to their non-cancerous, differentiated counterparts and also nuclear grade, which assesses the size and shape of the nucleus as well as a proliferative index. In breast cancer this system ranges from Grade 1, (low grade) well-differentiated, tumors with a low proliferative index to Grade 3 (high grade) undifferentiated, highly proliferative tumors (NCI, 2013). Generally, low-grade tumors have a better prognosis.

1.2.3 Genetic profiling of breast cancer

Advances in technology including, the emergence of global gene expression profiling have revolutionized cancer genetics. With it, the discovery that “breast cancer” does not constitute a single disease, but rather a collection of diverse disease entities, has profoundly enhanced our understanding of its heterogeneity. This notable heterogeneity is informative for understanding differences in patient outcome and response to therapy. The seminal studies by Dr. Charles Perou and Dr. Therese Sorlie identified 5 intrinsic breast cancer subtypes which exhibit distinct molecular profiles: luminal A, luminal B, HER2-positive, basal-like and normal-like breast cancer (Perou et al., 2000; Sorlie, 2004). Seven years after the initial classification, a new intrinsic subtype, the claudin-low subtype, was further identified (Herschkowitz et al., 2007). The subtypes are most broadly differentiated based on expression of ER. Their classification is maintained throughout chemotherapy and metastasis and also relates to prognosis (Perou et al., 2000; Sorlie et al., 2001; Weigelt et al., 2005). Although other signatures have since been developed, this “intrinsic subtype” classification still provides the most detailed biological and prognostic information. Further studies using independent data sets have shown similar clusters and prognostic associations (Hu et al., 2006; Sotiriou et al., 2003; Yu et al., 2004).

The luminal A subtype accounts for 50-60% of all breast cancer cases. It has the highest expression of ER as well as genes activated by ER and also highly expresses the transcription factor GATA binding protein 3 (GATA3). This subtype has low expression of
genes related to cell proliferation and the best prognosis of all the intrinsic subtypes (Sorlie et al., 2001). Molecular profiling revealed that all cases of LCIS are luminal A tumors. Luminal B tumors make up 10-20% of breast cancers and exhibit moderate expression of ER. The main difference between luminal A and luminal B is that the latter has a higher proliferative index, expressing elevated Ki67 and cyclin B1 which likely contribute to this subtype’s worse prognosis. Luminal B cancers also usually express HER2 (Perou et al., 2000; Sorlie et al., 2001). Of the ER negative subtypes, HER2-positive breast cancers account for 15-20% of breast cancers. They are characterized by amplification of the ERBB2 gene, as well as genes associated with this pathway and/or located on the 17q12 chromosome. This subtype also over-expresses genes related to cell proliferation as well as harbors p53 mutations in ~40% of cases. HER2-positive cancers have a poor prognosis, however the widespread clinical use of HER2-targeting therapies has significantly improved survival for patients with this subtype and will be discussed in further detail below (Slamon et al., 2001; Sorlie et al., 2001). Basal-like breast cancers (BLBC) also have a worse prognosis than luminal tumors. They express genes usually present in normal myoepithelial cells including CK5 and CK17 as well as CD44 and EGFR. This subtype also has a high proliferative index as well as frequent p53 mutations, which could account for the poor prognosis of this subtype. BLBC comprises 10-20% of breast cancer cases (Perou et al., 2000; Sorlie et al., 2001). Interestingly, tumors with BRCA1 germline mutations are classified as BLBC based on intrinsic subtyping (Sorlie et al., 2003). There is still some debate as to whether the normal-like represents a true subtype or whether it is an artifact of normal tissue contamination during microarrays. Based on the intrinsic molecular subtyping, the normal-like subtype accounts for 5-10% of cases and group with fibroadenomas and normal breast tissue (Perou et al., 2000). They express genes characteristic of adipose tissue, lack expression of ER, PR and HER2 and do not express CK5 or EGFR. Finally, the claudin-low subtype which accounts for 12-14% of tumors, is characterized by low expression of genes involved in tight junctions such as; claudin -3, -4, -7 and E-Cadherin (E-Cad). They have a poor prognosis despite low expression of proliferative genes. Conversely, they over-express genes indicative of mesenchymal differentiation and epithelial-mesenchymal transition (EMT) such as vimentin. These two features are associated with a cancer stem cell (CSC) phenotype (Prat et al., 2010).
The widespread clinical use of genetic profiling in guiding treatment still suffers from high cost and is somewhat limited in that many require fresh frozen tissue. Nonetheless, Oncotype DX® and MammaPrint® are clinically useful in directing treatment of ER-positive breast cancers and clinical trials are ongoing to validate these signatures (Paik et al., 2004; van de Vijver et al., 2002). Although they are useful in predicting relapse in ER-positive breast cancers, they have limited use in stratifying the HER2-positive and BLBC subtypes as they are assigned to the high-risk category in almost all cases (Desmedt et al., 2008). Moreover, recent identification of additional subtypes and alternative prognostic signatures indicates that further work needs to be done to standardize these classification systems before genetic profiling becomes common clinical practice (Curtis et al., 2012; Staaf et al., 2010; Weigelt et al., 2005).

1.2.4 Immunohistochemical classification of breast cancer

Perhaps a more clinically relevant method of stratifying breast cancer has been through immunohistochemical (IHC) analysis. This method utilizes protein expression as a surrogate for gene expression to attempt to identify intrinsic subtypes and although there is not 100% concordance, for the most part the intrinsic subtypes can be distinguished with considerable accuracy utilizing this technique. This section will explore the relationship between the intrinsic and IHC subtypes with a particular focus on triple-negative breast cancer.

**Luminal A.** The IHC profile of luminal A is characterized by the expression of ER, PR, Bcl-2, GATA3 and CK8/18, an absence of HER2 and low Ki67 staining. It also has a low histological grade (Eroles et al., 2012). Similar to intrinsic subtyping, IHC can also be utilized to predict outcome. Based on IHC classification, luminal A carcinomas were identified as having the lowest rate of relapse as well as the longest post-relapse survival compared to other subtypes. Moreover, each subtype displays distinct patterns of recurrence with luminal A preferentially metastasizing to the bone relative to other locations (Kennecke et al., 2010).

**Luminal B.** Compared to the luminal A subtype, luminal B has a worse prognosis and as such, the ability to reliably differentiate these two subtypes would be invaluable. Luminal B
cancers have a higher histological grade and higher proliferative index compared to luminal A. Bone is the most common site of distant recurrence with luminal B also having an increased incidence of recurrence to other sites. Additionally, the survival from time of relapse is lower for patients with luminal B cancers (Kennecke et al., 2010). Attempts to differentiate luminal A from B based on IHC for Ki67 are being investigated but a consensus on the cut-off point distinguishing “high Ki67” from “low Ki67” remains to be reached (Dowsett et al., 2011). Currently, this subtype is defined by either ER+/HER2- and high Ki67 or ER+/HER2+ although up to 6% of intrinsic luminal B tumors are ER+/HER2- (Cheang et al., 2009).

**HER2 positive.** The IHC profile of ER+/HER2+ does not correspond perfectly with the intrinsic subtype. Only 70% of intrinsic HER2+ tumors over-express HER2 by IHC. Moreover, some tumors over-expressing HER2 fall into other intrinsic categories such as luminal B (Eroles et al., 2012). HER2-positive cancers have a high histological grade and proliferative index. Bone was again the most common site for distant metastasis but HER2-positive tumors have a significantly higher rate of brain, liver and lung metastases than the luminal A subtype (Kennecke et al., 2010).

### 1.2.5 Triple-negative breast cancer (TNBC)

**Immunohistochemical Classification.** As the name suggests, TNBC is characterized by lack of expression of ER, PR and HER2. The terms TNBC and BLBC are often used interchangeably, and although there is considerable overlap between the two subtypes they do not represent the same disease. The molecular signature of TNBC usually overlaps with BLBC in about 70-90% of cases (Bertucci et al., 2008; Irvin and Carey, 2008; Kreike et al., 2007). However, the classification of TNBC also encompasses other intrinsic subtypes including claudin-low and normal-like (although as mentioned, this may be an artifact) (Foulkes et al., 2010). While BLBCs express the myoepithelial cytokeratins CK5/6, CK17 and CK14 as well as c-kit, EGFR, and mutant p53, the gene expression profiles of TNBC is more heterogeneous (possibly due to the inclusion of other intrinsic subtypes within TNBC) and may not express any of these markers (Bertucci et al., 2008; Rakha et al., 2009). Conversely, 18-40% of BLBCs do not have a triple-negative IHC phenotype (Bertucci et al., 2008). Various combinations of IHC surrogates have been proposed to try to identify
BLBCs including the addition of staining for CK5/6, CK14 and CK17 as well as EGFR and c-kit to ER, PR and HER2 which also add prognostic value (Cheang et al., 2008; Nielsen et al., 2004; Rakha et al., 2009). However, due to the heterogeneity in the staining of the cytokeratins as well as the absence of defined cutoffs for IHC staining there is currently no standardized panel of IHC markers to identify BLBC.

**Epidemiology and clinical characteristics.** TNBCs are a clinically aggressive subtype accounting for 10-25% of all invasive breast cancers and exhibiting a high proliferative index as well as high histological grade (Bauer et al., 2007; Carey et al., 2006; Eroles et al., 2012). Their aggressive nature is reflected in the fact that TNBCs have an early age of onset and have a larger tumor size upon presentation with only one third of tumors presenting < 2 cm in TNBC vs. two thirds of tumors from other subtypes being < 2 cm (Anders et al., 2011; Bauer et al., 2007; Dent et al., 2007). Unlike other cancer subtypes, there is no correlation between tumor size and node status. Even small tumors in TNBC have a high rate of node positivity and 55% of women with tumors ≤1 cm still had at least one positive lymph node (Dent et al., 2007). They also occur more frequently in African American women than other ethnic groups (Bauer et al., 2007; Bosch et al., 2010; Carey et al., 2006; Lund et al., 2009; Morris et al., 2007; Stead et al., 2009).

**Clinical Outcome.** TNBC is associated with poor prognosis, with shorter periods of relapse-free and overall survival (Bauer et al., 2007; Carey et al., 2006; Mersin et al., 2008; Nishimura and Arima, 2008). Patients with TNBC also have an increased risk of distant recurrence and death. This pattern of recurrence is only seen within the first 5 years after diagnosis with virtually no relapse occurring after 8 years (Dent et al., 2009; Dent et al., 2007). Paradoxically however, TNBCs exhibit a higher pathological complete response (pCR) rate to neoadjuvant chemotherapy than other breast cancer subtypes (22% vs. 11% respectively). If a pCR is achieved, both TNBC and non-TNBC patients have similarly good overall survival rates (Berry et al., 2006; Carey et al., 2007; Liedtke et al., 2008). In contrast, patients who do not achieve pCR have a worse overall survival if they have TNBC compared to non-TNBC patients (Liedtke et al., 2008). Metastatic TNBC is associated with a high proliferative index and a median survival of about 12 months, which is much shorter than other subtypes (Hugh et al., 2009). The pattern of metastatic relapse also differs from
the other subtypes affecting predominantly the visceral organs; lung, central nervous system and lymph nodes (Dent et al., 2009; Kennecke et al., 2010). Despite the heterogeneity in TNBC, it would be highly desirable to find a common molecular target among them.

**Genetics.** TNBC is a remarkably heterogeneous subtype exhibiting amplifications in numerous genetic regions, but with the prevalence of each being low (Turner et al., 2010). Similar to BLBC, TNBC is associated with BRCA-related breast cancers (Atchley et al., 2008). Eighty to ninety percent of all BRCA1-associated tumors are TNBC, however there is no association with BRCA2 mutation carriers (1997; Lakhani et al., 2002; Metzger-Filho et al., 2012). The incidence of BRCA1 mutations varies from 16-42%, however epigenetic mechanisms and BRCA1 suppressor up-regulation are also associated with TNBC and likely contribute to the development of BRCA-related cancers (Atchley et al., 2008; Gonzalez-Angulo et al., 2011; Murphy and Moynahan, 2010; Turner et al., 2004; Turner et al., 2007). The heterogeneity of this subtype is further reflected in recent identification of six biologically distinct TNBC subgroups based on gene expression profiling. These clusters are divided broadly by mesenchymal features, immune system–related genes, DNA damage response genes, and activated androgen receptor signaling (Lehmann et al., 2011; Metzger-Filho et al., 2012). As defined by Lehmann et al. these 6 groups include; 2 basal-like (BL) clusters, BL1 and BL2 (enriched in cell cycle and cell division components), an immunomodulatory (IM) cluster (enriched for genes involved in immune cell signaling, antigen presentation and cytokine signaling), a mesenchymal (M) cluster (enriched for genes involved in cell motility pathways), a mesenchymal stem–like (MSL) cluster (which also expresses cell motility genes, but uniquely include growth factor signaling pathway genes such as EGFR, PDGFR and ERK1/2, low levels of proliferative genes and claudins-3, -4 and -7, yet are enriched for stem-cell associated genes) and finally a luminal androgen receptor (LAR) cluster (negative for ER, but enriched for hormonally regulated pathways and androgen receptor). Independent research groups have also demonstrated the importance of these individual clusters. As described, the claudin-low subtype is characterized by mesenchymal features including, low expression of cell-cell junction proteins (claudins and E-Cad). The majority of intrinsic claudin-low tumors are TNBCs (Prat et al., 2010). The claudin-low subtype represents the most primitive tumors with regards to epithelial cell differentiation (Lim et al., 2009; Prat et al., 2010). Several research
groups have demonstrated that genes involved in immune system regulation can provide prognostic information (Metzger-Filho et al., 2012). A pooled analysis of microarray studies observed that high expression of an immune response gene module was significantly associated with better outcome among patients with TNBC (Desmedt et al., 2008). TNBC subgroups defined based on DNA repair genes could be useful in predicting response to DNA targeting agents as will be discussed below (Section 1.3.3). Finally, Gucalp et al. also identified a subset of TNBCs that express androgen receptor as well as gene expression patterns similar to ER-positive breast cancers (Gucalp and Traina, 2010). As the heterogeneity within TNBC is better defined, novel therapeutic targets are likely to emerge. Furthermore, identifying differences in signaling pathways within TNBC may help to predict response to various targeted therapies and should be considered when designing inclusion criteria for clinical trials.

Overall, one continuing limitation to using IHC as a surrogate for gene expression profiling is a paucity of standardization in cutoffs for what constitutes positive or negative expression for a particular marker. Additionally there is substantial discordance due to inter-observer and intra-observer variation. However, efforts are being made to standardize methodology and define thresholds, which offers promise for refining risk assessment (Hammond et al., 2010; Oakman et al., 2010; Wolff et al., 2007).

### 1.3 Breast Cancer Treatment

Gene expression profiling offers great promise to aid clinicians in predicting response to chemotherapy, indicating prognosis and guiding therapeutic decisions, however the clinical use of these platforms is still in its infancy and molecular classification is not yet the standard-of-care according to the National Comprehensive Cancer Network (NCCN) guidelines (NCCN, 2012; Paik et al., 2004; van de Vijver et al., 2002). Currently, therapeutic decisions are formulated according to tumor size, lymph node status, estrogen-receptor and progesterone-receptor levels and HER2/ERBB2 receptor status, menopausal status, and the general health of the patient. Treatment regimens consist of various combinations of local therapy including surgery and radiation and systemic therapies
depending on the stage of cancer. In general, local, non-invasive disease is treated with surgery with or without radiation. Local radiation following surgery is performed with the purpose of eliminating subclinical residual disease and reducing local recurrence from occurring. There has been a general movement towards breast-conserving surgery (BCS) in combination with radiation as opposed to radical mastectomy as the former results in equivalent survival rates while reducing the surgical complications (Clarke et al., 2005). Axillary lymph node status is the single most important prognostic variable in the management of patients with primary breast cancer (Carter et al., 1989; Dent et al., 2007; Jatoi et al., 1999). Lymph node positivity indicates that cancer has likely spread to other tissues and as such node-positive patients are indicated for adjuvant systemic treatments in addition to local treatments. Adjuvant therapy is given after surgery where all detectable disease has been removed, yet there remains a statistical risk of relapse due to occult disease. Neoadjuvant therapy is given prior to surgery with the purpose of reducing tumor size in order to facilitate a more effective surgery. Systemic therapies include: cytotoxic chemotherapy, hormonal blocking therapy and monoclonal antibody therapy, which will be discussed in more detail below.

1.3.1 Systemic therapies

Chemotherapy. The first use of drugs to treat cancer was not until early in the 20th century. History has named Sidney Farber “the father of modern chemotherapy” as he demonstrated for the first time that the induction of clinical remission of childhood acute lymphoblastic leukemia was achievable using a folate antagonist named aminopterin (Farber and Diamond, 1948; Miller, 2006). The discovery that drugs, in addition to surgery and radiation, could be used to treat cancer opened the door to the scientific community to a new approach to treating cancer. Chemotherapy works by impairing proliferation and so it elicits a selective effect on cells that divide rapidly, such as cancer cells. However, chemotherapeutic cytotoxicity is not exclusive to cancer cells and unfortunately highly proliferative, non-cancerous cells are also affected, resulting in unwanted side effects such a myelosuppression, immunosuppression, mucositis and alopecia. Chemotherapeutic agents can be broadly divided into three groups based of their mode and site of action: 1) antimetabolites, which interfere with nucleotide and DNA synthesis 2) genotoxic agents
which elicit a deleterious action on a cell’s genome and prevent its replication and 3) mitotic spindle inhibitors which prevent cytokinesis from occurring. The preferred chemotherapeutics currently used to treat breast cancer include: DNA-intercalating anthracyclines (doxorubicin and epirubicin), microtubule-stabilizing taxanes (docetaxel and paclitaxel) and the anti-metabolites (capecitabine and gemcitabine). Other agents include: cyclophosphamide (alkylating agent), cisplatin (DNA cross-linking agent) and 5-fluorouracil (pyrimidine analog). More recently, ixabepilone, a microtubule-stabilizing agent was approved for the treatment of metastatic breast cancer refractory to anthracycline and taxane-based therapy (NCCN, 2012; Thomas et al., 2007). Various combinations and dosing schedules of these compounds are indicated and may be used in combination with anti-HER2 therapies. There is no compelling evidence that concurrent combination regimens are superior to sequential single agent regimens however, the use of more than one agent overall demonstrates a significant survival advantage (Carrick et al., 2009; NCCN, 2012). Cytotoxic agents work best on cancer subtypes with a high proliferative index including most HER2 and basal-like subtypes. Patients with luminal B subtype also respond well to neoadjuvant chemotherapy, which again reiterates the need for reliable methods for stratifying patients with luminal A from luminal B so that the most beneficial treatment regimens can be administered (Eroles et al., 2012).

**Targeted Therapy.** The introduction of mechanism-based targeted therapies to treat human cancers has been heralded as one of the fruits of decades of remarkable progress in cancer research. The term “targeted therapy” has been coined to describe drugs/antibodies that interfere specifically with molecules critical to tumor growth and progression rather than broadly interfering with cell proliferation (i.e. conventional chemotherapy). This type of anti-cancer therapy exploits cancer-specific genetic addictions and/or growth pathway alterations and as such, is envisaged to be the optimal method of targeting cancer, as inhibiting the unique biological processes in cancer cells should ideally eliminate toxicity to normal cells. Indeed, many targeted agents have shown remarkable activity devoid of long-term serious adverse effects and are now the standard-of-care for patients with breast cancer (exemplified by anti-HER2 agents trastuzumab and lapatinib, discussed below). However, targeted therapy is not without its own challenges. Both acquired and intrinsic resistance
present challenges to achieving cures. Current targeted therapies for breast cancer work generally by three different mechanisms: (i) hormone receptor antagonists, (ii) monoclonal antibodies, and (iii) inhibitors of catalytic kinase domains. The first targeted therapy in oncology was the anti-hormonal (endocrine) compound tamoxifen, utilized to treat hormone-receptor positive breast cancer (Jordan, 1976; Jordan, 2008; Jordan and Allen, 1980; Jordan and Koerner, 1975). Monoclonal antibodies bind with high specificity to their target and in addition to inhibiting signaling can also induce complement-mediated phagocytosis (Park et al., 2010). Kinase inhibitors usually bind to the ATP-binding pocket of the enzyme (although some may bind other domains) and inhibit catalytic functions (Wicki and Rochlitz, 2012; Zhang et al., 2009).

Endocrine therapy. Endocrine therapies have been shown to improve treatment of ER and PR positive breast cancers (Davies et al., 2011b; Rao and Cobleigh, 2012). The presence of ER and PR are strong predictors of response to endocrine therapy (Berry et al., 2006). Tamoxifen is a selective estrogen receptor modulator (SERM) acting as an estrogen receptor antagonist in breast tissue. Tamoxifen is used to treat pre-menopausal women and the most recently published meta-analysis has further confirmed it reduces the risk of recurrence and breast cancer mortality (Davies et al., 2011b; NCCN, 2012). More recently, fluvestrant (an ER antagonist) and raloxifene (a SERM that has anti-estrogenic effects in the breast and uterus but estrogenic action in the bone thus preventing osteoporosis in post-menopausal women) are recommended. Ovarian suppression (OvS) and ovarian ablation (OA) are also recommended in the adjuvant treatment of pre-menopausal breast cancer (NCCN, 2012). OA can be accomplished via surgery (oophorectomy) or radiation whereas OvS is achieved via medications such as luteinizing hormone-releasing hormone (LHRH) agonists which inhibit the secretion of estrogen from the ovaries (Rao and Cobleigh, 2012). An alternate approach in hormonal therapy is to block the production of estrogen using aromatase inhibitors (anastrozole, letrozole and exemestane). Aromatase inhibitors (AIs) are the standard-of care for post-menopausal women (NCCN, 2012). When compared to tamoxifen, AIs further reduce the risk of recurrence by less than 5% with equivalent overall survival rates through multiple years of follow-up (Rao and Cobleigh, 2012).
Monoclonal antibodies. Trastuzumab is a humanized monoclonal IgG1 that binds to the juxtamembrane region of HER2/ERBB2/Neu receptor, uncoupling ligand-independent HER2-HER3 heterodimers and inhibiting downstream signaling (Junttila et al., 2009). It also induces antibody dependent cell-mediated cytotoxicity (Park et al., 2010). Several large randomized trials have shown the addition of trastuzumab to standard chemotherapy reduces the risk of recurrence and death compared to chemotherapy alone and subsequently the addition of trastuzumab to adjuvant chemotherapy has become the standard-of-care for patients with HER2-positive breast cancer (NCCN, 2012; Robert et al., 2006; Romond et al., 2005; Smith et al., 2007a; Vogel et al., 2002). Unfortunately, a large proportion of patients with HER2-positive tumors either do not respond to trastuzumab or develop acquired tolerance to the antibody, suggesting both intrinsic and acquired mechanisms of drug resistance (Garrett and Arteaga, 2011).

Kinase inhibitors. Finally, an alternate approach to targeting cell surface receptors has been to block activation of downstream kinase cascades through the use of kinase inhibitors. For example, several small molecule compounds have been developed to potently and selectively inhibit the activity of MEK, which is activated through Ras signaling. Although KRAS mutations are almost never observed in triple-negative breast cancer (Grob et al., 2012) gene expression profiles of TNBC cell lines are similar to those of KRAS-mutant cancers (Hoefflich et al., 2009; Mirzoeva et al., 2009). In these studies TNBC cells were sensitive to MEK inhibition in vitro warranting further evaluation of their efficacy against this subtype in the clinic. Unfortunately however, despite promising preclinical results, clinical trials have identified poor bioavailability as well as dose-limiting toxicities associated with many MEK inhibitors (Fremin and Meloche, 2010). An allosteric inhibitor of MEK, CI-1040, was the first MEK1/2 inhibitor to enter clinical trials (Lorusso et al., 2005). Although, it was well tolerated in patients, insufficient antitumor activity, poor solubility and low bioavailability of CI-1040 precluded further clinical development of this compound (Rinehart et al., 2004). A second generation MEK inhibitor, AZD6244, caused reversible blurred vision in a subset of patients and failed to improve upon standard-of-care regimens based on preliminary results from several phase II clinical trials across several cancer types (Adjei et al., 2008; Fremin and Meloche, 2010). Trametinib (GSK1120212), a
MEK1/2 inhibitor demonstrated significant clinical activity in patients with metastatic BRAF-mutant melanoma as a front-line therapy, but not in those who had been previously treated with a BRAF inhibitor (Kim et al., 2013). Additionally, both intrinsic and acquired resistance present major challenges to the development of MEK inhibitors. Suppressing MEK results in the release of negative feedback loops, attenuating drug response and contributing to the development of acquired resistance (Gysin et al., 2011). For example, an up-regulation in phosphatidylinositol 3-kinase (PI3K) signaling has been shown after MEK inhibition, specifically in breast cancer (Hoeflich et al., 2009; Mirzoeva et al., 2009). To-date, there are no MEK inhibitors recommended as front-line therapy for the treatment of breast cancer although several are currently undergoing clinical trial (Britten, 2013).

Activating PI3K mutations are also common in breast cancer leading to activation of Akt signaling and promoting oncogenesis, making this pathway another attractive therapeutic target (Kang et al., 2005; Vinayak and Carlson, 2013). The serine/threonine kinase mammalian target of rapamycin (mTOR) is an important component of PI3K/Akt signaling and regulates cell growth, survival as well as autophagy by enhancing protein synthesis in nutrient rich conditions and promoting survival during nutrient deprivation (Ma and Blenis, 2009). Rapamycin is an mTOR inhibitor that targets the activated mTOR-raptor complex 1 (mTORC1) and inhibits growth of cancer cell lines and xenografts, however increased signaling through upstream receptor tyrosine kinases, causes Akt activation and promotes cell survival (Fiebig et al., 1990; Shi et al., 1995). It is speculated that this is the reason for rapamycin’s limited clinical activity. Dual mTOR/PI3K inhibitors, such as NVP-BEZ235, have since been developed and demonstrate higher anti-proliferative activity than rapamycin in pre-clinical studies as well as activity against trastuzumab-resistant breast cancer (Serra et al., 2008). Dual mTOR/PI3K inhibitors are currently being tested in combination with endocrine therapies (exemestane and letrozole), chemotherapy (paclitaxel), and anti-HER2 therapy (trastuzumab) in breast cancer (reviewed in (Vinayak and Carlson, 2013)).
1.3.2 Treatment based on cancer stage

Upon disease presentation a biopsy is taken for tumor analysis regarding staging and receptor status (ER, PR and HER2) by IHC. The treatment for carcinoma *in situ* (LCIS and DCIS) was discussed in section 1.2.2. Invasive stage (TNM I and II) treatment includes surgery (lumpectomy or mastectomy) including surgical axillary lymph node staging with adjuvant radiation. Any women with positive lymph nodes or a tumor exceeding 1 cm should be considered for systemic treatment. Neoadjuvant chemotherapy may also be given in cases where the only contraindication for BCS (rather than mastectomy) is tumor size (NCCN, 2012). Histological assessment to identify hormone receptor status is then utilized to direct the type of systemic therapy. ER-positive and PR-positive cancers receive endocrine therapy. Recently, NCCN guidelines also recommends the use of the 21 gene score (Oncotype DX®) to help direct whether patients should also receive chemotherapy as it was found to help predict response to tamoxifen and chemotherapy (Paik *et al.*, 2004; Paik *et al.*, 2006). Patients with a low recurrence score can be treated with endocrine therapy alone and thus spared chemotherapy. Patients with HER2-positive cancers are indicated to receive trastuzumab as well as chemotherapy. Patients who do not express any hormone receptors are ineligible for hormonal therapies and their systemic treatment regimen relies on chemotherapy alone (NCCN, 2012). This includes patients with TNBC. Patients with TNM stage III cancer are indicated for preoperative, neoadjuvant therapy, including endocrine therapy or trastuzumab as receptor status indicates. If the tumor responds to therapy and becomes operable this may be followed by either lumpectomy or mastectomy depending on the residual tumor size (NCCN, 2012). Care for patients with TNM stage IV cancers is palliative. Metastatic breast cancer is considered an incurable disease with a poor prognosis of only ~20% 5-year survival (NCI, 2013; SEER, 2012). Chemotherapy is the standard first line treatment (NCCN, 2012). Endocrine therapy and trastuzumab may also be given however recurrent disease is often refractory to these therapies if they have been previously administered.

1.3.3 Emerging treatments for triple-negative breast cancer

No formal guidelines exist for the treatment of TNBC, however the lack of ER, PR and HER2 rules out treatment with endocrine and HER2-directed therapies. As such, treatment
for this enigmatic subtype relies upon conventional cytotoxic chemotherapy. However, our ever improving understanding of the molecular biology of TNBC is beginning to inform therapeutic development, leading to the evaluation of new agents that target TNBC-specific pathways. This section will discuss a few of the most recent novel therapeutic strategies currently in clinical trials for the treatment of TNBC.

**DNA Damaging Agents.** BRCA1/2 are critical regulators of DNA repair and maintenance of genomic stability (Thompson and Schild, 2001). Due to the association of TNBC with impaired BRCA-mediated DNA repair it was hypothesized that TNBC would be particularly sensitive to DNA damaging agents such as platinum agents (cisplatin and carboplatin) and poly(ADP-ribose) polymerase1/2 (PARP)-inhibitors (O'Shaughnessy et al., 2011). In the neoadjuvant setting, the addition of a platinum agent to anthracycline and/or taxane chemotherapy regimens has shown promising outcomes, achieving pCRs ranging from 30% to 62%. The improvement was limited to cisplatin as carboplatin did not improve pCR rates (Gelmon et al., 2012). The PARP inhibitor, olaparib, has shown promise in BRCA-related breast cancers of any subtype however, did not improve treatment of non-BRCA-related breast cancer. Moreover, maintaining dose intensity of combination paclitaxel and olaparib has been difficult due to myelosuppression (Gelmon et al., 2011; Tutt et al., 2010). The clinical utility of PARP inhibitors in unselected populations remains uncertain.

**Angiogenesis Inhibitors.** Intratumoral expression of vascular endothelial growth factor (VEGF) is significantly higher in TNBC than non-TNBC (Linderholm et al., 2009). Bevacizumab (Avastin) is a monoclonal antibody that binds to VEGF and prevents it from activating its receptor. Bevacizumab was FDA approved for breast cancer however it has recently come under question due to a poor risk-benefit ratio of the compound (Goozner, 2011). It has shown modest improvement in pCR in the neoadjuvant setting however hypertension and cardiotoxicity remain concerns (von Minckwitz et al., 2012). An alternative approach to inhibit angiogenesis is to inhibit the function of receptor tyrosine kinases (RTK) via receptor tyrosine kinase inhibitors (RTKIs). Sorafenib has demonstrated improved overall outcomes for sorafenib-chemotherapy combinations in both first and second-line treatment of metastatic breast cancer in phase II clinical trials (Gelmon et al.,
However, sorafenib combinations were associated with higher rates of grade 3 & 4 toxicities.

**Epidermal-Growth Factor Receptor (EGFR) Inhibition.** TNBCs also frequently over-express EGFR based on IHC (27-57% of cases) and as such EGFR inhibitors have been investigated as targeted therapies for TNBC (Kreike et al., 2007; Rakha et al., 2007; Tan et al., 2008; Viale et al., 2009). The efficacy of the EGFR inhibitors, cetuximab, which is a monoclonal antibody and the RTKIs gefitinib, erlotinib and lapatinib have been investigated in randomized clinical trials in the context of TNBC. The addition of cetuximab to irinotecan and carboplatin in metastatic breast cancer patients improved response rate in a subset of TNBC patients (n= 72; overall response rate, 3% vs. 49%). Unfortunately no improvements in progression-free survival (PFS) or overall survival (OS) were apparent (Gelmon et al., 2012). A small, randomized phase II trial assessing the combination of erlotinib with carboplatin and docetaxel in the neoadjuvant setting demonstrated an increase in pCR (40%) with minimal side effects (Gelmon et al., 2012). Retrospective studies revealed modest activity with gefitinib in combination with standard neoadjuvant chemotherapy and a lack of activity for lapatinib in combination with paclitaxel in patients with TNBC (Finn et al., 2009; Rakha et al., 2007). The role of RTKIs in TNBC is an ongoing area of investigation however overall the effect of EGFR inhibitors to-date is unremarkable.

The development of many targeted therapies in TNBC has been hindered by the inability to define patient groups that would preferentially benefit from a particular targeted agent. It has only relatively recently been fully appreciated that each targeted therapy needs to be matched to patients whose cancers display the mutations/pathway alteration to which the therapy was designed against in order to gain maximum therapeutic benefit. For example, non-small cell lung carcinoma patients with EGFR mutations respond to EGFR inhibitors while patients with ALK translocations respond to ALK inhibitors (Yap and Workman, 2012). Additionally, the current clinical definition of ER⁺, PR⁻ and HER2⁻ for TNBC does not take other differences of this extremely heterogeneous subtype into account. Limiting clinical trial enrolment to target-selected populations will likely be required in order to assess therapeutic efficacy of many of these targeted agents. Indeed, Lehmann et al. found
that TNBC cell lines expressed differential sensitivity to targeted therapies depending on which subtype they were clustered in. Cell lines that fell into the LAR cluster were most sensitive to the androgen receptor antagonist, bicalutamide, whereas cell lines with gene expression most similar to the BL1 and BL2 clusters, and having higher expression of cell cycle genes and BRCA1/2 mutations preferentially responded to cisplatin (Lehmann et al., 2011). This in vitro data supports the idea that mechanism-based targeted therapies demonstrate optimal efficacy when utilized within relevant populations. However, an alternative and perhaps superior strategy to developing therapies that only target a subset of TNBC patients is to identify targets that are critical across several TNBC subtypes. This would prevent novel targeted therapies from being limited to one TNBC subtype and expand the spectrum of patients likely to experience therapeutic benefit.

1.3.4 Drug resistance
Cancer cells live fiercely and inventively, evolving into progressively successful invaders and colonizers. They exploit the fundamental logic of evolution to survive and adapt to treatment, becoming the ultimate product of Darwinian selection. It is for this reason that, although many therapies prolong survival times, drug resistant populations often expand and drive recurrence causing many people to succumb to this disease. While therapeutic resistance can be influenced at the level of the host (low absorption, rapid metabolism, poor drug tolerance, and reduced drug delivery to the tumor) this review will focus on some of the most prominent cellular mechanisms that mediate resistance to therapy.

Resistance to Targeted Therapy. An ongoing challenge is that although drugs may demonstrate therapeutic efficacy in clinical trials, resistance frequently emerges. In fact, development of refractory disease is not only likely in advanced metastatic breast cancer, but is considered inevitable (Howell and Wardley, 2005; Wong and Goodin, 2009). There are two explanations for this: a) some tumor cells are inherently drug resistant, possibly due to unique genetic/epigenetic characteristics which allow them to survive and expand or b) cells may adapt to become drug resistant following exposure to the drug. These two mechanisms of drug resistance are not mutually exclusive and likely work in concert to progress drug refractory disease. Primary resistance (intrinsic resistance) may occur due to lack of target dependency, in which case patients do not respond to initial treatment with a
given targeted therapy. Alternatively, cancer may initially respond well to targeted therapies but then through selective or drug-mediated adaptive pressure acquire resistance. Targeted therapies can fail when tumor cells circumvent the action of a single agent through a variety of mechanisms, thereby facilitating resistance. Resistance to molecularly targeted agents can be due to activating mutations of the target itself, as in the case of kinase gatekeeper mutations, which simultaneously prevent targeted agents from binding as well as maintain the kinase in a constitutively activated conformation (Gibbons et al., 2012). Acquired resistance can also occur via activation of adaptive/compensatory feedback loops within the targeted pathway (Rodrik-Outmezguine et al., 2011) or activation of alternative oncogenic pathways (Johannessen et al., 2010; Nazarian et al., 2010). Moreover, cancer cells may become refractory to targeted therapies through loss of expression of the target (Mittendorf et al., 2009) or through mutations that act downstream of the target which restore pathway activation (Berns et al., 2007). Interestingly, in most cases, the resistance mechanism preserves the original overall pathway addiction, e.g. to the Ras/MAPK pathway (Hoelder et al., 2012). Therefore, combinations of compounds targeting the same pathway through distinct mechanisms of action, or that inhibit different levels of pathway activation may reduce if not eliminate acquired resistance (e.g. Combining an EGFR inhibitor with a p90 ribosomal S6 kinase (RSK) inhibitor).

**Resistant to Chemotherapy.** As previously described, chemotherapy acts by impairing proliferation and inducing apoptosis selectively, but not exclusively in cancer cells. Chemoresistance is a persistent problem in the treatment of a wide variety of blood cancers and solid tumors. Even if tumors are not intrinsically resistant to a specific anti-cancer treatment, genetic and epigenetic heterogeneity in the face of powerful selection imposed by anti-cancer drugs results in expansion of drug-resistant populations and development of refractory cancers. In fact, most patients who initially respond to chemotherapy invariably show a loss of response later on, resulting in tumor re-growth (Luqmani, 2005). Some mechanisms of resistance such as; loss of a cell surface receptor/transporter, specific metabolism of a drug, or alterations by mutation to the drug target result in resistance to only a small number of closely related chemotherapeutics (Longo-Sorbello and Bertino, 2001). In such cases, the use of multiple drugs with different modes of entry into the cell and distinct cellular targets reduces the development of chemoresistance and results in
higher cure rates (Gottesman, 2002). However, cancer cells often employ mechanisms of resistance that confer simultaneous insensitivity to a variety of chemically related or unrelated anti-cancer drugs in a phenomenon termed multidrug resistance (MDR) (Gottesman, 2002; Gottesman et al., 2002). In such cases, utilizing multiple cytotoxic agents offers no therapeutic benefit. Multidrug resistance can occur by increased broad-spectrum drug efflux, which reduces intracellular drug levels to sub-lethal concentrations, preventing them from interacting with their target thus allowing cell survival. Induction of cell cycle arrest and/or cellular death pathways are the underlying pharmacological causes for cytotoxicity in tumor cells and so alterations to these processes can also lead to the development of drug resistance and will also be discussed (Lage, 2008).

*Increased Drug Efflux* is a significant mechanism of resistance to anti-neoplastic agents. The ubiquitous superfamily of membrane-embedded, ATP-dependent, multidrug transporters known as ATP-binding cassette (ABC) transporters, represent the most widely observed mechanism of transport-associated MDR. This family consists of 7 subclasses ranging from ABCA to ABCG (Dean et al., 2001; Kast and Gros, 1997; Kast and Gros, 1998). An intriguing characteristic distinguishing the ABC transporters from other mammalian transporters is their highly promiscuous substrate specificity allowing them to extrude a number of structurally diverse, mainly hydrophobic compounds including many commonly used chemotherapies such as doxorubicin, daunorubicin, paclitaxel and vinblastine (reviewed in (Gottesman et al., 2002; Lage, 2008)). The *MDR1*/*ABCB1* gene, which encodes P-glycoprotein (P-gp/ MDR1/ABCB1) (Chen et al., 1986; Gottesman et al., 1995; Juliano and Ling, 1976; Ueda et al., 1987) is the most well documented mechanism of transport-associated multidrug resistance (Endicott and Ling, 1989; Gottesman et al., 2002; Gottesman et al., 1996; Higgins, 1992). The first specific association between cell membrane transporters and a drug-resistant phenotype was demonstrated in mid-1970s where P-gp expression was shown to correlate with drug resistance to several chemotherapeutics in cell lines (Juliano and Ling, 1976). Since then, evidence that P-gp plays a role in clinical resistance has been extensively examined and can be summarized as follows: 1) the levels of P-gp are high enough in many tumors to confer chemoresistance and the presence of P-gp correlates with drug resistance in several cancers (Goldstein et al., 1989; Kartner et al., 1983), 2) acquisition of drug resistance after chemotherapy is
associated with increased P-gp levels (Goldstein et al., 1989) 3) expression of P-gp in some tumors predicts poor response to chemotherapy with drugs that are transported by P-gp (Chan et al., 1991) and 4) P-gp has prognostic significance in certain types of neoplasms (Penson et al., 2004; van den Heuvel-Eibrink et al., 2000; Wuchter et al., 2000). However, P-gp is not the only efflux protein that mediates MDR. Other ABC transporters have also been associated with multidrug resistance, such as the MDR-associated protein 1 (MRP1, ABCC1) and the mitoxantrone resistance protein/breast-cancer resistance protein (MXR1/BCRP, ABCG2) (Borst et al., 2000; Cole and Deeley, 1998; Cole et al., 1994; Gottesman et al., 2002; Litman et al., 2001). The use of ABC transport inhibitors to overcome drug resistance remains challenging as many normal cells also depend on these pumps to maintain cellular integrity. Moreover, simultaneous involvement of several efflux pumps in contributing to resistance suggests that targeting one may not be sufficient to reduce MDR.

*Modulation of Cellular Death* pathways constitute an alternate mode of broad-spectrum therapy resistance (Dive and Hickman, 1991). Both apoptotic and non-apoptotic mechanisms such as autophagy and senescence, may contribute to a drug-resistant phenotype (reviewed in (Gimenez-Bonafe et al., 2009; Letai, 2008)). Apoptosis is the main type of programmed cell death and is regulated by a plethora of cellular proteins. Alterations in the function of any of the proteins involved in maintaining homeostasis between pro-apoptotic and anti-apoptotic pathways may contribute to the development of a drug-resistant phenotype if the overall effect is a decreased propensity for cells to enter apoptosis. This could include activation of anti-apoptotic factors (Bcl-2, Bel-X₁), inactivation of pro-apoptotic factors (p53, p53 pathway) or reinforcement of survival signals (survivin, NF-κB). For example, increased expression of anti-apoptotic proteins is associated with a decreased susceptibility for cancer cells to trigger apoptosis in response to drug exposure. This general “pro-survival” phenotype can cause resistance to a wide range of anti-cancer drugs (Buchholz et al., 2003; Kupryjanczyk et al., 2003; Lage, 2008; Letai, 2008).

*Enhanced DNA Repair* is another mechanism by which cells can become resistant to DNA damaging agents. Since DNA is the main target of many classical cytotoxic anti-cancer
drugs including anthracyclines, alkylating agents or platinum-containing compounds, the activation of DNA repair pathways represents one of the most important target repair mechanisms causing drug resistance. Up-regulation of proteins that are involved in various types of DNA repair effectively reverse the damage induced by genotoxic agents and allow cells to survive. For example, the excision repair cross-complimenting protein (ERCC1) is a rate-limiting factor involved in nucleotide excision repair (NER). Repair of platinum-containing drug-induced DNA damage is predominantly performed via this process (Rosell et al., 2007). Various in vitro studies have demonstrated that enhanced activity of ERCC1 is associated with platinum drug resistance (Melton et al., 1998; Youn et al., 2004). Furthermore, high levels of ERCC1 correlate with poor response to platinum-based chemotherapy in patients (Lord et al., 2002; Metzger et al., 1998; Shirota et al., 2001).

1.4 Tumor-Initiating Cells

1.4.1 Cancer stem model versus clonal evolution model
An axiom in cancer research is that tumors exhibit significant heterogeneity in terms of morphology, cell surface markers, genetic lesions and cell proliferation kinetics. Several factors contribute to tumor heterogeneity including, intrinsic factors such as genetic/epigenetic changes and extrinsic factors which occur through interactions with the microenvironment. Additionally the presence or absence of a cellular hierarchy can influence tumor heterogeneity. Two theoretical models have been proposed to explain the phenotypic and functional heterogeneity that exists in diverse cancer types: the clonal evolution theory and the cancer stem cell model (Figure 1.2). The acquisition of genetic (or epigenetic) alterations underpins the clonal evolution theory in which all cells within the dominant clonal population possess similar tumorigenic potential (Nowell, 1976). This model involves a stochastic component. Conversely, the cancer stem cell model postulates a hierarchical organization of cells such that only a small subset of cells is responsible for sustaining tumorigenesis and maintaining the heterogeneity within tumors. The CSC is always located at the apex of this hierarchy and possesses the ability to both self-renew and “differentiate” into non-tumorigenic cells (which have limited replicative potential and
cannot become tumorigenic) to recapitulate the heterogeneity of the tumor from which it was derived (Figure 1.2)
Figure 1.2 Schematic of clonal evolution and cancer stem cell models.

A) The clonal evolution model is a non-hierarchical model where accumulating mutations confer a selective growth advantage producing a dominant clone. Tumor cells arising from the dominant clone (green) all have similar tumorigenic capacity while other cells (pink) may lack tumorigenic potential due to stochastic events. Heterogeneity results from the diversity of cells generated through mutations.  

B) The cancer stem cell model predicts a hierarchical organization where only the cancer stem cell (CSC) (black) has tumorigenic potential and generates heterogeneity through differentiation. Differentiated progeny of the CSC do not possess tumorigenic potential. A tumor-initiating cell (TIC) differs from a CSC in that although it has unique tumor initiating capabilities, it is not necessarily organized in
a hierarchy and can arise from non-TICs. Neither model addresses the cell of origin. The transforming event can occur in cells at any stage of differentiation in the mammary hierarchy.

The advent of flow cytometry brought with it the ability to prospectively isolate CSCs and evaluate their tumorigenic potential. The first strong evidence in support of CSCs came from Dr. John Dick and colleagues in an acute-myeloid leukemia (AML) model where they demonstrated that the CD34⁺CD38⁻ cell fraction exclusively possesses the ability to initiate leukemia, self-renew and differentiate in vivo indicating a hierarchal organization (Bonnet and Dick, 1997; Lapidot et al., 1994). Since then there has been mounting evidence indicating that some solid tumors also follow the CSC model (Al-Hajj et al., 2003; Visvader and Lindeman, 2008). However, although the existence of CSCs is supported in some cancers, not all cancers are sustained by CSCs. Melanomas contain a high proportion (up to 50%) of tumorigenic cells when assayed in permissive xenograft models which can be identified based on a wide spectrum of markers arguing against a CSC hierarchy (Quintana et al., 2012).

To confirm the existence of CSCs, it is necessary to identify markers that reproducibly distinguish tumorigenic from non-tumorigenic cells. In some cases it has proven difficult to confirm markers that originally appeared to robustly distinguish these population. For example, studies have demonstrated tumorigenic activity in what was originally thought to be the non-tumorigenic population (CD133⁻) of brain tumor cells (Beier et al., 2007). Moreover, the CSC cell surface marker phenotype may differ between patients even within the same cancer type making generalizability of the model uncertain. Since the original publication in 1997, Dr. John Dick’s group has determined that leukemogenic activity is not always restricted to the CD34⁺CD38⁻ fraction of cells between patients somewhat dampening the evidence for hierarchal organization (Eppert et al., 2011). Validation of a hierarchal organization is further complicated by the fact that the clonal evolution and the CSC models are not mutually exclusive. CSCs themselves are dynamic and subject to genetic evolution indicating that the two models likely act in concert. Thus, it is becoming evident that CSC cell surface markers are not universal for any cancer subtype (reviewed in
(Magee et al., 2012; Visvader and Lindeman, 2012). Without any consistently expressed markers of CSCs the best way to validate the CSC model may be through the use of lineage tracing in combination with clonality studies, a technique that has been utilized to demonstrate the cell of origin in colon cancer (Barker et al., 2009). This technique would allow a single cell to be tracked throughout tumor progression and shown to be uniquely capable of generating heterogeneous tumors (similar to the one from which it was derived) and subsequently serially passaged in xenotransplant models. Conversely, tracking of the non-CSCs could demonstrate that more “differentiated” cells do not possess or acquire tumor-forming ability. This type of experiment would provide strong evidence that a hierarchal organization contributes to tumor heterogeneity.

Although the terms CSC and tumor-initiating cell (TIC) are often used interchangeably, the term TIC more aptly denotes the cell(s) with increased ability to initiate and sustain tumor growth. Thus, in an attempt to more accurately describe the population being studied, many researchers now utilize the term tumor-initiating cell or in some cases tumor-propagating cell rather than CSC. **TICs are defined by** two fundamental elements 1) having the ability to serially propagate tumor formation with superior efficacy to other populations of cancer cells 2) exhibiting resistance to conventional chemotherapy and radiotherapy. An important difference between CSCs and TICs is that although TICs exhibit a differential ability to initiate tumors, they do not necessarily follow a one directional hierarchal organization. Non-TICs can become TIC-like (discusses in further detail in section 1.4.7.) (Figure 1.2). A consequence of their second property is that TICs can survive treatment to conventional therapy and subsequently drive relapse. This property of TICs makes them perhaps more clinically relevant than CSCs as it is only through complete elimination of cells with the ability to survive treatment and re-establish tumors (i.e. TICs) that we will achieve the ultimate goal of developing curative therapies with durable, long-term remissions.

**Cell of Origin.** It is important to note that none of these models address the cell of origin. The cell of origin is the cell that acquires the transforming event and it can arise from either the normal tissue resident stem cells (Barker et al., 2009; Yang et al., 2008) or restricted progenitors and differentiated cells which acquire ectopic activation of stem-cell associated pathways (Huntly et al., 2004; Krivtsov et al., 2006; Zhao et al., 2010). Moreover, most
inferences have been based upon experimentally induced cancers, as the cell of origin that arises spontaneously has not been accurately identified in patients.

1.4.2 Evidence for normal mammary stem cells

The extensive, cyclical expansion of the mammary gland during development and pregnancy suggests the existence of mammary stem cells (MaSCs), with remarkable regenerative capacity, capable of dividing and differentiating into both myoepithelial and luminal cells. Indeed, it has been demonstrated in mice that a single mammary stem cell is capable of regenerating an entire functional mammary gland in vivo providing unequivocal proof for their existence in mice (Shackleton et al., 2006). In humans, identical chromosomal alterations in contiguous regions of human breast epithelium, implies the presence of clonal outgrowth from a mammary stem cell (Deng et al., 1996; Lakhani et al., 1996; Tsai et al., 1996). Several elegant studies have since elucidated the human mammary hierarchy and identified markers that can distinguish cells at each stage of differentiation. A subset of human breast cells defined by CD49f\(^+\)/EpCam\(^-\) has been demonstrated to have mammary regenerative capacity in vivo (Eirew et al., 2008; Lim et al., 2009). The structures formed in these experiments contained lobular regions reminiscent of TDLUs and were capable of terminal differentiation into both luminal and myoepithelial lineage cells via a series of lineage-restricted intermediates. Mammary stem cell populations also lack expression of steroid hormone receptors and ERBB2/HER2, yet express high levels of EGFR, CK5/6 and p63 (Carey et al., 2007; Lim et al., 2009; Visvader, 2009). Both bipotent and unipotent progenitors have been identified. Bipotent progenitors and stem cells both display a phenotype CD49f\(^+\)/EpCam\(^-\) (also MUC1\(^-\)CD24\(^-\)CD133\(^-\)Thy1\(^-\)CD10\(^+\)). Myoepithelial-restricted progenitors were demonstrated to lie downstream of bipotent progenitors (Stingl et al., 2001). Conversely, committed luminal-restricted progenitors express EpCam\(^-\)CD49f\(^+\)MUC1\(^-\)CD24\(^-\)CD133\(^+\)Thy1\(^-\)CD10\(^+\) with abundant EpCam expression on fully differentiated luminal epithelial cells (Eirew et al., 2008; Lim et al., 2009; Raouf et al., 2008; Stingl et al., 2001). Interestingly, in addition to expressing luminal-specific cytokeratins, luminal progenitor cells can also express CK5/6 indicating these markers are not specific to the basal lineage (Lim et al., 2009). Conversely, CD24 is exclusively a luminal cell marker. Cell surface marker expression throughout differentiation
is summarized in Figure 1.3. It is important to note that, although these markers significantly enrich for MaSCs, markers that exclusively identify these cells have yet to be defined. Demonstration of a single human MaSC with differentiating capacity remains challenging due to less than ideal xenotransplant environments.

Figure 1.3 Schematic diagram of normal mammary gland hierarchal organization.
Model of differentiation of normal mammary epithelial cells with the mammary stem cell located at the apex, progressing to lineage-restricted progenitors and finally terminally differentiated myoepithelial or luminal cells. Cell surface markers for each cell type are indicated (grey box) as well as key regulatory pathways (blue box). Potential relationships between different tumor subtypes and their closest normal epithelial counterpart are also indicated.
**Breast cancer subtypes and putative cells of origin.** The ability to identify and sort populations of mammary cells throughout various stages of development has allowed for the derivation of gene signatures for human MaSC, luminal progenitors and mature luminal and myoepithelial cells. Interrogation of the different breast cancer subtypes with mammary epithelial signatures has revealed some interesting similarities (Figure 1.3). The basal-like subgroup shares remarkable similarity with the luminal progenitor signature while the MaSC signature had the greatest overlap with the claudin-low and normal-like subtypes. Further, the signature of differentiated luminal cells exhibited the most overlap with the luminal A and luminal B subtypes. The HER2 subgroup showed no clear association with any of the epithelial cell types (reviewed in (Visvader, 2009)). When taken together, it is tempting to speculate that the cellular context in which the transforming mutation occurs may influence the subtype of cancer that develops. Conversely, specific driver mutations can influence the frequency of progenitor cell populations. For example, breast tissue of BRCA1 mutation carriers exhibited expanded luminal progenitor populations with aberrant growth characteristics (Lim et al., 2009). These data indicate that the luminal progenitor population is likely the target for transformation in BRCA1-associated basal tumors.

1.4.3 Evidence for breast tumor-initiating cells

As previously mentioned, TICs can be defined by two properties 1) having the ability to serially propagate tumor formation with superior efficacy to other populations of cancer cells 2) exhibiting resistance to conventional chemotherapy and radiotherapy. Although there is extensive evidence demonstrating TICs in other cancer types, this section will review the evidence supporting the first property of TICs (increased tumor-initiating capacity) in breast cancer specifically. The relationship between TICs and drug resistance will be discussed in section (1.4.4).

TIC is an operational term and as such TICs must be validated in functional assays with the “gold standard” being *in vivo* xenotransplantation into immunocompromised mice. Two methods that have proven successful at enriching for TICs are fluorescence-activated cell sorting (FACS) based on antibody staining for cell surface marker expression, aldehyde
dehydrogenase (ALDH) activity or Hoechst33342 exclusion and non-adherent cell culture (also referred to as mammosphere culture in the case of breast tissue). The most well established cell surface markers for identifying breast TICs are CD44+/CD24− and were originally identified by Al-Hajj and colleagues. In this influential study, tumorigenic cells were prospectively isolated from primary human breast cancer based on CD44+/CD24−/ESA+ phenotype. When injected into the mammary fat pads of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, as few as 200 cells of this phenotype could consistently initiate tumors whereas 20,000 CD44+/CD24+ always failed to form tumors and 10,000 unsorted cells only formed tumors in 25% of cases. Moreover, the TIC population could be serially passaged in vivo with the CD44+/CD24− fraction uniquely retaining tumorigenic potential (Al-Hajj et al., 2003). Confirming and extending these findings, Ponti et al. reported that, in addition to being more tumorigenic in vivo, CD44+/CD24− human breast cancer cells could be propagated in vitro as tumor mammospheres, a property that was described previously to enrich for normal mammary stem/progenitor cells (Dontu et al., 2003; Farnie et al., 2007; Ponti et al., 2005). Analogous to normal mammary stem cells, TICs are enriched in non-adherent culture conditions. Anoikis-resistant cells were found to have increased mammosphere forming ability and in vivo tumor formation capabilities thereby validating mammosphere culture as a technique for expanding for TICs. Further, these data suggest, but do not formally prove that mammospheres and TICs are overlapping populations (Harrison et al., 2010). Finally, CD44+/CD24−/ESA+ cells are able to form mammospheres more efficiently than un-enriched populations in both cell lines and primary samples (Harrison et al., 2010; Li et al., 2008). Conversely, oncolytic adenovirus-mediated killing of primary CD44+/CD24− cells from pleural effusions prevented tumor initiation by this population whereas un-infected cells efficiently formed orthotopic xenografts (Eriksson et al., 2007). Cell surface markers can also be utilized to enrich for TICs in cell lines. As few as 100 CD44+/CD24−/ESA+ cells (SUM149 and SUM159 cell lines) could initiate tumors in mice and could be serially passaged as mammospheres while cells with alternate phenotypes failed at both (Fillmore and Kuperwasser, 2008). MCF-7 cells cultured as mammospheres were able to establish tumors in 3/4 mice when 10^3 cells were injected whereas 10^6 cells were required to form tumors from monolayer cultured MCF-7 cells. In this study, the authors found that α6 integrin (CD49f) is highly expressed in mammosphere-
derived cells and that it is necessary for the tumorigenicity of these cells (Cariati et al., 2008). These and other data indicate the existence of populations of cells with differential tumor-initiating capacity and validate the use of some cell lines for the study of TICs (Tanaka et al., 2009). CD49f was also found to define a xenograft-initiating population in primary ER-negative breast cancers (Meyer et al., 2010). Thus, in addition to enriching for normal mammary stem cells, CD49f can also be utilized to identify TICs.

**Alternate Techniques for Identifying TICs.** Other methods of enriching for TICs have been established. Both normal stem cells and TICs can be identified from primary mammary epithelia or breast cancer samples based on increased aldehyde dehydrogenase activity (ALDH) using the ALDEFLUOR assay. In breast carcinomas, high ALDH activity identifies the tumorigenic cell fraction, capable of self-renewal and expression of ALDH1 detected by immunostaining correlated with poor prognosis (Ginestier et al., 2007). Studies in cell lines demonstrated that the ALDEFLUOR-positive population showed increased mammosphere-forming capacity and serial tumorigenicity with as few as 500 ALDEFLUOR-positive cells from MDA-MB-453 being capable of initiating tumors (Charafe-Jauffret et al., 2009).

Another population found to be enriched for TICs is the flow-cytometry-based side population of cells. This population does not accumulate an appreciable amount of dye when incubated with Hoechst33342 and is therefore identified as a Hoechst<sup>lo</sup> side population (SP). It is highly enriched in normal stem/progenitors of various tissues. The side-population phenotype is mediated by the ABC family of transporter proteins. One of the major mediators seems to be ABCG2/BCRP which effluxes multiple chemotherapeutic drugs and xenobiotics (Doyle and Ross, 2003; Meyer et al., 2010). Purified side populations from breast cancer cell lines (MCF-7 and T47D) were shown to be more tumorigenic in mice and more resistant to ionizing radiation than non-SP fractions (Han and Crowe, 2009; Patrawala et al., 2005). When taken together, it is clear that none of the techniques uniquely enriches for TICs and that elucidation of markers that further distinguishes TICs need to be established. Nonetheless, these methods have proven to be useful for obtaining enriched populations of cells with increased tumorigenicity.
**TICs in Syngeneic Models.** Importantly, TICs have been identified in mouse models of breast cancer, which can be enriched through cell-sorting techniques. The use of syngeneic transplant models excludes any bias associated with xenotransplantation models and lends support to the notion that TICs truly represent a population of cells with increased tumor forming capacity. Several reports have identified populations of cells with differential capacity to initiate tumors when transplanted into syngeneic backgrounds. Breast tumor cells from the mouse mammary tumor virus (MMTV)-Wnt1 mice that are sorted for Thy1⁺CD24⁺ were highly enriched for cells capable of regenerating tumors when injected into FVB/NJ female syngeneic mice and could be serially passaged in vivo (Cho et al., 2008). Further studies indicated these cells also contained lower levels of ROS and exhibited resistance to irradiation in vivo (Diehn et al., 2009). In a distinct mouse model of spontaneous breast cancer, BALB-neuT mice containing an activated form of the HER2/Neu oncogene identified that the Sca-1⁺ population of tumor cells is responsible for initiating tumors in vivo (Grange et al., 2008). These cells also displayed increased sphere-generating capacity in culture as well as resistance to doxorubicin.

**TICs and basal-like breast cancer.** An interesting association between TICs and the basal-like subtype has been noted. In pre-chemotherapy primary biopsy specimens (n=92) a higher proportion of either CD44⁺/CD24⁻ or ALDH1-positive cells was associated with higher histological grade (P=0.002 and P=0.007, respectively) and the basal-like subtype (Lee et al., 2011). This was supported by an independent study of 240 cases of breast cancer which again found that based on IHC that CD44⁺/CD24⁻ phenotype was most common in the basal-like subgroup (Honeth et al., 2008). Moreover, cell lines with high CD44⁺/CD24⁻ cell content correlates with basal/mesenchymal marker expression but not luminal markers (Fillmore and Kuperwasser, 2008; Sheridan et al., 2006). Additionally, prospectively isolated CD44⁺/CD24⁻ cells from patient samples and cell lines exhibit basal-like gene expression signatures and cell surface marker expression (Harrison et al., 2010; Shipitsin et al., 2007).

**Frequency of TICs.** Evaluating the true frequency of TICs has been difficult due to a lack of markers that exclusively identify this population. Additionally, dissociation techniques may affect the immunophenotype of some markers. It is also likely that the frequency of
TICs has been underestimated in many cases due to barriers imposed by xenotransplantation models. Species-specific differences in growth factors, tissue architecture, stromal composition and residual immune activity in mouse models are factors that create a less than ideal environment for human cancer cell survival (Visvader and Lindeman, 2012). Opponents of the TIC model argue that assessing TIC frequency in a permissive niche cannot provide an accurate quantification of TICs. Indeed, the level of host immunodeficiency affects the ability of cells to initiate tumors and in some cases the frequency of TICs increases dramatically in more permissive xenotransplantation conditions. For example, using NOD/SCID mice lacking the interleukin-2 receptor gamma chain (NSG mice) which are more immunodeficient that than the NOD/SCID increased the proportion of cells capable of initiating tumors (Quintana et al., 2008). However, TICs in many solid tumors have been shown to be relatively infrequent even when measured under permissive conditions (Ishizawa et al., 2010; Stewart et al., 2011). Furthermore, dissociation methodology, transplantation site and recipient mouse sex and strain can also affect the ability of tumorigenic cells to engraft adding confounding variables to accurately quantifying TICs. Yet, although xenotransplant models do not perfectly recapitulate the microenvironment of cancer cells in humans making absolute quantification of TICs unlikely, they still provide a relative measure of a cell’s tumorigenic potential.

1.4.4 Evidence for drug resistance in tumor-initiating cells
With regards to the second characteristic of TICs, a plethora of studies have shown that the percentage of TICs increases after chemotherapy (reviewed in (Lacerda et al., 2010)). Clinical evidence from Dr. Jenny Chang’s group comparing paired core biopsies before and after neoadjuvant chemotherapy (n=31 pairs) observed that the percentage of CD44+/CD24- cells increased after chemotherapy based on flow cytometry analysis (Li et al., 2008). Moreover, there was a statistically significant increase in mammosphere forming ability in cells post-chemotherapy compared to their matched samples and the number of xenografts that grew from post-chemotherapy samples was double that of pre-chemotherapy indicating TICs preferentially survive treatment. This increase in TIC component was observed regardless of breast cancer subtype. Interestingly, HER2-positive patients that were treated with the dual HER2/EGFR inhibitor, lapatinib, experienced a non-significant decrease in
CD44+/CD24− cell content suggesting some targeted therapies may be effective at reducing the TIC population (Li et al., 2008). Subsequent studies from the Chang lab also found that residual breast tumors in patients after conventional chemotherapy were enriched for both TIC and mesenchymal features based on a CD44+/CD24−-mammosphere associated gene signature (Creighton et al., 2009). Other groups comparing paired human breast cancer biopsies before and after neoadjuvant chemotherapy (including doxorubicin and docetaxel or cyclophosphamide) showed an increase in CD44+/CD24− and ALDH1+ cells (P=0.013 and P=0.018, respectively) based on IHC (n = 79), which again occurred regardless of cancer subtype (Lee et al., 2011). An enrichment for TICs (based on CD44+/CD24− phenotype, mammosphere formation and increased ability to form tumors in vivo in NOD/SCID mice) after chemotherapy has been observed by other groups in both patient samples (Yu et al., 2007) and cell lines further indicating these cells preferentially survive chemotherapy (Calcagno et al., 2010; Fillmore and Kuperwasser, 2008; Lacerda et al., 2010; Tanaka et al., 2009).

Radioresistance. In addition to being resistant to chemotherapy, TICs also exhibit radioresistance. Phillips et al. demonstrated that mammospheres from MCF-7 and MDA-MB-231 cell lines were more radioresistant than cells grown in monolayer culture. Moreover, they found the CD44+/CD24− population of MCF-7 cells increased after fractioned doses of irradiation (Phillips et al., 2006). They attributed the radioresistance to reduced levels of reactive oxygen species (ROS). Similarly, Diehn et al. found that primary human breast TICs (defined by CD44+/CD24−) contain lower levels of ROS due to up-regulated free radical scavenging genes when compared to their non-TIC counterparts, reducing the DNA damaging effects and preferentially sparing them after irradiation (Diehn et al., 2009). Depletion of ROS scavengers using pharmacological inhibitors resulted in radio-sensitization. Together these studies suggest that TICs are endowed with properties which confer radioresistance and these cells are thereby liable to re-seed tumors after radiotherapy.

1.4.5 Mechanisms of drug resistance in tumor-initiating cells

TICs exploit many mechanisms of drug resistance. This is perhaps why this population of cells has proven difficult to eliminate. This section will provide evidence that TICs
specifically up-regulate several of the main pathways mediating drug resistance. Interfering with specific mechanisms of resistance may be an effective strategy to re-sensitize TICs to conventional therapies.

*Increased DNA repair.* Evidence for increased DNA repair in breast TICs has been demonstrated. CD44+CD24− mammospheres from cell lines exhibited lower levels of double strand breaks as indicated by γ-H2AX after ionizing radiation (Phillips et al., 2006). In addition, mammosphere cells benefited from increased cellular protection relative to that seen in monolayer cells, through a more active DNA single-strand break repair (SSBR) pathway, possibly due to a higher level of expression of the key SSBR protein, human AP endonuclease 1 (APE1) (Karimi-Busheri et al., 2010). Breast TICs which, demonstrated resistance to radiation-induced apoptosis, were arrested in the G2 phase of the cell cycle while non-TICs were prone to radiation-induced apoptosis (Lagadec et al., 2010). Further evidence from primary breast cancer samples exposed to various apoptosis-inducing stimuli (UV, cisplatin, etoposide) also indicated CD44+ cells had a significantly lower rate of apoptosis, and a significantly higher proportion of cells in the G2 phase of the cell cycle (Harper et al., 2010). The extended G2 phase may be utilized by these cells as a mechanism to prolong repair of DNA damage.

*Enhanced drug efflux.* Breast TICs display an increased ability to efflux drugs due to high expression of ABC transporters, which is why they can be identified as a side population (Hirschmann-Jax et al., 2004; Patrawala et al., 2005). Preventing the intracellular accumulation of drugs may inherently protect TICs from the cytotoxic effects of many chemotherapeutics. The side population of breast TICs exhibits an increase in chemoresistance (to mitoxantrone) when compared to non-side population cells. They can be re-sensitized to mitoxantrone by combining treatment with doxquidar fumarate, an inhibitor of both ABCB1/P-gp and ABCC1/MRP1 (Katayama et al., 2009). Resistance may also be acquired. Prolonged, doxorubicin-selected (MCF-7/ADR) cells exhibited an increased CD44+/CD24− population as well as higher expression of ABCB1 mRNA (Calcagno et al., 2010). Thus, inhibiting these transporters may provide a means to re-sensitize TICs to conventional therapy.
**Increased Resistance to Apoptosis.** Resistance to apoptosis is another mechanism of resistance that TICs exhibit to a greater extent than non-TIC populations. Based on immunohistochemistry of patient samples, CD44+ cells have higher expression of the anti-apoptotic protein Bcl-2 (Madjd et al., 2009). Further, expression of the germline stem cell protein Piwi2, which maintains Stat3/Bcl-XL and cyclin D pathway, is predominantly expressed in the CD44+/CD24− fraction isolated from cell lines (Lee et al., 2010).

**Quiescence.** Quiescence has been attributed to endow chemoresistance to TICs since a lack of proliferation would spare cells from the effects of chemotherapy and radiation. It is a property of at least some TICs in leukemia, however there is little data to support this assertion in solid tumors and in fact contrary, emerging evidence suggests that this may not be the case (Lathia et al., 2011). In a mouse model of breast cancer, Cicalesa et al. found that TICs underwent more frequent self-renewal divisions than their normal mammary stem cell counterparts based on limiting dilution assays in a syngeneic background suggesting TICs are actually more proliferative than normal stem cells (Cicalesa et al., 2009). However, when compared to the CD44+/CD24+/ESA+ cancer cell population, TICs in cell lines did cycle more slowly based on BrdU retention (Fillmore and Kuperwasser, 2008). Thus quiescence may offer a level of protection but it is likely not the only mechanism for chemoresistance of TICs. Overall, the mechanisms governing drug resistance in breast TICs need to be further explored.

### 1.4.6 Prognostic/predictive value of tumor-initiating cells

The prognostic value of TICs pre-chemotherapy has been hard to define and several studies have found no association between CD44+/CD24− cell prevalence and clinical outcome (Madjd et al., 2009). Specifically, Abraham et al. found no association between CD44+/CD24− cell percentage and event-free or overall survival nor did the percentage of TICs influence the response to different treatment modalities in this study (Abraham et al., 2005). TIC content was however, associated with distant metastasis. Immunohistochemical staining for CD44+/CD24− content pre-chemotherapy found it was not predictive of pCR, which is an independent prognostic factor in patients with locally advanced breast cancer (Tanei et al., 2009). Nor, was CD44+/CD24− cell content (or ALDH1 expression) associated with disease-free survival (Lee et al., 2011). However, patients who displayed an increase
in CD44+/CD24− content after chemotherapy also exhibited a high Ki67 index and shorter disease-free survival times (Lee et al., 2011). Further complicating the elucidation of CD44 as a prognostic marker is the fact that some splice variants, such as CD44s, are associated with prolonged patient survival (Berner et al., 2003; Diaz et al., 2005) whereas other variants, CD44v3 and CD44v7-v8 correlate with metastasis, and significantly poorer overall and disease-free survival (Rys et al., 2003; Watanabe et al., 2005). Since TICs may comprise only a small subset of the entire tumor, sampling through biopsy might not be effectively capturing this population of cells making correlations between TIC content and patient outcome difficult to identify. Furthermore, utilizing marker expression alone to define TICs may be insufficient to provide prognostic value and gene expression profiling may more accurately define this population of cells. Supporting this idea, the molecular analysis of functionally defined leukemic stem cell populations generated a stem-cell signature that was a strong predictor of poor prognosis (Eppert et al., 2011). Additionally, a gene signature derived from CD44+/CD24− primary breast cancer cells correlated with decreased patient survival (Shipitsin et al., 2007). Importantly, this signature was generated using expression data from bulk tumors.

1.4.7 Targeting signaling pathways in tumor-initiating cells

As described above there is good evidence that standard therapies do not effectively eliminate the TIC population. It is vital to develop agents that target TICs specifically and the combination of TIC-targeted therapies with standard therapy holds great promise for both de-bulking of tumors as well as preventing recurrence. One approach to eliminating TICs is through the inhibition of signaling pathways critical to maintaining a TIC phenotype, thereby inducing differentiation or apoptosis. Alternatively, targeting TIC mechanisms of resistance may sensitize them to current treatments. Therapies that interfere with the stem cell niche is an alternate approach proposed to eliminate TICs but is beyond the scope of this review. The ability to separate tumorigenic and non-tumorigenic populations of cells allows for molecular characterization and elucidation of pathways that account for their tumorigenic potential. Interestingly, many signaling pathways that regulate embryonic development are reactivated or persistent in TICs and thus provide attractive therapeutic targets.
**Notch Pathway.** The Notch pathway has been implicated in the maintenance of both normal mammary stem cell and TIC activity (Dontu et al., 2004). Of the 4 different isoforms (Notch1-4), the Notch4 isoform in particular is crucial for mammary stem cell maintenance. Notch binds to ligands called Delta-like and Jagged, which trigger proteolytic cleavage of Notch by γ-secretase, releasing the intracellular domain to translocate to the nucleus and transcriptionally activate genes which maintain an undifferentiated cell state. Aberrant activation of Notch4 signaling in normal mammary epithelial cells prevents differentiation and ultimately induces mammary carcinomas in mice (Gallahan et al., 1996). In breast cancer cell lines and patient samples, anoikis-resistant and CD44+/CD24-/ESA+ sorted TICs expressed higher levels of activated Notch4 (N4ICD) than their non-TIC counterparts. Knockdown of Notch4 specifically, significantly reduced mammosphere formation and xenograft initiation in mice (Harrison et al., 2010). Pharmacological, inhibition of this pathway using γ-secretase inhibitors also significantly reduces TIC activity by preventing the activating cleavage from occurring thus reducing Notch signaling (Farnie et al., 2007; Harrison et al., 2010). Furthermore, the γ-secretase inhibitor, MRK003, was recently shown to inhibit tumor initiation in mice using an ERBB2 model of mammary tumorigenesis and mice treated with MRK003 had sustained, long-term, relapse-free survival (Kondratyev et al., 2012). Two γ-secretase inhibitors (GSI) are currently in phase I/II clinical trials for the treatment of breast cancer (Al-Hussaini et al., 2011). Early indications suggest the combination of the GSI, MK-0752 and docetaxel may reduce the TIC burden in patients (Schott et al., 2013). In this small cohort of patients (n=30), a decrease in CD44+/CD24−, ALDH+ and mammosphere-forming efficiency was observed in tumor samples of patients undergoing serial biopsies. It will be interesting to see whether these surrogate assays for TICs are predictive of long-term relapse in these patients.

**Wnt/Frizzled/β-Catenin Pathway.** The Wnt pathway has also been implicated in normal breast development and tumor formation. The Wnt ligands bind to the cell-surface Frizzled family of receptors, which in turn activate Dishevelled proteins that inhibit the proteolytic degradation of β-Catenin. β-Catenin then is available to translocate to the nucleus and transcribes genes involved in cell polarity, cytoskeletal activity and cell differentiation (Nusse et al., 2008). Over-expression of Wnt pathway components in transgenic mice leads to an expansion of progenitor cells in pre-neoplastic tissue and promotes breast tumor
formation (Li et al., 2003; Lindvall et al., 2006; Liu et al., 2004). The dietary extract, sulforaphane, down-regulates the Wnt pathway and decreases the ALDH-positive cell population in primary human breast cancer cells. Treatment of mice with sulforaphane eliminated TICs in vivo and subsequently abrogated tumor growth upon re-implantation into secondary mice indicating this pathway as a critical mediator of breast TICs (Li et al., 2010).

Hedgehog Pathway. Hedgehog (Hh) proteins stimulate target cells that express Patched-1, the Hh receptor, and are involved in proliferation, differentiation and cell fate determination. Patched-1 then activates downstream effectors such as the Gli transcription factors and Bmi1, a polycomb gene. Hedgehog signaling is up-regulated in both MaSCs and TICs and is gradually down-regulated as cells differentiate (Liu et al., 2006). Components of the hedgehog pathways are highly expressed in TICs identified by CD44+CD24- marker expression or side population sorting while the suppression of Hh activity inhibited growth of TICs (Tanaka et al., 2009). Derivatives of the Hh inhibitor, cyclopamine, are now in clinical trials.

CD44. Although CD44 is typically viewed as a marker for identifying TICs, it may also have therapeutic implications. CD44 is a family of transmembrane glycoproteins that interact with components of the extracellular matrix, namely hyaluronic acid (hyaluronan) and osteopontin (Ponta et al., 2003; Screaton et al., 1992). As such, it can regulate a TICs response to its niche. Interaction of CD44 with hyaluronan and RHAMM sustains ERK1/2 signaling to promote anchorage-independent breast cancer cell growth, survival, and migration, thereby promoting metastatic spread (Gotte and Yip, 2006; Hamilton et al., 2007). Further, it can act as a platform for growth factors or matrix metalloproteinase (MMPs) to promote invasion and also act as a co-factor for growth factor receptors within the ERBB receptor tyrosine kinase family (reviewed in (Ponta et al., 2003)). Accordingly, interest in CD44 as a therapeutic target has grown. Inhibiting CD44 using siRNA decreases mammosphere growth in breast cancer cell lines (To et al., 2010). Marangoni et al. demonstrated that an antibody targeting CD44 (P245) was effective at reducing human breast tumor xenografts in mice when given alone but the tumors re-grew when the treatment was stopped. However, a much more sustainable suppression on tumor re-growth
was seen when P245 was given in combination with chemotherapy (Marangoni et al., 2009). These data suggest targeting CD44 may be effective at eliminating TICs.

Due to the significant overlap between normal stem cell and TIC signaling, an important consideration when developing TIC-directed therapies is to determine whether they elicit an effect on normal stem cells. However, targeting TIC pathways is feasible if the genetic programs governing a TIC phenotype are differentially activated between normal and malignant stem cells, thereby opening up a therapeutic window. There is evidence in hematopoietic malignancies that TICs can be selectively targeted without ablating normal stem cell function (Guzman et al., 2005). Evaluating the efficacy of TIC-directed therapies in the clinic likely will require novel biomarkers, as the standard tumor response criteria (decrease in tumor volume) may not apply if TICs do indeed represent only a small proportion of the overall tumor.

1.4.8 Tumor-initiating cell “plasticity”

As previously noted, unlike CSCs, TICs do not necessarily follow a hierarchal organization. Non-TICs can become TICs under certain selective pressures or ectopic expression of certain stem-cell-associated pathways. In fact, even without any selective pressure, breast cancer cell lines were shown to transition stochastically between a TIC and non-TIC state to maintain a stable phenotypic equilibrium (Gupta et al., 2011). Additionally, TICs can arise de novo from transformed breast epithelial cells (Chaffer et al., 2011). Interestingly, the induction of epithelial-mesenchymal transition (EMT) has also been shown to confer TIC properties. Constitutive expression of either Snail or Twist in transformed human mammary epithelial cells (HMLE) increased the proportion of CD44+/CD24- cells, increased colony formation in non-adherent conditions and drastically increased the ability of cells to initiate tumors in immunodeficient mice (Mani et al., 2008). Similar results were seen when EMT was induced via exposure to TGFβ or activation of the Ras/MAPK pathway (Morel et al., 2008; Scheel et al., 2011). Finally, knockdown of E-Cad in HMLE cells also induces a TIC phenotype by increasing the percentage of CD44+/CD24- cells, mammosphere formation and ability to initiate tumors in mice as well as enhancing drug resistance to paclitaxel and doxorubicin (Gupta et al., 2009).
1.5 Cell Signaling in Breast Cancer

One method in which cells respond to their extracellular environment is by using cell surface receptors. Transmembrane receptors detect extracellular ligands and transmit signals into the cell by initiating cell-signaling cascades. Receptor tyrosine kinases are one type of transmembrane receptor. The EGF (ERBB) family of receptor tyrosine kinases is particularly important in breast cancers. It includes EGFR/ERBB1/HER1, ERBB2/HER2/Neu, ERBB3/HER3 and ERBB4/HER4. These receptors can either homodimerize or heterodimerize to differentially activate signaling cascades including the Raf/MEK/ERK pathway and the PI3K/Akt pathway (Roskoski, 2004). The activation of these pathways results in growth, differentiation and survival signaling. EGFR and HER2 are frequently over-expressed in breast carcinomas resulting in hyperactivated growth and proliferation of cancer cells. This section will focus on two proteins that are activated through ERBB signaling cascades. p90 ribosomal S6 kinase (RSK), a kinase that is activated via Raf/MEK/ERK and Y-box binding protein-1 (YB-1), a transcription/translation factor, which is downstream of both the Raf/MEK/ERK and the PI3K/Akt pathway. An overview is represented in (Figure 1.4).
Figure 1.4 Schematic representation of YB-1 activation through the Raf/MEK/ERK/RSK pathway and the PI3K/Akt pathway. YB-1 is predominantly activated via phosphorylation by RSK at Ser^{102}, at which point it translocates to the nucleus to transcriptionally up-regulate several genes important in breast cancer growth (grey boxes).

1.5.1 EGFR and Raf/MEK/ERK signaling in triple-negative breast cancer

Since TNBCs do not express HER2, EGFR signaling represents an important signaling pathway in this subtype. Upon stimulation by extracellular signals, EGFR activates the GTPase, Ras, which in turn initiates a kinase signaling cascade and sequential activation of Raf, MEK1/2 and ERK1/2 (Figure 1.4). EGFR expression is highest in TNBC compared to other subtypes and its expression predicts adverse patient outcome (Stratford et al., 2007; Viale et al., 2009). EGFR signaling is also implicated in TICs. The mammosphere-forming efficacy of high-grade primary DCIS treated with gefitinib (an EGFR inhibitor) was lower than that of high-grade DCIS treated with mammosphere medium lacking gefitinib (Farnie et al., 2007). Moreover, breast cancer patients treated with the dual EGFR/HER2 inhibitor,
lapatinib, displayed a decrease in CD44⁺/CD24⁻ and mammosphere-forming cells after 12 weeks indicating this may be an effective strategy for eliminating these cells (Li et al., 2008). Further evidence for activation of this pathway in TNBC came from Dr. Carlos Arteaga’s lab. Gene expression profiling of primary breast tumors revealed that gene expression patterns of activated Raf/MEK/ERK signaling as well as low levels of an ERK phosphatase, DUSP4, was associated with the basal-like subtype (Balko et al., 2012). Moreover, ERK activation after neoadjuvant chemotherapy correlated with treatment refractory, high Ki67 scores and shorter recurrence-free survival. Low DUSP4 was also associated with a significantly shorter time to relapse in an independent study interrogating the Wang et al. microarray dataset (P=0.0014, n=286) (Martin et al., 2008; Wang et al., 2005). This intriguing evidence identifies the Raf/MEK/ERK pathway in TNBC and particularly in proliferating cells that survive chemotherapeutic regimens. However, inhibiting Raf/MEK/ERK signaling through inhibition of up-stream targets has proven difficult. Single agent pathway inhibition can lead to the release of negative feedback loops on the PI3K/Akt (reviewed in (Gysin et al., 2011)). Additionally, downstream effectors may become activated and circumvent up-stream pathway suppression (Normanno et al., 2008). Therefore an appealing strategy is to target kinases further downstream (such as RSK) as this may reduce the ability of cancer cells to compensate through redundant pathways. However, this hypothesis remains to be tested.

1.5.2 p90 ribosomal S6 kinase structure and activation

The RSK family of serine/threonine kinases consists of four human isoforms RSK1-RSK4 which share ~80% sequence homology as well as two structurally related homologues, RSK-like protein kinase (RLPK) and mitogen-and stress-activated kinase 1 (MSK1) and MSK2 (Anjum and Blenis, 2008; Romeo et al., 2012). They are activated predominantly through the Raf/MEK/ERK pathway, which is activated by receptor tyrosine kinases such as (EGFR), fibroblast growth factor receptors (FGFR) and insulin-like growth factor receptors (IGFR) all of which are commonly activated in TNBC (Carriere et al., 2008; Kang et al., 2007; Law et al., 2008; Stratford et al., 2008). Despite their similarity, there is mounting evidence that each isoform has distinct biological functions. RSK was first purified by Erikson and Maller in 1985 and was named based on its rpS6 kinase activity,
hence, ribosomal S6 kinase (Erikson and Maller, 1985). It was later named p90 RSK to incorporate an aspect pertaining to its size (~90 kDa) (Jones et al., 1988).

**Structure and Activation.** RSK protein structure is unique compared to other kinases as it contains two functional but non-identical kinase domains (Fisher and Blenis, 1996). As such, RSKs belong to two protein kinase families. The C-terminal domain (CTKD) belongs to the calcium/calmodulin-dependent protein kinase (CAMK) family while the N-terminal kinase domain (NTKD) belongs to the protein A, G and C (AGC) family of kinases. Full activation of RSK requires a series of sequential phosphorylations with the four essential sites being Ser\(^{221}\), Ser\(^{363}\), Ser\(^{380}\) and Thr\(^{573}\). The process is initiated through mitogen stimulation of the Raf/MEK/ERK pathway resulting in ERK1/2 docking to the CTKD (Roux et al., 2003). Once docked, ERK phosphorylates Ser\(^{363}\) and Thr\(^{359}\) in the linker region and also Thr\(^{573}\) in the CTKD resulting in the translocation of RSK to the plasma membrane (Dalby et al., 1998; Richards et al., 2001; Richards et al., 1999; Smith et al., 1999; Sutherland et al., 1993a; Sutherland et al., 1993b). Ser\(^{380}\) then gets phosphorylated via autophosphorylation from the CTKD (Vik and Ryder, 1997). Phosphorylated Ser\(^{380}\) serves as a docking site for PDK1 (Frodin et al., 2002), which phosphorylates RSK at Ser\(^{221}\) in the NTKD resulting in full activation of the kinase (Jensen et al., 1999; Richards et al., 1999). Autophosphorylation of a serine residue near the ERK docking site prompts dissociation of ERK allowing RSK to translocate throughout the cell (Roux et al., 2003). Once activated, RSK recognizes target proteins through the minimal phosphorylation motifs Arg/Lys-Xaa-Arg-Xaa-Xaa-pSer/Thr or Arg-Arg-Xaa-PpSer/Thr (Leighton et al., 1995). Only one phosphatase has been found to be associated with RSK proteins. Protein phosphatase-2C\(\delta\) decreases RSK2 phosphorylation, thereby reducing its kinase activity (Doehn et al., 2004).

**Inhibitors of RSK.** Currently, three selective RSK inhibitors have been identified: dihydropteridinone (BI-D1870), SL0101 and fluoromethyl ketone (FMK). BI-D1870 is an ATP competitive inhibitor for the NTKD of RSK. It is specific to RSK relative to other AGC kinases and remarkably potent, having an in vitro IC\(_{50}\) of 15-30 nM at an ATP concentration of 100 µM (Bain et al., 2007; Sapkota et al., 2007). However, BI-D1870 also inhibits PLK1 with a slightly higher IC\(_{50}\) than RSK (Bain et al., 2007). SL0101 is also an ATP competitive inhibitor of the NTKD. This natural product is a kaempferol glycoside
with less potent RSK-inhibitory activity than BI-D1870 (Smith et al., 2005). Alternatively, FMK is an irreversible inhibitor, which covalently modifies the CTKD of RSK. It too is highly specific to RSK (Cohen et al., 2005). It is important to note that none of these inhibitors are isoform specific nor have they been tested in the clinic.

### 1.5.3 p90 ribosomal S6 kinase biological functions

RSK is involved in phosphorylation of a wide range of targets, which are involved in various cellular functions such as; cell cycle progression, proliferation, cell growth, protein synthesis, cell migration and cell survival. RSK mediates these processes both directly (e.g. through activation/inactivation of proteins that govern these pathways) and indirectly (e.g. through regulation of transcription factors). This section will highlight some of the key processes regulated by RSK. RSK2 is the most studied of all the isoforms and although each isoform likely has some unique targets, further work elucidating isoform-specific targets needs to be done.

**Role in Development.** Expression of RSK1-3 transcript is ubiquitous and expressed in every human tissue (Zeniou et al., 2002). Conversely, RSK4 expression is low in both embryonic and adult tissue. In humans, inactivation of the RSK2 gene Rps6ka3, results in Coffin-Lowry syndrome (CLS), which is an X-linked disease characterized in male patients by severe psychomotor retardation and facial hand and skeletal malformations. It occurs as a consequence of a developmental defect during embryogenesis (Pereira et al., 2010; Trivier et al., 1996). RSK2-knockout mice somewhat mimic the phenotype of CLS. They are viable but exhibit cognitive impairment and poor coordination as well as reduced size compared to their littermates (Dufresne et al., 2001; Poirier et al., 2007). They also develop osteopenia, a progressive skeletal disease, due to impaired osteoblast function and normal osteoclast differentiation (David et al., 2005). Additionally, despite having normal levels of B and T cells, RSK2 knockout mice exhibited delayed T-cell activation (Lin et al., 2008a). RSK1/RSK2/RSK3 triple-knockout mice are also viable but no phenotype information has been reported for these animals (Dumont et al., 2005). A RSK4 knockout mouse has not yet been reported.
Role in Growth and Translation. RSK plays a role in translation by regulating a variety of translation factors and studies show that it associates with actively translating polysomes and phosphorylates several ribosome-associated proteins (Angenstein et al., 1998). The 40S ribosomal subunit component, rpS6 is phosphorylated by RSK1/2, which promotes assembly of the cap-binding complex and correlates with increased cap-dependent translation (Roux et al., 2007). Additionally, RSK phosphorylates eEF2K (Wang et al., 2001) and eIF4B (Shahbazian et al., 2006) both of which positively regulate protein synthesis. Further, association of inactive RSK1 with 4E-BP1 (an inhibitor of eIF4F) and eIF4E prevents initiation of translation. Phosphorylation of inactive RSK1 results in its dissociation from 4E-BP1 at the same time that 4E-BP1 dissociates from eIF4E to allow initiation of cap-dependent translation (Kroczyńska et al., 2011). Finally, RSK1-mediated phosphorylation of GSK3β inhibits its kinase activity thereby releasing inhibition on the translation factor eIF2B (Cohen and Frame, 2001). Additionally, RSK regulates the mTOR signaling which regulates cell growth, survival and autophagy. For example, RSK phosphorylates the tumor suppressor, liver kinase B1 (LKB1), inhibiting its ability to activate AMP-dependent protein kinase (AMPK), which in turn prevents cells cycle arrest (Zheng et al., 2009). Furthermore, RSK regulates mTOR signaling by inhibiting tuberin (TSC2). RSK1 phosphorylation of Ser^{1798} inhibits the tumor suppressor function of the tuberin/hamartin complex, resulting in increased mTOR signaling to S6K1 and increased translation (Roux et al., 2004).

Role in Proliferation. RSK mediates cell cycle progression and cellular proliferation both directly and indirectly through a variety of mechanisms. It phosphorylates several transcription factors some of which are involved in immediate early gene response including c-Fos (David et al., 2005). C-fos itself regulates expression of cyclin D1, which promotes G_{1}/S transition. Additionally, activation of either RSK1 or RSK2 positively regulates G_{1} progression by phosphorylation and inactivation of p27^{kip1}, a negative regulator of cyclin-dependent kinase 2 (CDK2) (Fujita et al., 2003).

Cell Survival. RSK regulates cell survival through both direct and transcriptional-mediated mechanisms. For example, RSK phosphorylates CREB, a transcription factor responsible for the expression of many anti-apoptotic, survival-promoting genes such as Bcl-2 and Bcl-
RSK also promotes survival through activation of the transcription factor NF-κB. NF-κB transcribes several anti-apoptotic genes such as Bel-X₁, cellular inhibitor of apoptosis (c-IAP) and X chromosome-linked inhibitor of apoptosis (XIAP) (Escarcega et al., 2007). Although NF-κB is not a direct target, RSK promotes its activation by phosphorylating IκBα and targeting it for degradation. This results in the dissociation of the IκBα/NF-κB complex and un masks the nuclear localization signal within NF-κB, allowing its rapid migration into the nucleus, where it activates the transcription of anti-apoptotic target genes (Ghoda et al., 1997; Schouten et al., 1997). In addition to up-regulating anti-apoptotic proteins, RSK also inhibits proteins that promote apoptosis. RSK2 phosphorylates the pro-apoptotic protein BAD at Ser¹¹², resulting in its inactivation. This process was shown to suppress BAD-mediated apoptosis in neurons (Bonni et al., 1999). RSK-mediated inactivation of the pro-apoptotic protein DAPK also results in increased cell survival (Anjum et al., 2005).

**Role in Cell Motility.** A role for RSK in mediating cell migration has been demonstrated by several groups. RSK is a principle effector of Raf/MEK/ERK-mediated motility and invasion in both non-transformed epithelial and carcinoma cells (Doehn et al., 2009). A genome-wide screen using RNAi revealed RSK as a common effector for multiple migratory stimuli (Smolen et al., 2010). Pharmacological inhibition of RSK dramatically suppressed epithelial cell migration induced through several different pathways, suggesting a convergence of diverse migratory signaling on this kinase. Thus, RSK plays a significant role in mediating migration through the Raf/MEK/ERK pathway. Moreover RSK2 inhibition attenuates both *in vitro* migration and *in vivo* metastasis of the highly invasive head and neck squamous cell carcinoma (HNSCC) (Kang et al., 2010).

**Role in Cancer.** The prominent role of RSK in a variety of pathways that mediate cell proliferation and survival, positions it as a key factor for regulating cancer. RSK1 and RSK2 isoforms exhibit tumor-promoting activity while RSK3 and RSK4 are indicated as tumor suppressors (reviewed in (Roux et al., 2003)). RSK1 and RSK2 are highly expressed in a wide variety of cancers including prostate (Clark et al., 2005), multiple myelomas (Cuadrado and Nebreda, 2007; Kang et al., 2007), T-cell lymphoma (Kang et al., 2007; Kang et al., 2009) and melanoma (Mirmohammadsadegh et al., 2007) as well as head and
neck cancers (Kang et al., 2010). In breast cancer (n=48), mean levels of RSK1 and RSK2 are statistically higher than that in the normal tissues (n=12) ($P<0.05$, Student's t test) based on densitometry of immunoblots. Moreover, knockdown of either isoform using siRNA or inhibition with SL0101 reduced growth in MCF7 cells, but not in the normal breast cell line MCF10A (Smith et al., 2005). Conversely, RSK3 and RSK4 have been shown to have antiproliferative, tumor-suppressive functions and are often found in lower abundance in tumor tissue than in normal tissue (Bignone et al., 2007; Thakur et al., 2008). Studies from several groups have identified RSK2 as being essential for transformation. Stable expression of RSK2 in mouse epidermal cells (JB6) significantly enhanced colony formation in anchorage-independent transformation assays (Cho et al., 2007) and transformation of hematopoietic cells by RSK2 through FGFR3-mediated activation has also been shown (Kang et al., 2007). RSK2-mediated activation of c-Fos endows oncogenic properties and is essential for osteosarcoma formation. In the absence of RSK2, osteosarcoma formation is impaired (David et al., 2005). Interestingly, although RSK2 plays a key role in carcinogenesis, as of yet there is no evidence that RSK2 is mutated in human cancer (Kang and Chen, 2011).

In addition to promoting tumorigenesis, RSK is critical for maintaining cancer once it is established. Inhibition of RSK induces cell death in FGFR1-transformed MCF10A mammary cells but not in the parental non-transformed cell line (Xian et al., 2009). Studies from several groups have recently identified the RSK2 isoform in particular is important in cancer (reviewed in (Kang and Chen, 2011)). Kang et al. demonstrated that inhibition of RSK2 but not RSK1 by siRNA as well as treatment with FMK induced apoptosis in FGFR3-expressing human myeloma cell lines and primary myeloma cells (Kang et al., 2007). In breast cancer, functional viability screening identified RSK2 as one of a limited number of genes that was required to sustain growth in a panel of TNBC cell lines (Brough et al., 2011). This study did not perform further functional validation assays to address the effect of RSK inhibition in TNBC. Importantly, although immortalized cell lines express RSK, their viability is not affected by RSK inhibitors suggesting targeting RSK may have minimal toxicity to normal cells (Smith et al., 2005), an assertion that is further supported by the fact that RSK1/RSK2/RSK3 knockout mice are viable (Dumont et al., 2005).
Recent emerging evidence also indicates a role for RSK in mediating drug resistance. Notably, RSK1 and RSK2 phosphorylate the tumor-specific transcription factor YB-1 (Stratford et al., 2008). Activation of YB-1 leads to up-regulation of TIC-associated genes, CD44 and CD49f as well as promotes growth and drug-resistance in TNBC cell lines (To et al., 2010). RSK1 also phosphorylates the transcription factor ERα, at Ser\textsuperscript{167}, which subsequently increases ERα-mediated transcription (Joel et al., 1998; Yamnik and Holz, 2010). Phosphorylation of Ser\textsuperscript{167} correlates with resistance to tamoxifen (Campbell et al., 2001) and is a prognostic marker for disease progression and poor survival (Jiang et al., 2007) thus RSK-mediated phosphorylation of this residue could have important implications for mediating tamoxifen resistance in breast cancer. Mechanistic insight regarding RSKs role in drug resistance came from Dr. Ray-David et al. This study demonstrated that RSK phosphorylates checkpoint kinase 1 (Chk1) at an inhibitory site, thereby preventing the G\textsubscript{2} DNA checkpoint induced by DNA damaging agents and allowing for proliferation. Conversely, RSK inhibition sensitized melanoma cells to DNA damaging agents (Ray-David et al., 2012). The essential role for RSK in sustaining tumor growth and the fact that it is activated by several RTKs which are over-expressed in cancer, positions it as potentially a more effective target than its upstream activators.

1.5.4 Y-box binding protein-1 structure and activation

An important target of RSK is YB-1. YB-1, encoded by the YBX1 gene, is a transcription/translation factor that lies downstream of both the Raf/MEK/ERK/RSK pathway and the PI3K/Akt pathway (Figure 1.4). YB-1 proteins fall into three subfamilies. The first subfamily (YB-1) includes the human YB-1. These proteins are characteristic of somatic cells and this review will focus primarily on this family. The second subfamily, contains human YB-2, and is specific to germ cells. Finally, the YB-3 family contains human dbpA and consists of proteins that are synthesized during embryonic development but disappear at birth (Eliseeva et al., 2011; Lu et al., 2006). YB-1’s amino acid sequence is around 40 kDa yet the protein migrates as a 50 kDa protein (Minich et al., 1993). It was discovered in 1988 by two groups nearly simultaneously. Dr. Dider et al. sequenced a DNA-binding protein interacting with the promoter of the major histocompatibility complex II and named it YB-1 (Didier et al., 1988). Later that same year, Dr. Sakura et al. found that
the protein called DNA binding protein (dbpB) had an identical amino acid sequence to YB-1 (Sakura et al., 1988).

**Structure and Activation.** The basic peculiarities of the YB-1 protein are: 1) it contains a cold shock domain which is flanked by 2) the N-terminal domain, containing a high alanine and proline amino acid content and 3) the elongated C-terminal domain which contains clusters of positively and negatively charged amino acid residues. The cold shock domain is highly conserved between subfamilies sharing ~90% homology (Wolffe, 1994). It also contains the RNP-1 and RNP-2 consensus sequences, which are involved in interactions with both RNA (Tafuri and Wolffe, 1992) and DNA (Bouvet et al., 1995; Ladomery and Sommerville, 1994). The N-terminal domain interacts with actin and contributes to mRNA localization (Ruzanov et al., 1999). The C-terminal domain provides a high affinity of YB-1 to nucleic acids likely stabilizing the interaction between YB-1 and DNA/RNA (Matsumoto et al., 1996). Although, all three domains have been shown to interact with proteins, the C-terminal domain has by far the most established protein interactions (reviewed in (Eliseeva et al., 2011)). As of yet, there has not been any success in determining YB-1’s three-dimensional structure. YB-1 is activated through phosphorylation, resulting in its nuclear translocation and transcriptional activation. It is phosphorylated at Ser$^{102}$ predominantly by RSK (Stratford et al., 2008), however Akt is another common activating kinase (Evdokimova et al., 2006; Sutherland et al., 2005). Importantly, RSK1/2 demonstrated ~10x greater activity to phosphorylate the Ser$^{102}$ residue of YB-1 than Akt (Stratford et al., 2008). ERK and GSK3β have also been shown to phosphorylate YB-1 but to a much lesser extent (Coles et al., 2005). YB-1 is completely degraded after ubiquitination during programmed cell death (Lutz et al., 2006).

### 1.5.5 Y-box binding protein-1 biological functions

YB-1 is involved in almost all DNA and RNA-dependent processes including DNA-replication and repair, transcription, pre-mRNA splicing and mRNA translation. This section will highlight some of the key processes regulated by YB-1.

**Role in Development.** A high YB-1 content is typical of all mouse organs both in prenatal and early post-natal stages, however protein levels gradually decrease with age.
Specifically, the amount of YB-1 protein in the mouse brain, heart and muscles decreases throughout several weeks after birth, while in testicles, spleen, kidney and lungs the decrease occurs with aging (Miwa et al., 2006). Although heterozygous offspring (YB-1+/−) appear normal and fertile, knockout of YB-1 (YB-1−/−) in mice results in embryonic lethality between E14.5-E18.5. These mice exhibit exencephaly indicating impaired neural tube closure, however other major organs remained intact (Uchiumi et al., 2006).

**Regulation of Expression.** The YB-1 gene promoter does not contain a TATA box or CCAAT element, but it does contain several E-boxes a well as GATA motifs, which are required for its transcription (Makino et al., 1996). The E-box is required for cisplatin-induced transcriptional up-regulation of YB-1, which is activated by a p73 and c-Myc complex binding (Uramoto et al., 2002). YB-1 mRNA synthesis is also up-regulated upon Twist binding to the E-box (Shiota et al., 2008). Further work demonstrated that inhibition of ILK signaling also decreased YB-1 mRNA and protein levels likely in a Twist-mediated mechanism (Kalra et al., 2010). The transcription factor Foxo3a has also been shown to decrease YB-1 and Twist1 expression (Shiota et al., 2010). In a more recent study it was found that the majority of YB-1 mRNA is stored as free, un-translated mRNPs in the cytoplasm (Lyabin et al., 2012). Its expression correlates with a cells proliferative capacity (determined by confluence) and could be suppressed by the mTOR inhibitor, PP242, in a 5'-UTR dependent manner.

**Transcription Factor.** YB-1 has been shown to regulate the transcription of many genes involved in cell division, apoptosis, immune response, multidrug resistance, stress response and tumor growth. It can both stimulate and inhibit transcription. It was originally discovered to interact with the inverted CCAAT boxes known as YB-1 responsive elements (YRE’s) to activate or repress transcription (Didier et al., 1988) however, it has since been shown to bind to a great variety of DNA sequences (Grant and Deeley, 1993; Hasegawa et al., 1991; Zasedateleva et al., 2002). Transcriptional regulation by YB-1 can occur through several different mechanisms. Firstly, YB-1 can bind directly to the promoters of genes containing YREs to promote or repress their expression. For example, phosphorylation at Ser102 enhances promoter binding and expression of EGFR (Stratford et al., 2007), PIK3CA (Astanehe et al., 2009), HER2 (Wu et al., 2006) and MET (Finkbeiner et al., 2009) all of
which have implications in cancer. Conversely, YB-1 binding to the TP53 promoter decreases its transcription (Lasham et al., 2003). Secondly, YB-1 can interact with other transcription factors to act as a co-activator or a co-repressor independent of Y-boxes. In human embryonic kidney cells, YB-1 stimulates the transcription of the MDRI gene encoding P-gp in an APE1-dependent manner whereby, APE1, p300 and YB-1 form a complex prior to binding to the MDR1 promoter (Sengupta et al., 2011). Inhibiting the formation of this complex impaired MDR1’s expression and sensitized cells to cisplatin or etoposide. Finally, YB-1 can bind to single-stranded DNA to enhance or inhibit binding of other transcription factors to DNA. An example of transcriptional inhibition is YB-1 binding to the collagen type α1(I) (COL1A1) gene (Norman et al., 2001). Stabilization of the single strand state prevents binding of transcription factors that interact with double-stranded DNA and promote transcription.

**DNA Repair.** YB-1 is involved in almost every type of DNA repair including; base excision repair, nucleotide excision repair, mismatch repair, double-stranded break repair and recombination repair. It interacts with proteins such as PCNA (Ise et al., 1999) and p53 (Okamoto et al., 2000), DNA polymerase δ (Gaudreault et al., 2004), APE-1 (Sengupta et al., 2011) and DNA ligase IIIα (Das et al., 2007) to name only a few. YB-1 has a high affinity for DNA with abasic sites (Hasegawa et al., 1991; Lenz et al., 1990) as well as cisplatin-damaged DNA (Ise et al., 1999) and DNA mismatches (Gaudreault et al., 2004). Moreover, it exhibits weak 3’-5’ exonuclease activity on single-stranded DNA (Izumi et al., 2001) and weak endonuclease activity on double-stranded DNA in a sequence dependent-manner (Gaudreault et al., 2004; Izumi et al., 2001).

**Translation.** YB-1 is involved in many aspects of mRNA translation. It is a universal packing protein of messenger ribonucleoprotein (mRNPs) associated with all or many mRNAs in both translated and un-translated states (Blobel, 1972; Morel et al., 1973). As is known, the entire mRNA in the cytoplasm of eukaryotic cells exists in the form of mRNPs, which are complexes consisting of individual transcripts bound by a changing repertoire of proteins that mediate the post-transcriptional events of gene expression including selective translational control, regulating mRNA lifetimes and intracellular distribution (Hieronymus and Silver, 2004). Further analysis revealed that at a low YB-1/mRNA ratio, mRNA is
accessible for interaction with translation initiation factors whereas at high YB-1/mRNA ratios the mRNA becomes inaccessible (Skabkin et al., 2004). Unfortunately the authors did not indicate whether these levels of YB-1 were physiologically relevant in either normal or cancerous cells. Inhibition of translation is observed only at the initiation stage whereby YB-1 prevents association of mRNA with the small ribosomal subunit by displacing all subunits of translation initiation factor eIF4F (eIF4G, eIF4E and eIF4A) (Bader et al., 2003; Evdokimova et al., 2001; Nekrasov et al., 2003). At the other extreme, removing YB-1 from lysates entirely halts translation but it can be activated upon addition of YB-1 (Matsumoto et al., 1996; Minich and Ovchinnikov, 1992). And so YB-1 exerts a dual effect on translation depending on its concentration relative to mRNA. Several mechanisms for YB-1 regulation of translation have been proposed. YB-1 regulated transcription of growth factors and proteins involved in various stress conditions, (or so-called “weak” templates) appears to be mediated in a phosphorylation-dependent manner. Akt phosphorylation of Ser\textsuperscript{102} leads to the dissociation of YB-1 from the cap-structure, allowing for translational initiation (Evdokimova et al., 2006). Finally, YB-1 can also mediate translation in a cap-independent manner. For example, it positively regulates translation of IRES-containing mRNA of the myc family of protooncogenes (Cobbold et al., 2008). In addition to regulating translation, YB-1 can stabilize mRNA, preventing its degradation ((Evdokimova et al., 2001) and reviewed in (Eliseeva et al., 2011)). Interestingly, YB-1 has also been shown to bind to actin (Ruzanov et al., 1999) and microtubules (Chernov et al., 2008) indicating it may localize mRNA within the cell.

**Secretion.** Another property of YB-1 is that it can be secreted under inflammatory stress (Frye et al., 2009). YB-1’s secretion depends on acetylation at its lysine residues 301 and 304. Interestingly, secretion of YB-1 does not occur via the classical mechanism (i.e. the golgi apparatus) but rather, through endolysosomal vesicles. Extracellular YB-1 stimulates proliferation and migration of rat mesangial cells and human kidney cells. In a follow-up study, it was discovered that extracellular YB-1 interacts with the Notch3 receptor, promoting its cleavage and up-regulation of Notch downstream target genes (Rauen et al., 2009). In these studies, YB-1 could be detected in the urine of afflicted animals suggesting it could be used as a diagnostic biomarker for mesangioproliferative diseases. However overall, little is known about the role of extracellular YB-1.
**Role in Cancer.** YB-1 is an oncogenic transcription/translation factor that is considered to be one of the most indicative markers of malignant tumors. It is documented to transcribe genes involved in drug resistance. Moreover, its role in DNA repair can promote resistance to DNA damaging agents and ionizing radiation. Finally, it has been shown to promote EMT and increase cell migration. In fact, YB-1’s multifunctional role in facilitating nearly every one of Hanahan and Weinberg’s “hallmarks of cancer” earns it a status as a master regulator of malignancy (Lasham et al., 2013). This section will highlight some of the most relevant findings regarding YB-1 in cancer with a particular emphasis on breast cancer.

The amount of YB-1 mRNA and protein are frequently increased in a variety of cancers including breast (Bargou et al., 1997; Gluz et al., 2009; Habibi et al., 2008; Huang et al., 2005; Janz et al., 2002; Wu et al., 2006), glioblastoma multiforme (Faury et al., 2007), colorectal (Shibao et al., 1999), non-small cell lung cancer (Gu et al., 2001; Kashihara et al., 2009; Shibahara et al., 2001), ovarian (Kamura et al., 1999; Yahata et al., 2002), prostate (Gimenez-Bonafe et al., 2004), multiple myeloma (Chatterjee et al., 2008), B-cell lymphoma (Xu et al., 2009), osteosarcoma (Oda et al., 1998), synovial sarcoma (Oda et al., 2003), embryonal rhabdomyosarcoma (Oda et al., 2008) and melanoma (Hipfel et al., 2000; Schitteke et al., 2007). Moreover, all of these studies demonstrated that expression of YB-1 is correlated with poor patient prognosis. In breast cancer, YB-1 is a stronger predictor of relapse and disease-specific survival than ER or HER2 across all subtypes based on microarray data from over 4,000 patients (Habibi et al., 2008). Furthermore, it was expressed in ~70% of patients with the most aggressive subtypes (HER2 over-expressing and TNBC).

Several studies have indicated that YB-1 may also play a role in carcinogenesis. In breast tissue YB-1 is a *bona fide* oncogene and transgenic mice expressing YB-1 in the mammary gland develop tumors with 100% penetrance (Bergmann et al., 2005). The authors discovered that YB-1 over-expression leads to genetic instability that emerges from mitotic failure and centrosomal amplification. A similar effect is seen in human mammary epithelial cells with induced YB-1 expression. Despite acquired numerical and structural chromosomal abnormalities, YB-1 promotes slippage through the G1/S cell cycle checkpoint, prompting proliferation of genomically compromised cells (Davies et al.,...
Thus, YB-1 may indeed be a driver of early preneoplastic progression towards neoplastic disease. Established cancers are also highly dependent on YB-1 for continued growth and survival. Numerous groups have found YB-1 to be either increased or more frequently localized to the nucleus in transformed cells and that suppressing it eliminates transformed cells’ ability to grow in anchorage-independent soft agar colonies (Gao et al., 2009; Shiota et al., 2011b). Interestingly, YB-1 regulates the expression of several growth receptors particularly important in breast cancer such as EGFR (Stratford et al., 2007), MET (Finkbeiner et al., 2009), and HER2 (Wu et al., 2006). It is essential for sustained growth and silencing it inhibits proliferation in cell lines representative of several subtypes including HER2 over-expressing and TNBC (Law et al., 2010; To et al., 2010; Wu et al., 2006; Finkbeiner et al., 2009; Lee et al., 2008; Stratford et al., 2007). Importantly, YB-1’s regulation of cancer cell growth is dependent upon its nuclear translocation. Thus, YB-1 plays a critical role in both malignant transformation as well as sustaining mammary carcinomas. Furthermore, YB-1 may facilitate metastatic spread of breast cancer. Upon inhibition of YB-1, the capacity for breast cancer cells to migrate and invade is reduced (Astanehe et al., 2009). This effect may be due to YB-1 mediated modulation of EMT. Dr. Evdokimova et al. found that MCF10AT breast epithelial cells induced to express YB-1 as well as activated Ras/MAPK signaling underwent EMT. When injected into the mammary fat pads of mice, cells with high YB-1 produced metastases while cells with normal levels of YB-1 were localized to the injection site (Evdokimova et al., 2009). Moreover, over-expression of YB-1 in MCF7 breast cancer cells promotes invasion in collagen and pulmonary metastatic spread and lethality in vivo (Lovett et al., 2010).

**Drug Resistance.** As discussed previously, drug-resistance presents a major obstacle in treating oncological diseases. YB-1 is a key mediator of drug resistance. The probability of curing various types of cancer including breast, without relapse after chemotherapy is significantly lower if YB-1 is elevated or localized to the nucleus (Gessner et al., 2004; Janz et al., 2002; Kamura et al., 1999; Kashihara et al., 2009; Shibahara et al., 2001; Xu et al., 2009). One possible mechanism contributing to resistance is YB-1-mediated drug efflux via ABC transporters. Many researchers have found that nuclear localization of YB-1 or its over-expression correlates with P-gp in various cancers (Gimenez-Bonafe et al., 2004; Oda et al., 2007; Oda et al., 2003; Oda et al., 1998; Xu et al., 2009) and in breast cancer.
specifically (Bargou et al., 1997; Fujita et al., 2005). It is postulated that YB-1 is involved in the increased expression of P-gp, however the exact mechanism by which this occurs remains to be determined. Bargou et al. found that nuclear, but not cytoplasmic YB-1 was associated with P-gp expression in patient samples and MCF-7 cells. Moreover, increased expression of YB-1 in drug-sensitive cells induced P-gp and resistance to doxorubicin (Bargou et al., 1997). YB-1 also positively regulates expression of other ABC transporters including LRP/MVP (Stein et al., 2005), MRP1 (Oda et al., 2003; Stein et al., 2001) and BCRP (Vaiman et al., 2006) but again the mechanism of action is not yet understood.

YB-1 may also contribute to drug resistance via its DNA repair activity. YB-1 has a higher affinity for cisplatin-modified DNA or DNA containing abasic sites or mismatches and its binding has been shown to promote efficient repair (Gaudreault et al., 2004; Grant and Deeley, 1993; Hasegawa et al., 1991; Ise et al., 1999; Izumi et al., 2001; Lenz et al., 1990; Zasedateleva et al., 2002). Accordingly, it has been demonstrated that mouse embryonic stem cells from heterozygous knockout mice (YB-1+/−) have an increased sensitivity to DNA damaging agents, cisplatin and mitomycin C (Shibahara et al., 2004). A similar effect was seen in epidermoid cancer cells whereby, treatment of cells with siYB-1 increased sensitivity to cisplatin, mitomycin C as well as UV radiation (Ohga et al., 1996).

Finally, YB-1 may also contribute to drug resistance by mediating a TIC phenotype. The YB-1 oncoprotein regulates CD44 and CD49f marker expression both in vitro and in vivo. Further, transfecting YB-1 into cells enhanced mammosphere formation even in the presence of paclitaxel in TNBC cell lines (To et al., 2010). In a HER2 over-expressing model of breast cancer, trastuzumab-resistant cell lines (HR5 and HR6) were demonstrated to have elevated P-YB-1S102 and CD44 when compared to the drug-sensitive BT474. Conversely, transfection of BT474 cells with constitutively active YB-1D102 conferred trastuzumab resistance to these cells as well as increased their mammosphere-forming capacity (Dhillon et al., 2010). One explanation for this was elevation of the YB-1 target gene MNK1, however ChIP-seq of the resistant cells revealed there is likely many genes involved in this process (Astanehe et al., 2012). These studies demonstrate YB-1 promotes drug-resistance and provide tantalizing hints that inhibiting YB-1 in combination with current front-line therapies for breast cancer may be effective at reducing drug-resistance.
and relapse, yet these experiments remain to be performed. The critical role for YB-1 in sustaining cell viability and promoting drug-resistance in breast cancer positions it as a promising candidate for overcoming these processes. However, due to the challenges of inhibiting transcription factors directly, researchers may look to inhibit upstream activators of YB-1 as more tractable therapeutic targets.
1.6 Hypothesis and Aims

The overall objective of this study is to identify novel targeted therapeutic strategies for the treatment of triple-negative breast cancer with the ability to eliminate chemoresistant cell populations that survive current standard-of-care chemotherapy. YB-1’s critical role in mediating drug resistance and a TIC phenotype make it an ideal target for overcoming the challenge of chemoresistance. However, due to the difficulties of inhibiting transcription factors, we sought to prevent YB-1’s activation through RSK inhibition, which itself may have added therapeutic benefits. **Hypothesis:** Blocking the activation of YB-1 via RSK inhibition will overcome chemotherapeutic resistance and suppress growth in TNBC.

**Chapter 2** will identify RSK2 as a novel target in TNBC and demonstrate that preventing the activation of YB-1 via RSK inhibition is effective at eliminating TICs. Based on our discovery that RSK2 is critical for the survival of TNBC, we endeavored to identify “off-patent” compounds with RSK-inhibitory activity utilizing both bioinformatics and *in vitro* kinase screening in **chapter 3.** Finally, in **chapter 4** we assess the utility of inhibiting YB-1 in combination with current front-line chemotherapeutics utilized to treat TNBC to determine whether inhibiting YB-1 can sensitize cells to these treatments and would be an effective strategy to overcome drug resistance.
CHAPTER 2. TARGETING P90 RIBOSOMAL S6 KINASE (RSK) ELIMINATES TUMOR-INITIATING CELLS BY INACTIVATING Y-BOX BINDING PROTEIN-1 (YB-1) IN TRIPLE-NEGATIVE BREAST CANCER

2.1 Overview

Y-box binding protein-1 (YB-1) is the first reported oncogenic transcription factor to induce the tumor-initiating cell (TIC) surface marker CD44 in triple-negative breast cancer (TNBC) cells. In order for CD44 to be induced, YB-1 must be phosphorylated at Ser\textsuperscript{102} by RSK. We therefore questioned whether RSK might be a tractable molecular target to eliminate TICs. In support of this idea, MDA-MB-231 cells that stably express Flag-YB-1 had increased growth \textit{in vivo} as well as enhanced CD44 expression. Despite enrichment for TICs, these cells were sensitive to RSK inhibition when treated \textit{ex vivo} with BI-D1870. Targeting RSK2 with siRNA or small molecule kinase inhibitors (SL0101 and BI-D1870) blocked TNBC monolayer cell growth by \textasciitilde100\%. In a diverse panel of breast tumor cell line models RSK2 siRNA predominantly targeted models of TNBC. RSK2 inhibition decreased \textit{CD44} promoter activity, \textit{CD44} mRNA, protein expression and mammosphere formation. \textit{CD44\textsuperscript{+}} cells had higher P-\textit{RSK1/2}\textsuperscript{S221/7}, P-\textit{YB-1}\textsuperscript{S102} and mitotic activity relative to \textit{CD44\textsuperscript{-}} cells. Importantly, RSK2 inhibition specifically suppressed the growth of \textit{CD44\textsuperscript{+}}/\textit{CD24\textsuperscript{-}} cells and triggered cell death. Moreover, silencing RSK2 delayed tumor-initiation in mice. In patients, RSK2 mRNA was associated with poor disease-free survival in a cohort of 244 women with breast cancer that had not received adjuvant treatment, and its expression was highest in the basal-like breast cancer subtype. Taking this further, we report that P-\textit{RSK1/2}\textsuperscript{S221/7} is present in primary TNBCs and correlates with P-\textit{YB-1}\textsuperscript{S102} as well as CD44. In conclusion, RSK2 inhibition provides a novel therapeutic avenue for TNBC and holds the promise of eliminating TICs.
2.2 Introduction

The treatment of triple negative breast cancer (TNBC) suffers from the lack of targeted therapies. Unlike estrogen receptor positive or HER2-positive breast cancers, which can be treated with anti-estrogens and Herceptin respectively, treatment options rest entirely upon conventional chemotherapies. While these tumors often initially respond very well to chemotherapy they commonly become resistant in the long-term leading to relapse (Dent et al., 2007; Liedtke et al., 2008). Tumor initiating cells (TICs), which are CD44+/CD24−, are more frequent in TNBC than other breast cancer subtypes (Charafe-Jauffret et al., 2009; Honeth et al., 2008; Nakshatri et al., 2009; Park et al., 2010) and have been linked to tumor recurrence. This is, in part, due to that fact that they are intrinsically resistant to traditional chemo- and radiotherapy (Creighton et al., 2009; Li et al., 2008; Phillips et al., 2006) but also the percentage of TICs increases following chemotherapy (Creighton et al., 2009; Fillmore and Kuperwasser, 2008). The loss of CD44 suppresses mammosphere formation, growth and branching morphogenesis (To et al., 2010). Thus, identifying targeted therapies that are uniquely able to eliminate TICs, as well as the bulk of the tumor, is critical (Stratford et al., 2010) as they are the most likely to improve TNBC patient outcome.

TICs are characterized as having the ability to self-renew, grow as mammospheres, invade and initiate tumor formation in mice (Dontu and Wicha, 2005; Ponti et al., 2005; Stratford et al., 2010). Using this battery of assays, new inhibitors are being sought to eliminate TICs. Compounds that are effective against both TIC and non-TIC populations within tumors are optimal as they would eliminate the bulk population as well as the drug-resistant TICs. Promising examples include targeting EGFR/HER2 with lapatinib or the Notch receptor with gamma secretase inhibitors (Li et al., 2008); however, these leads only provide modest growth suppression. The NF-κB (Murohashi et al., 2010) and TGFβ (Blick et al., 2010; Shipitsin et al., 2007) pathways are also implicated in TICs based on gene expression analysis of CD44+/CD24− cells. Most targeted therapies are directed toward kinases that mediate signal transduction (Sawyers, 2009); therefore, perhaps an alternative strategy to targeting individual receptor tyrosine kinases is to suppress a common convergence point farther downstream.
The p90 ribosomal S6 kinase (RSK) family of kinases are activated by receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR), fibroblast growth factor and insulin-like growth factor receptors which are commonly activated in TNBC (Carriere et al., 2008; Kang et al., 2007; Law et al., 2008; Stratford et al., 2008). This allows RSK to phosphorylate downstream targets involved in tumor growth, invasion and epithelial-mesenchymal transition (Anjum and Blenis, 2008; Carriere et al., 2008). These include transcription factors such as Y-box binding protein-1 (YB-1) (Stratford et al., 2008), CREB and c-fos (Chen et al., 1993) as well as inhibits the pro-apoptotic protein BAD (Shimamura et al., 2000), the translation factor GSK3β (Sutherland et al., 1993c) and histone H3 (Lau and Cheung, 2011; Sassone-Corsi et al., 1999). More specifically, RSK phosphorylates YB-1 at Ser102 leading to nuclear translocation and transcriptional activation (Stratford et al., 2008). Here, YB-1 induces a TIC phenotype by up-regulating CD44 and CD49f (To et al., 2010). Conversely, we have shown that knocking down YB-1 using siRNA suppresses CD44 expression and mammosphere formation (To et al., 2010). Of clinical relevance, RSK is also activated by commonly used chemotherapies such as paclitaxel leading to the phosphorylation of YB-1 and ultimately to the induction of CD44 (To et al., 2010). One strategy in the pursuit of TIC ablation is to inhibit P-YB-1S102, which leads to reduced CD44 and TICs, however, this has only been achievable with small interfering RNAs (siRNAs) as there are no small molecules available to block YB-1 directly. Therefore, we addressed whether blocking the activation of YB-1 via RSK inhibition could be an alternative approach to combating relapse by eliminating TICs.

2.3 Results

2.3.1 RSK inhibitor BI-D1870 is effective at suppressing growth of a YB-1-induced CD44+ population

We have previously identified YB-1 as an oncogenic transcription factor with the ability to regulate a TIC phenotype including; TIC markers CD44 and CD49f as well as mammosphere formation and drug resistance (To et al., 2010). Using YB-1 as the driver to
induce a CD44-high population, we created stable cell lines expressing a Flag-YB-1 transgene in MDA-MB-231 cells (Figure 2.1A). When injected into the mammary fat pads of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (500 cells/mammary fat pad), cells overexpressing YB-1 demonstrated a significantly increased growth rate when compared to empty vector control cells (Figure 2.1B). The tumors were isolated, cell lines established and characterized for TIC markers. We noted that the tumors arising from Flag-YB-1 cells had increased CD44 expression as indicated by immunoblotting (Figure 2.1C). A second YB-1 target gene, CD49f was also confirmed to be induced in these explanted cell cultures (Figure 2.1C). P-YB-1S102 was elevated in the Flag-YB-1 cells although it is still present in the EV cells (Figure 2.1C). The induction of CD44 was further confirmed in a second pair of explanted tumors from MDA-MB-231 cells expressing the empty vector as compared to Flag-YB-1 (Figure S2.1). Interestingly, despite having an increased CD44+ population, BI-D1870, a small molecule RSK ATP competitive inhibitor, was capable of inhibiting growth in the Flag-YB-1 cell population ex vivo suggesting that Flag-YB-1 relies on RSK for enhanced growth (Figure 2.1D). In both ex vivo cell lines the target P-YB-1S102 was eliminated following treatment with BI-D1870 (Figure 2.1D). DMSO alone had no effect on cell growth in either cell line (Figure S2.2). To further elucidate the role of RSK1 and RSK2 separately on YB-1 activation, an in vitro kinase assay was performed using a YB-1 peptide as the substrate (Law et al., 2010; Stratford et al., 2008). Both RSK1 and RSK2 directly phosphorylate YB-1 at Ser102, which can be inhibited with BI-D1870 (Figure S2.3A). Additionally, BI-D1870 was shown to block activation of a second downstream RSK substrate GSK3β (Figure S2.3B). Thus, YB-1 drives tumorigenesis in vivo and the tumors that arise have higher level of CD44+/CD49f+ cells. While CD44 is high in the emergent tumors they are sensitive to RSK inhibition.
Figure 2.1 YB-1-induced CD44\textsuperscript{high} cells remain sensitive to RSK inhibition by BI-D1870.

A) Stable incorporation of Flag-YB-1 into the MDA-MB-231 cell line was validated by immunoblot and 500 cells were injected into the 4\textsuperscript{th} inguinal mammary fat pads of NOD/SCID mice (n=5). B) Tumors from cells expressing Flag-YB-1 had a significantly increased growth rates compared to empty vector control tumors ($P=0.009$) Error bars represent the standard error of the mean (SEM). C) Cells isolated from tumors expressing Flag-YB-1 had higher expression of the TIC markers CD44 and CD49f, as well as P-YB-1\textsuperscript{S102} as assessed by immunoblot. D) RSK inhibition via BI-D1870 (10 µM) suppressed growth and P-YB-1\textsuperscript{S102} in the Flag-YB-1 population with comparable efficacy as in the control empty vector cells (n=5; *$P<0.05$). Control immunoblot demonstrates P-YB-1\textsuperscript{S102} suppression by BI-D1870 in both cell lines.
2.3.2 RSK2 siRNA suppresses growth of TNBC cells

Next we asked if there was a specific RSK isoform that supports the growth of TNBCs. We treated TNBC cell lines SUM149 with RSK siRNAs which led to a >90% loss in their target protein expression after 72 hrs (Figure 2.2A inset). This corresponded with a similar decrease in P-YB-1$^{S102}$ (Figure 2.2A inset). Targeting RSK2 with siRNA every 72 hrs for a total period of 10 days inhibited the growth of the TNBC cell line SUM149 by 90% (Figure 2.2A). A consistent growth inhibition was observed at 72 hrs using two distinct siRNAs against both RSK 1 and 2 (Table S2.1). RSK2 inhibition was far more effective at suppressing tumor cell growth than RSK1 as loss of the former suppressed growth by almost 100% and the latter by only 50% (Figure 2.2A). The importance of RSK2 in the growth of TNBC cells was further confirmed in an unbiased screen of siRNAs targeting >700 kinases in 20 breast cancer cell line models representing the major subtypes of the disease (Brough et al., 2011). In the primary screen, siRNA targeting of RSK2 selectively suppressed the triple negative cell line models ($P<0.05$ permutation t test) and was one of a very limited number of genes that was able to elicit such a TNBC-specific effect (Figure 2.2B). This screen result was validated using multiple different siRNAs targeting RSK2 in a panel of 20 genetically diverse cell lines (Figure S2.4) where RSK2 siRNAs preferentially inhibited TNBC models but was not particularly effective against non-TNBC cell lines.
Figure 2.2 Inhibiting RSK suppresses growth of TNBC cell lines.

A) SUM149 cells were transfected with siRNA against RSK1 and/or RSK2, scramble control (Ctrl) or media only (No Tx) for 10 days (n=5; *P<0.05). Immunoblot demonstrates loss of protein after 72 hrs. B) Heat map showing the results of a supervised clustering of siRNA Z-scores. Negative values (blue) indicates a loss of viability and Z=0 (white) represents no effect on viability. Breast tumor cell lines were clustered based on TNBC status and differential effects between TNBC and non-TNBC groups identified using the median permutation test. Statistically significant effects (P<0.05) are shown. siRNA targeting RSK2 is indicated by the red arrow. C) Doses of BI-D1870 as low as 2 µM results in 80% reduction of SUM149 cell growth (n=5; *P<0.05). Immunoblot demonstrates decreased P-YB-1\(^{S102}\) across a range of BI-D1870 concentrations at 96 hrs. D) The effect of BI-D1870 on SUM149 cell growth could be partially rescued through expression of an activated YB-1 mutant (D102) (n=5 *P<0.05). Transgene expression was validated by immunoblot (inset). E) Cells which survived 72 hr BI-D1870 treatment were seeded at low density in the presence of the RSK inhibitor and allowed to grow for 10 days. Treated cells did not grow in this clonogenic assay (n=3; *P<0.05). F) Treatment of SUM149 cells with BI-D1870 resulted in the induction of apoptosis as measured by PI uptake (n=5; *P<0.05).
2.3.3 BI-D1870 blocks the growth of TNBC cells

We then sought to determine whether we could achieve similar growth suppression by treating TNBC cell lines with small molecule RSK inhibitors. Inhibiting pan RSK kinase activity with BI-D1870 (2 μM or above) reduced the growth of SUM149 cells by >90% after 10 days, with repeated dosing every 3 days (Figure 2.2C). Suppression of P-YB-1S102 was confirmed by immunoblotting at 96 hrs (Fig. 2C inset). Further, BI-D1870 (0.1-10 μM) or SL0101 (25-100 μM), a second RSK inhibitor, suppressed P-YB-1S102 and tumor cell growth by up to >90% after only 72 hrs (Figure S2.5A-B). To establish that P-YB-1S102 was a mediator of the effect observed following treatment with BI-D1870 we transfected SUM149 cells with activated YB-1 (D102) or empty vector (EV) and after 24 hrs exposed these cells to BI-D1870 (5 and 10 μM) for 72 hrs. Cell growth was then measured and as expected in the EV transfected cells BI-D1870 killed >80% of the cells (Figure 2.2D). This phenotype was partially rescued in the D102 transfected cells (50% growth inhibition) (Figure 2.2D). Transgene expression was validated by immunoblotting (Figure 2.2D inset).

Next, we asked if the few cells which remain following treatment with BI-D1870 are in fact resistant to the drug. Cells which remained after 72 hrs BI-D1870 treatment were plated at low density along with control treated cells. While the control treated cells formed colonies in this clonogenic assay, there was 100% growth suppression of the BI-D1870 treated cells, indicating that these cells have not developed resistance to the drug (Figure 2.2E). Having demonstrated a growth suppressive effect following RSK inhibition we then assessed induction of apoptosis. Treating the SUM149 cells with both BI-D1870 and RSK siRNA resulted in the induction of apoptosis. This is demonstrated by PI uptake (Figure 2.2F), P-H2AXS139 (Figure S2.5C) and PARP cleavage (Figure S2.5D). Additionally, both SUM149 and MDA-MB-231 cells stained positively for the apoptotic marker Annexin-V when treated with BI-D1870 (1, 5 or 10 μM) for 48-72 hrs (Figure S2.5E-F). Thus, TNBCs are dependent upon RSK signaling to sustain tumor growth and survival.
2.3.4 Inhibiting RSK decreases CD44 expression

Keeping in mind that the frequency of TICs is higher in TNBC than in other breast cancer subtypes and that RSK inhibition decreases the growth of TNBC cell lines we suspected it would also have an effect on TICs. As previously mentioned, a TIC phenotype is induced by the RSK substrate YB-1 through binding to the CD44 promoter in a phosphorylation dependent manner (Stratford et al., 2008; To et al., 2010). Site-directed mutants that prevent YB-1S102 phosphorylation stop nuclear trafficking and over-ride YB-1’s ability to induce TICs (To et al., 2010). BI-D1870 was therefore used as a chemical probe to mirror this effect. BI-D1870 inhibited the nuclear translocation of P-YB-1S102 in SUM149 cells (Figure 2.3A) and CD44 promoter activity (Figure 2.3B). Moreover, decreases in CD44 transcript levels were observed following treatment with RSK1/2 siRNA or BI-D1870 (Figure 2.3C and S2.6) with a concomitant reduction in the number of cells expressing high levels of CD44 (Figure 2.3D). Interestingly, as with the effect on growth, suppression of RSK2 with siRNA resulted in a much larger decrease in CD44 transcript levels than that of RSK1 (Figure 2.3C). A fundamental problem with many chemotherapeutic agents is that they enrich for CD44+ cells (Creighton et al., 2009; Fillmore and Kuperwasser, 2008; Li et al., 2008) and this is thought to be involved in drug resistance and recurrence. Herein we show that treatment of SUM149 cells with paclitaxel increases the percentage of CD44+ cells relative to DMSO-treated cells however this did not occur upon treatment with BI-D1870 (Figure 2.3E). More importantly, combining paclitaxel treatment with BI-D1870 prevented the enrichment for CD44+ cells by the former (Figure 2.3E).
Figure 2.3 RSK inhibition decreases CD44 expression.

A) Treating SUM149 cells with BI-D1870 decreases nuclear localization of P-YB-1$^{S102}$ (n=5). Immunofluorescence shows P-YB-1$^{S102}$ (green) and Hoechst33342 (blue). Scale bar is 20 µm. B) Inhibiting RSK with BI-D1870 decreases CD44 promoter activity (n=3). C)
CD44 transcript levels decrease after treatment with RSK siRNA in the SUM149 and MDA-MB-231 cell lines (n=3). D) The CD44^{high} fraction in cell populations was reduced upon RSK inhibition with BI-D1870 (10 µM; n=5). E) Paclitaxel, but not BI-D1870 increases the percentage of CD44{^+} cells. Further, the combination of BI-D1870 and paclitaxel prevented enrichment for CD44{^+} cells (n=5). Differences were considered statistically significant when \*P<0.05.
2.3.5 Targeting RSK inhibits TIC growth

While conducting high content screening, we unexpectedly noticed that CD44\(^+\) cells were more proliferative than the CD44\(^-\) cells, having a greater number of mitotic figures based on Hoechst33342 staining (Figure S2.7A). To further validate this we stained for a second marker of mitosis, P-histone H3\(^{S10}\), an early M-phase marker. Consistently, we found that CD44\(^+\) cells had higher P-histone H3\(^{S10}\) expression and were more actively undergoing mitosis (Figure 2.4A). The replicative capacity of CD44\(^+\) cells was blunted by exposing the cells to increasing amounts of BI-D1870 (Figure 2.4B). There were also fewer CD44\(^+\) cells in total (Figure 2.4C). As, P-histone H3\(^{S10}\) is downstream of RSK signaling, the suppression in growth in the CD44\(^+\) population may be in part due to a perturbed mitotic process (Lau and Cheung, 2011; Sassone-Corsi et al., 1999). Thus, RSK inhibition repressed CD44-positive cells’ ability to replicate. Taking this further we examined the impact of RSK inhibition on mammosphere growth. CD44\(^+\) cells have a higher capacity to form mammospheres as compared to CD44\(^-\) cells as previously reported by our group and several others (Calcagno et al., 2010; Dontu and Wicha, 2005; Fillmore and Kuperwasser, 2008; Li et al., 2008; Ponti et al., 2005; To et al., 2010) and reproduced herein (Figure S2.7B). Additionally, it has been shown that knocking down CD44 reduces the ability of cells to form mammospheres (To et al., 2010). As a functional readout of TICs we therefore measured the ability of MDA-MB-231 and SUM149 cells to form mammospheres in culture. In line with the decreased CD44, RSK inhibition markedly suppressed mammosphere formation by 80-100% (Figure 2.4D and Figure S2.7C). There was such a substantial effect it was not possible to serially passage the spheres. Further the compound caused regression of established mammospheres (Figure 2.4E).
Figure 2.4 Targeting RSK suppresses growth of TICs.

A) CD44<sup>+</sup> cells express high levels of P-histone H3<sup>S10</sup>. Immunofluorescence staining shows CD44 (red), P-histone H3<sup>S10</sup> (green) and Hoechst33342 (blue). Scale bar is 15 µm. B-C) Treatment with BI-D1870 (1-10 µM) reduces P-histone H3<sup>S10</sup> and CD44 protein levels respectively (n=5) after 48 hrs. D) MDA-MB-231 cells treated with BI-D1870 or RSK1/2 siRNA had a significantly reduced ability to form mammospheres after 7 days (n=3). E) Mammosphere number was reduced following treatment of established spheres with BI-D1870 (10 µM)(n=3). Differences were considered statistically significant when *P<0.05.
Next, TICs (CD44+/CD24- cells) that were isolated by FACS were found to be enriched for P-RRSK1/2S221/7 and P-YB-1S102 compared to CD44-/CD24+ cells suggesting that this pathway may be particularly important in CD44+/CD24- cells (Figure 2.5A-B). These TICs were dependent upon RSK signaling because exposing them to BI-D1870 reduced cell growth by >90% after 72 hrs at doses as low as 1 µM (Figure 2.5C). Apoptosis was also induced as indicated by increased PI uptake (Figure 2.5D). When compared to their CD44-/CD24+ counterparts, TICs were found to have increased PI uptake after treatment with BI-D1870; perhaps, due to an increased dependence on RSK in this population (Figure S2.7D). Knock down of RSK2 expression using siRNA similarly decreased growth and induced apoptosis in CD44+/CD24- cells (Figure 2.5E and F, respectively). In parallel with our findings in unsorted cells, RSK2 inhibition was more effective at suppressing growth and inducing apoptosis in TICs than RSK1 inhibition. The studies thus far indicate that RSK2 inhibitors are exciting therapeutic leads for inhibiting growth in TNBC including TIC-enriched populations.

One important consideration is the effect of inhibiting RSK on normal stem cells. To address this question we assessed the growth and differentiation of primary human hematopoietic stem cells when dosed with a range (0.015-15 µM) of BI-D1870. Treatment with BI-D1870 did not suppress the growth or differentiation of normal human hematopoietic stem cells at doses that inhibited growth in TNBC (1-2 µM) (Table S2.2, Figure S2.8A-B). We subsequently investigated the effect of inhibiting RSK on normal breast epithelial cells (184htert). In accordance with the data on the hematopoietic stem cells we observed no effect on the growth of 184htert cells at concentrations up to 2 µM BI-D1870, a dose which suppressed growth of TICs by 90% (Figure S2.9A). Interestingly, neither SL0101 (50 µM) or RSK siRNA, in particular RSK2 siRNA, had any effect on growth of normal mammary epithelial 184htert cells (Figure S2.9B-C, respectively).
Figure 2.5 Inhibiting RSK suppresses growth in CD44\(^+\) cells.

A) CD44\(^+\)/CD24\(^-\)-sorted cells have a higher level of activated RSK and YB-1 than CD44\(^-\)/CD24\(^+\) cells. B) Quantification of the level of P-RSK1/2\(^{S221/7}\) in CD44\(^+\) compared to CD44\(^-\) cells along with representative images of P-RSK1/2\(^{S221/7}\) staining. C) Treatment of CD44\(^+\)/CD24\(^-\) sorted cells with a single dose of BI-D1870 (1 \mu M-10 \mu M) results in a ~90\% decrease in growth after 72 hrs (n=5). D) Treatment of CD44\(^+\)/CD24\(^-\) sorted cells with BI-
D1870 induces apoptosis in TICs as indicated by PI uptake \( (n=5) \). E) Knockdown of RSK2 in CD44\(^+\)/CD24\(^-\) sorted SUM149 cells resulted in a ~70% decrease in growth after 96 hrs \( (n=5) \). Immunoblot demonstrates loss of protein. F) Suppression of RSK2 with siRNA led to an increase in apoptosis as demonstrated by elevated PI uptake \( (n=5) \). Differences were considered statistically significant when \( *P<0.05 \).
2.3.6 RSK inhibition delays tumor initiation

To directly assess the effects of RSK knockdown on tumor initiation we performed a transient RSK2 knockdown in MDA-MB-231 cells which homogeneously express high CD44 and low CD24 (Figure 2.6A). RSK2 was silenced for 48 hrs and loss of expression was confirmed by qRT-PCR and immunoblotting (Figure 2.6B). We observed >80% decrease in RSK2 expression (Figure 2.6B). This decrease in RSK lead to a subsequent loss of CD44 protein expression (Figure S2.10A). NOD/SCID mice were injected with MDA-MB-231 cells transfected with either the scrambled control siRNA or RSK2 siRNA (1x10^6 cells/MFP). Given the short half-life of siRNAs (~10 days; Figure S2.10B-C) high cell numbers were required. Based on our prior experience, we knew that this number of cells would initiate tumor formation within approximately two weeks and that the siRNAs would remain active within this timeframe (Table S2.3). It was interesting to find that 100% of the mice (4/4) that received the MDA-MB-231 cells exposed to the scrambled control developed palpable tumors beginning at 17 days post-injection. In contrast, only 40% of the mice (2/5) developed tumors following RSK2 inhibition at the same time interval and this trend continued out until 24 days post-injection (Figure 2.6C). The delay in tumor-initiation correlated with RSK2 expression in that after three weeks all of the mice eventually developed tumors (day 27) (Figure 2.6C). At this point we validated RSK2 expression in all tumors and it was found to be equal to the controls (Figure S2.10D). Once RSK2 was re-expressed, tumors grew at equivalent rates, however the average size of the RSK2 siRNA tumors was approximately half of the control tumors (Table S2.3). This is the first proof-of-concept study to show that RSK inhibitors block the growth of TNBC cells in vitro and in vivo in part through the loss of TICs.
Figure 2.6 Inhibiting RSK2 delays tumor initiation.

A) MDA-MB-231 cells are ubiquitously CD44⁺/CD24⁻ as demonstrated by flow cytometry.

B) A reduction in RSK2 transcript and protein (inset) was achieved at 48 hrs in MDA-MB-231 cells (n=3; *P<0.05).

C) Transient RSK2 knockdown inhibited tumor initiation of MDA-MB-231 cells (1x10⁶ cells/injection) in NOD/SCID mice (n=5⁺; P=0.058). *the siCtrl group had n=4 as one mouse was euthanized.
2.3.7 Activated RSK is expressed in patient samples

As we found RSK2 to have the most significant effect on TNBC and TIC survival, we then investigated whether it was also important in patient samples. We assessed RSK2 mRNA levels in 244 breast cancer patients who had not received adjuvant therapy. Patients with high RSK2 expression had significantly worse survival outcomes (Log rank test $P=8.3 \times 10^{-6}$; Cox proportional hazards $P=1.01 \times 10^{-5}$) (Figure 2.7A left). Analysis of the entire cohort of 771 patients showed that interestingly, RSK2 expression was highest in tumors of the basal-like subtype (Figure 2.7A centre) and in those with the highest grade (grade 3; Figure 2.7A right). In support of this data we demonstrated that RSK2 expression levels were consistently significantly higher in TNBC cell lines compared with non-TNBC cell lines (Figure S2.11). It is important to note that while the basal-like subtype is defined by gene expression, the majority of basal-like breast cancers are also triple negative in terms of expression of cell surface receptor proteins (Foulkes et al., 2010).

Finally, we obtained a focused collection of 18 high-grade breast cancers to address whether RSK was active in TNBC and if it correlated with either P-YB-1$^{S102}$ or CD44. P-RSK1/2$^{S221/7}$ was highly expressed in 85% (11/13) of TNBC (Figure 2.7B). Activated RSK significantly correlated with P-YB-1$^{S102}$ ($P=0.0002$, Spearman’s correlation 0.771) (Supplemental Table 4). Furthermore, in more than half the cases CD44 expression tracks with P-RSK1/2$^{S221/7}$ and P-YB-1$^{S102}$ ($P=0.0333$, Spearman’s correlation 0.5031, and $P=0.0109$, Spearman’s correlation 0.5840 respectively) (Table S2.4). In contrast to the high frequency of RSK and YB-1 activation in TNBC, P-RSK1/2$^{S221/7}$ and P-YB-1$^{S102}$ were not expressed in primary normal mammary ducts (Figure 2.7C). To expand this finding, ten additional normal breast tissues were examined and RSK was consistently not expressed (Figure 2.7C two examples shown). We thus conclude that the RSK/YB-1/CD44 pathway is activated in primary TNBC.
Figure 2.7 Activated RSK is expressed in TNBC patient samples.

A) RSK2 mRNA was associated with poor survival in 244 breast cancer cases who had not received chemotherapy (log-rank $P=8.3\times10^{-6}$; Cox proportional hazards $P=1\times10^{-5}$) (left). When examining RSK2 mRNA expression in a cohort of 771 breast cancer cases representing all subtypes it was highest in the basal-like subtype (centre) \(n=771;\)
**P<0.005) and in those of high grade (right) (n=771; P<0.05). B) P-RSK1/2S221/7 was detected in 13/18 aggressive breast tumor samples. P-YB-1S102 and CD44 expression also correlated (Table S2.4). C) Expression of activated RSK and YB-1 was not detected in normal breast tissue. Scale bars represent 100 µm.
2.4 Discussion

Currently, the treatment of TNBC is limited to conventional chemotherapy as targeted therapies are not available. YB-1 is a signature feature of highly aggressive breast cancers, such as TNBC, and is associated with poor clinical outcome and disease relapse (Habibi et al., 2008). It is possible that this is in part, due to the high proportion of TICs in TNBC. YB-1 is an early driver in breast cancer formation and evokes cancer susceptibility by promoting genetic instability leading to tumorigenesis (Davies and Dunn, 2011). We have previously demonstrated YB-1 as a critical regulator of drug resistance and TIC phenotype in breast cancer (To et al., 2010). In the brain, YB-1 overexpression promotes expression of stem-cell genes and prevents differentiation of both normal neural stem cells and also brain tumor-initiating cells (Fotovati et al., 2011). Furthermore, YB-1 promotes castration-resistant prostate cancer cell growth, thus its influence in drug-resistant cell populations extends beyond breast cancer (Shiota et al., 2011a). However, the lack of molecular therapies specific to YB-1 limits the ability to target it directly.

Herein, we have identified that targeting RSK is a novel strategy for specifically inhibiting growth of TNBC but not normal breast epithelial cells. These findings are consistent with those observed by Smith et al. in an ER positive model, MCF-7 cells, where growth suppression was demonstrated following treatment with SL0101 (Smith et al., 2005). Immortalized normal breast cells, MCF-10A cells were also not effected. In this study they did not assess the effects of targeting RSK using a model of TNBC. It is noteworthy that the TNBC models that we assessed were exquisitely sensitive to RSK inhibition, more so than that reported for MCF-7 cells (Smith et al., 2005). For example, Smith et al. reported that loss of RSK1 or RSK2 suppressed tumor cell growth by ~40% (Smith et al., 2005). Yet we show that the growth of TNBC cells is inhibited by 90-100% when RSK is inhibited with RSK2 siRNA, SL0101 or BI-D1870. Importantly, upon subsequent treatments with BI-D1870 cells do not acquire resistance to the compound. Currently, this represents the most striking effect of a kinase inhibitor on TNBC growth.

TNBCs have a higher percentage of TICs - as defined by CD44 expression - compared to other breast cancer subtypes, which could partially account for their increased propensity to relapse (Park et al., 2010). The growth of the CD44+ cells is notably different in that the
number of mitotic figures and the levels of P-histone H3^S10 are considerably higher in those cells compared to the CD44^− cells. One way of explaining this growth advantage is through the activation of signaling in CD44 positive cells by way of an autocrine loop where its ligand hyaluronan is produced. The MDA-MB-231 cells are described as having an autocrine production of hyaluronan, which activates cell signaling through recruitment of RHAMM and ERK, this in turn would engage the MAPK pathway (Hamilton et al., 2007). Consistent with this model, the MEK1 inhibitor PD098059 disrupts this pathway. While the authors attribute the CD44/RHAMM/ERK pathway to increased cell motility which is of course an important aspect of the spread of TICs from the primary site this signaling network would also fuel cellular proliferation and drug resistance. While CD44 is used as a means to isolate tumor-initiating cells it too serves important functions in maintaining cell growth and invasion. Thus, eliminating the TIC subpopulation alongside the rest of the tumor may help overcome the challenge of relapse.

We previously identified the RSK downstream target YB-1 as a critical regulator of a TIC phenotype (To et al., 2010). Knockdown of YB-1 using siRNA in TNBC resulted in decreased growth and mammosphere formation (To et al., 2010). Herein we demonstrate that inhibiting RSK, particularly RSK2, is sufficient to suppress growth in the CD44^+CD24^− population within TNBC. Additionally, the ability to suppress mammosphere formation indicates inhibition of proliferation of TIC-enriched populations. One major challenge in the development of TIC-directed therapies is that many of the critical signaling pathways in TICs are also crucial for normal stem cell survival. Thus, inhibiting these pathways could result in severe toxicity to normal stem cells. We found that RSK inhibition demonstrated specific/increased toxicity to breast TICs when compared to normal hematopoietic stem cells. This is in line with a study by Kang et al. which showed that loss of RSK2 via knockout also had no effect on the hematopoietic stem cell subpopulation (Kang et al., 2009). Collectively these data indicate that RSK inhibition is effective at eliminating breast cancer TICs but unlike conventional chemotherapies, has little effect on normal stem cells. We conclude that RSK is uniquely linked to promoting the proliferation of CD44^+ cells and as such targeting this pathway has important implications in the management of TNBC. Furthermore, CD44 is a cancer stem cell biomarker for many other types of cancer including leukemia (Jin et al., 2006), prostate (Patrawala et al., 2007), pancreatic (Li et al.,
Therefore we propose that RSK inhibitors may be used to inhibit the growth of cancer stem cells from a wide range of tumor types.

RSK2 has therefore become a subject of interest as an emerging therapeutic target (Stratford and Dunn, 2011). In part because, RSK2 has been linked to numerous cancer types (Kang et al., 2010) such as head and neck squamous cell carcinoma (Kang et al., 2010) and those of hematopoietic origin (Kang et al., 2007). More recently, a study to determine therapeutic targets for specific breast cancer subtypes by a siRNA screen identified RSK2 as one of three genes with potential for the TNBC subtype (Brough et al., 2011). Our exciting and timely breakthrough brings forth RSK, and RSK2 in particular, as a druggable molecular target for TNBC. Moreover, RSK inhibitors have the highly desirable property of inhibiting TICs and given this it stands out as a cutting-edge opportunity to potentially improve the treatment of TNBC.

2.5 Experimental Procedures

i) Cell Lines and reagents. SUM149 (Asterand, Detroit, MI) and MDA-MB-231 (ATCC; Manassas, VA) cells were used as models of TNBC and cultured as previously described (Stratford et al., 2008). For high-throughput cell-based screening, breast tumor cells lines were purchased from either Asterand or ATCC and maintained according to the distributor’s instructions. 184htert, immortalized normal breast epithelial cells, were cultured as previously described (To et al., 2010). RSK specific inhibitors SL0101 (Toronto Research Chemicals Inc., North York, ON) was dissolved in methanol (Smith et al., 2005), and BI-D1870, a kind gift from Dr. Ching-Shih Chen was dissolved in DMSO. RSK1 and RSK2 siRNA were obtained from (Qiagen, Mississauga, ON).

ii) siRNA transfections. Cells were transfected with siRNA (20 nM) (Qiagen; Madison, WI) with RNAiMAX (Invitrogen; Burlington, ON) using the fast forward protocol. All experiments were performed once the cells had been transfected for 72 hrs unless otherwise stated and repeated in triplicate.
iii) RNAi screening. Breast tumor cell line models were siRNA screened as described in (Brough et al., 2011), using Cell Titre Glo reagent (Promega) to estimate cell survival after five population doublings. For high-throughput screening and subsequent validation experiments, siARRAY siRNAs (Dharmacon) were used, transfecting cells and estimating cell viability in a 96 well plate format. Validation of screen effects was performed as described in (Brough et al., 2011) with the effect of each siRNA being defined as a normalized percent inhibition (NPI) score. To calculate NPI scores, which scale cell viability effects according to maximal and minimal effects, we defined the maximal inhibitory effect in each cell line as that caused by siRNA targeting PLK1 and the minimal effect as that caused by non-targeting control siRNAs.

iv) Semi-quantitative real-time PCR. RNA was extracted from cells and xenograft tissue (RNeasy mini kit; Qiagen) and converted into cDNA (superscript III; Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed to detect CD44, RSK1, RSK2 and 18s ribosomal subunit using Taqman gene expression assays (Applied Biosystems; Carlsbad, CA, USA).

v) Immunoblot analysis. Immunoblotting was performed as previously described (Wu et al., 2006). Antibodies were used as listed: RSK1; 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), RSK2; 1:500 (Santa Cruz Biotechnology), YB-1; 1:2000 (Cell Signaling Technology, Boston, MA), YB-1; 1:1000 (Epitomics, Burlingame, CA), CD44; 1:1000 (Abcam, Cambridge, MA), Flag; 1:2000 (Sigma, Oakville, ON), P-RSK1/2S221/7; 1:1000 (Invitrogen), P-YB-1S102; 1:1000 (Cell Signaling Technology), P-histone H2AXS139; 1:1000 (Abcam), P-GSK3βS9; 1:1000 (Cell Signaling Technology) α/β-Tubulin; 1:1000 (Cell Signaling Technology), Vinculin; 1:1000 (Upstate, MA) and Pan-actin; 1:1000 (Cell Signaling Technology).

vi) Mammosphere assay. Cells were seeded (SUM149: 20,000 cells/well; MDA-MB-231: 10,000 cells/well) in triplicate in ultra-low adherent 6 well plates (StemCell Technologies; Inc. Vancouver, BC) and grown in MammoCult® (StemCell Technologies) supplemented with hydrocortisone and heparin. Spheres were counted after 7 days. For post-treatment of established spheres, BI-D1870 was added in fresh media to wells after 72 hrs.
vii) Growth and Apoptosis Assays. Cells were seeded (3,000 – 5,000 cells/well) in 96 well plates in replicates of 5 then treated with inhibitors and allowed to grow for a further 72 hrs – 10 days (siRNA refreshed every 3 days). Cells were stained as previously described (Law et al., 2008). Growth and PI uptake was quantified on the Cellomics, ArrayScan VTI. Propidium iodide (1 µg/ml) was added to the medium of unfixed cells and incubated at 37°C for 1 h before analysis. Cell number was quantified based on nuclear staining for DAPI. The average of 20 fields/well was taken to determine cell number and/or fluorescence intensity per replicate. All experiments were thrice repeated. Apoptosis was also analyzed based on: P-H2AX\dS139, PARP cleavage and caspase-3 cleavage which were assessed by immunoblotting.

viii) Annexin V Staining. SUM149 and MDA-MB-231 cells were treated with BI-D1870 and collected at 48 hrs and 72 hrs respectively. Cells were trypsinized and a single cell suspension was obtained. PE Annexin V Apoptosis Detection Kit (BD Pharmingen) was used to detect apoptosis as per the manufacturer’s protocol. Cells were stained for Annexin V (1:20) on ice for 20 minutes then analyzed by flow cytometry.

ix) Clonogenic Assay. SUM149 cells were seeded in a 6 well plate (4x10^5 cells/well) and treated with DMSO or BI-D1870 (10 µM) for 72 hrs. Surviving cells were counted and re-seeded in 6 well plates (1000 cells/well). After 7 days clonal colonies were visualized with crystal violet and manually counted.

x) CD44 Promoter Assay. SUM149 cells were transfected with a CD44 promoter construct, as previously described (To et al., 2010). Cells were treated with BI-D1870 (10 µM) 6 hrs prior to harvest.

xi) Immunofluorescence. Staining for nuclear localization of P-YB-1\dS102 and P-histone H3\dS10 was performed using SUM149 (5,000 cells/well) seeded in a 96-well plate and treated with BI-D1870 at 24 hrs then allowed to grow for a further 48 hrs. Primary antibodies used were P-histone H3\dS10 (Cell Signaling, 1:200), P-YB-1\dS102 (Cell Signaling, 1:100), CD44-PE conjugated and Hoechst33342 dye (1 µg/ml). Secondary antibody used was Alexafluor 488 anti-rabbit. Cells were mounted with Prolong Gold antifade media with DAPI (Invitrogen)
and signal was quantified on the Cellomcs, ArrayScan VTI, as previously described (Law et al., 2008).

\textit{xii) YB-1^{D102} rescue.} SUM149 cells were seeded in 6 well plates (4x10^5 cells/well) and transfected 24 hrs later with 5\mu g 3xflag:EV or 3xflag:YB-1^{S102D} (D102) using FuGene HD (Roche, Laval, QC). After 24 hrs cells were reseeded in 96-well plates (5,000 cells/well). Remaining cells were collected for protein analysis. Plated cells were treated with BID1870 for 72 hrs and then stained for Hoechst33342 and quantified using the Cellomcs ArrayScan VTI.

\textit{xiii) FACS analysis.} A single cell suspension of SUM149 cells was obtained as previously described (To et al., 2010). Cells were stained with CD44-PE conjugated (BD Pharmingen), CD24-FITC conjugated (StemCell Technologies), and 7-aminoactinomycin D (7-AAD) viability dye (BD Pharmingen) and sorted for the top 10\% CD44^+/CD24^- population.

\textit{xiv) In vivo tumor growth xenograft model.} All experimentation involving mice were conducted in accordance with the standard protocol approved by the University Committee on the Use and Care of Animals at the University of British Columbia.

\textit{a) YB-1 over-expressing cells.} Injections were performed using stable cell lines containing either Flag-YB-1 or control empty vector in the MDA-MB-231 created as previously described (To et al., 2010). Cells (500 cells/injection) were resuspended in PBS containing 25\% matrigel (BD biosciences) and injected into the 4\textsuperscript{th} inguinal mammary gland of 6-8 week old female NOD/SCID mice (Charles River). Tumors were measured using a digital caliper and volume was calculated using V = W^2 x L/2. Student’s \textit{t}-test was utilized to assess difference in final tumor volume \textit{in vivo} and data are represented as a mean ±SEM tumor volume at a given time point.

\textit{b) Effect of RSK2 on tumor initiation.} To test the effects of transient RSK2 knockdown on tumor initiation MDA-MB-231 cells were transfected with either RSK2 siRNA or control siRNA for 48hrs. Knockdown was validated using both qRT-PCR and immunoblot analysis. Orthotopic mammary fat pad injections were performed in a similar manner as described above. In order to achieve tumor initiation at a time that would capture the effects
of RSK2 knockdown mice were injected with 1 x 10^6 cells/injection. A tumor was considered to be anything measuring r > 2.5 mm. Student’s t-test was utilized to determine statistical significance between tumor initiation in the Ctrl scramble and the siRSK2 groups.

**xv) Dissociation of tumors.** Mice were humanely euthanized when tumors reached 500 mm³. Tumors were harvested, dissociated mechanically and digested in DMEM containing collagenase/hyaluronanidase (StemCell Technologies) at 37°C for 4 hrs. Red blood cell lysis was performed by incubating the suspension in ammonium chloride. To ensure a single cell suspension the cells were passed through a 40 µM nylon mesh. This was then used to perform flow cytometry, immunoblotting and drug treatment experiments.

**xvi) Flow analysis.** Cultured or xenograft cells were resuspended in FACS buffer (PBS containing 2% FBS and 5mM EDTA then stained with CD44-PE conjugated (BD Pharmingen), CD24-FITC conjugated (StemCell Technologies), and 7-AAD viability dye (BD Pharmingen). Cells were washed once and resuspended at ~10 x 10^6 cells/mL in FACS buffer and collected using a FACS Calibur. Analysis was performed using Flowjo software.

**xvii) RSK2 survival and subtype analyses.** RSK2 mRNA levels were assessed using robust multi-array average (RMA)-normalized Affymetrix HG-U133A or HG-U133PLUS2 microarray data from 771 clinically annotated breast tumors drawn from five breast cancer cohorts as previously described (Lasham et al., 2012). Using the only probe set for RSK2 (RPS6KA3- 203843_at), expression levels were plotted against histological grade and subtype for all 771 patients, followed by one-way analysis of variance and Tukey’s Honestly Significant Difference Test to determine the statistical significance, using the R statistical environment. In addition Kaplan-Meier survival analysis with both log-rank significance tests (Harrington and Fleming, 1982) (comparing above -vs- below RSK2 median expression) and significance tests using Cox proportional hazards models (http://cran.r-project.org/web/packages/survival/) were performed on those 244 patients who had not received any kind of adjuvant treatment. Patients with events ≥ 12 years were excluded, since over 82% of these patients came from a single cohort (Desmedt et al., 2007).

**xviii) Transcript profiling.** RNA was extracted from cell lines and hybridized to Illumina
human-6 v2 chips. Expression profiles were generated for each cell line and compared between subtypes as described in (Brough et al., 2011)

**xix) Immunohistochemistry.** A breast cancer TMA of 18 patients with high-grade infiltrating ductal tumors was obtained and stained. The sections were stained with P-YB-1S102 (1:100), P-RSK1/2S221/7 (1:200), or CD44 (1:100) antibodies. Statistical analyses were performed using JMP version 8.0.2 (SAS Institute Inc). Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients. Differences were considered statistically significant for \( P<0.05 \). Scale bars on images represent 100 µM.

**xx) Hematopoietic Stem Cell Assay.** Hematopoietic stem cell differentiation and growth was assessed following treatment with BI-D1870 (0.015 µM-15 µM) as performed by StemCell Technologies.

**xxi) RSK kinase assays.** RSK kinase assays were conducted with and without BI-D1870 as previously described using 50 mM ATP (Stratford et al., 2008).

**xxii) Statistical analysis.** Unless indicated otherwise, all *in vitro* data is presented as mean ± SD of at least three individual experiments. Significance was evaluated using a paired Student’s *t*-test to assess difference between test and control samples and were considered statistically significant when \( *P<0.05 \) and \( **P<0.005 \).
2.6 Supplementary Data

2.6.1 Supplemental figures

Figure S2.1 CD44 is elevated in a second *ex vivo* Flag-YB-1 cell line relative to Flag-EV control cell line.

Flow cytometry analysis of CD44 expression in a second pair of *ex vivo* cell lines.
Figure S2.2 Effect of DMSO on cell growth in ex vivo cell lines.

DMSO had no significant effect on cell number after 72 hrs growth in monolayer relative to no treatment (No Tx)/media only. There was a significant difference in cell number after 72 hrs between the Flag-EV and Flag-YB-1 cell lines (n=5; *P<0.05).
Figure S2.3 BI-D1870 inhibits RSK activity.

**A)** RSK1 and RSK2 kinase activity is inhibited with BI-D1870 (10 µM) when using a YB-1 peptide including the Ser^{102} residue as the substrate. Similar results were observed using a peptide to S6 kinase, a known RSK substrate. BI-D1870 is more potent against RSK1. **B)** BI-D1870 (10 µM) prevents activation of two downstream RSK substrates P-YB-1^{S102} and GSK3β^{S9}.
Figure S2.4 Growth is preferentially suppressed in triple-negative breast cancer cell lines utilizing three distinct siRNA targeting RSK2.

siRNA against RSK2 decreases viability of TNBC cell lines (red) more consistently than non-TNBC cell lines (black) (n=3). $P<0.002$ TNBC inhibition vs. non-TNBC model inhibition for two different RSK2 siRNA species and $P<0.007$ for the RSK2 siRNA pool, permutation t test. NPI, normalized percent inhibition compared to non-silencing control siRNA and siRNA targeting PLK1.
**Figure S2.5** RSK inhibitors suppress growth and induce apoptosis in triple-negative breast cancer.

**A)** BI-D1870 (10 µM) inhibits growth of SUM149 and MDA-MB-231 cell lines after 72 hrs (n=5; *P<0.05). P-YB-1S102 is decreased at this time point (inset). **B)** SL0101 inhibits
growth of SUM149 cells after 72hrs (n=5; *P<0.05). Immunoblot demonstrates pharmacological inhibition of P-YB-1^{S102}. C) Treatment of SUM149 cells with BI-D1870 induces apoptosis as shown by induction of P-H2AX^{S139} and D) PARP cleavage. E) Apoptosis was also assessed by Annexin-V staining after treatment with BI-D1870 in the SUM149 cells at 48 hrs and F) in the MDA-MB-231 cells at 72 hrs using flow cytometry.
Figure S2.6 BI-D1870 reduces CD44 mRNA.

Treatment of SUM149 cells with BI-D1870 (10 µM) resulted in a decrease in CD44 transcript levels after 48 hrs as quantified by qRT-PCR (n=3; *P<0.05).
**Figure S2.7** CD44+ cells are highly proliferative but inhibiting RSK activity or reducing its expression decrease mammosphere formation and induce apoptosis.

A) CD44+ cells are more actively undergoing cell division and express a higher proportion of mitotic figures and P-histone H3\(^{S10}\) staining when compared non-CD44+ cells (n=5; \(*P<0.05\)).  
B) Sorted CD44+/CD24- SUM149 cells had increased mammosphere-forming ability compared to CD44-/CD24+ cells (n=3; \(*P<0.05\)).  
C) Mammosphere formation in SUM149 cells was inhibited by both BI-D1870 (10 µM) and siRNA against RSK1/2 (n=3; \(*P<0.05\)).  
D) PI uptake was increased in CD44+ compared to CD44- SUM149 cells (n=5; \(*P<0.05\)).
Figure S2.8 Growth response curve for hematopoietic progenitor cells treated with BI-D1870.

Colony formation ability of hematopoietic progenitor cells treated with increasing doses of BI-D1870. IC_{50} for erythroid and myeloid progenitors were 6 µM and 4.6 µM respectively.
Figure S2.9 The effect of RSK inhibition on normal mammary epithelial cells.

A) BI-D1870 dose response on immortalized normal breast epithelial 184htert cells after 72 hrs (n=5; *P<0.05). B) SL0101 (50 µM) inhibited growth of SUM149 cells by 90% (n=5; *P<0.05) but had no effect on 184htert cells (n=5). C) Suppression of RSK using siRNA had no effect on 184htert cell growth after 72 hrs (n=5).
Figure S2.10 Validation of RSK2 knockdown in the MDA-MB-231 cells pre- and post-xenotransplantation.

A) RSK2 knockdown, but not RSK1, resulted in a decrease on CD44 expression in the MDA-MB-231 cells at 96 hrs by immunoblotting. B-C) MDA-MB-231 cells were transfected with RSK2 siRNA for 7, 10 and 14 days. Knockdown was measured by immunoblotting or qRT-PCR (n=3) respectively. D) RSK2 mRNA was measured by qRT-PCR (n=3) in all tumors taken from the mice upon termination of the experiment to confirm RSK2 re-expression.
Figure S2.11 Comparison of RSK2 mRNA expression between TNBC and non-TNBC cell lines.

Box/whiskers plots of RSK2 mRNA expression in triple-negative (TN) and not-TN cell lines. RSK expression levels are significantly higher in TNBC cell lines compared with non-TNBC cell lines based on 4 specific probes (P-values are as indicated). P-values were calculated using Student’s t-test.
2.6.2 Supplemental tables

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Table S2.1 Growth inhibition by two different oligonucleotides against RSK1 and RSK2.

Mean percent growth inhibition (±SD) in two distinct oligonucleotide sequences against RSK1 and RSK2 inhibited growth in the SUM149 cells at 72 hrs (n=5; *P<0.05, when compared to a second unique sequence targeting the same RSK isoform).
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**T-Test Results**

- $x < 0.0001$  
- $0.0001 \leq x < 0.0005$  
- $0.0005 \leq x < 0.001$  
- $0.001 \leq x < 0.005$  

**STANDARD AND SOLVENT CONTROLS**

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**BI-D1870**

Table S2.2 Colony formation ability of hematopoietic progenitor cells treated with increasing doses of BI-D1870.

The ability of hematopoietic progenitors to mature and differentiate was assessed by StemCell Technologies. The bolded box indicates doses where significant impairment of differentiation was observed.
**Tumor Volume (mm$^3$)**

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Table S2.3 *In vivo* tumor volumes following treatment of MDA-MB-231 cells with RSK2 siRNA.

Raw data indicating tumor volumes 20, 24 and 27 days post-injection.
### Table S2.4 Correlation between P-RSK1/2$^{S221/7}$, P-YB-1$^{S102}$ and CD44 in patient samples.

Statistical analyses were performed using JMP version 8.0.2 (SAS Institute Inc). Bivariate correlations between study variables were calculated by Spearman's rank correlation coefficients.

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CHAPTER 3. LUTEOLIN IS A NOVEL P90 RIBOSOMAL S6 KINASE (RSK) INHIBITOR THAT SUPPRESSES NOTCH4 SIGNALING BY BLOCKING THE ACTIVATION OF Y-BOX BINDING PROTEIN-1 (YB-1)

3.1 Overview

Triple-negative breast cancers (TNBC) are notoriously difficult to treat because they lack hormone receptors and have limited targeted therapies. Recently, we demonstrated that p90 ribosomal S6 kinase (RSK) is essential for TNBC growth and survival indicating it as a target for therapeutic development. RSK phosphorylates Y-box binding protein-1 (YB-1), an oncogenic transcription/translation factor, highly expressed in TNBC (~70% of cases) and associated with poor prognosis, drug resistance and tumor initiation. YB-1 regulates the tumor-initiating cell markers, CD44 and CD49f however its role in Notch signaling has not been explored. We sought to identify novel chemical entities with RSK inhibitory activity. The Prestwick Chemical Library of 1120 off-patent drugs was screened for RSK inhibitors using both in vitro kinase assays and molecular docking. The lead candidate, luteolin, inhibited RSK1 and RSK2 kinase activity and suppressed growth in TNBC, including TIC-enriched populations. Combining luteolin with paclitaxel increased cell death and, unlike chemotherapy alone, did not enrich for CD44+ cells. Luteolin’s efficacy against drug-resistant cells was further indicated in the primary x43 cell line, where it suppressed monolayer growth and mammosphere formation. We next endeavored to understand how the inhibition of RSK/YB-1 signaling by luteolin elicited an effect on TIC-enriched populations. ChIP-on-chip experiments in SUM149 cells revealed a 12-fold enrichment of YB-1 binding to the Notch4 promoter. We chose to pursue this because there are several reports indicating that Notch4 maintains cells in an undifferentiated, TIC-like state. Herein we report that silencing YB-1 with siRNA decreased Notch4 mRNA. Conversely, transient expression of Flag:YB-1WT or the constitutively active mutant Flag:YB-1D102 increased Notch4 mRNA. The levels of Notch4 mRNA and the abundance of the Notch4 intracellular domain (N4ICD) correlated with activation of P-RSK1/2S221/7 and P-YB-1S102 in a panel of
TNBC cell lines. Silencing YB-1 or RSK reduced Notch4 mRNA and this corresponded with loss of N4ICD. Likewise, the RSK inhibitors, luteolin and BI-D1870, suppressed P-YB-1$_{S102}$ and thereby reduced Notch4. In conclusion, inhibiting the RSK/YB-1 pathway with luteolin is a novel approach to blocking Notch4 signaling and as such provides a means of inhibiting TICs.

3.2 Introduction

Therapeutic intervention relies on conventional chemotherapeutics for patients with triple-negative breast cancer (TNBC). Since this subtype does not express estrogen receptor (ER), progesterone receptor (PR) or HER2 patients are ineligible for targeted agents to these molecules such as tamoxifen or trastuzumab. Compared to other subtypes, TNBC has an aggressive clinical course and women with this subtype are faced with the highest recurrence and death rates within the first five years after diagnosis, underscoring the imperative need for new treatments (Dent et al., 2007; Liedtke et al., 2008; Marotta and Polyak, 2011; Stratford et al., 2010).

The p90 ribosomal S6 kinases (RSK), particularly RSK1 and RSK2, are associated with breast cancer growth. This family of serine/threonine kinases is part of the MAPK pathway and is responsible for activating a wide range of substrates involved in cell proliferation, motility and survival (Carriere et al., 2008; Stratford et al., 2008). Moreover, RSK signaling deregulation may play a role in pre-neoplastic progression to neoplastic disease (Davies and Dunn, 2011). RSK1 is primarily known for its role in promoting cancer cell invasion and metastasis (Larrea et al., 2009; Smolen et al., 2010). Importantly, RSK2 has recently been identified as a lead molecular target for TNBC (Brough et al., 2011; Stratford et al., 2012). In an unbiased, genome-wide screen for breast cancer subtype-specific inhibitors, RSK2 was one of only three molecules found to be important for sustaining the growth of TNBC (Brough et al., 2011). Building on this, we demonstrated that suppressing RSK2 inhibited growth of TNBC cell lines and delayed tumor initiation in mice, providing the first proof-of-concept for RSK2 inhibitors in TNBC (Stratford et al., 2012). As such, RSK is positioned as a molecular target that could individualize therapy for patients with this breast
cancer subtype. However, currently there are no clinically available RSK inhibitors although a few small molecules have been identified through screening efforts in the past five years (Berghe et al., 2011; Liu et al., 2011; Smith et al., 2007b; Xu et al., 2006). Considering the poor prognosis for patients with TNBC, this new information indicating that RSK2 inhibitors could improve treatment of this disease makes a focus in this area timely.

RSK is the predominant kinase that phosphorylates Y-box binding protein-1 (YB-1) at its Ser\textsuperscript{102} site (Stratford et al., 2008). YB-1 is an oncogenic transcription/translation factor that promotes breast cancer growth and drug resistance. Upon phosphorylation at Ser\textsuperscript{102}, P-YB-1\textsuperscript{S102} translocates to the nucleus and promotes the induction of growth factors such as EGFR, HER2, and the MET receptor as well as the tumor-initiating-cell (TIC)-associated genes CD44 and CD49f (Stratford et al., 2010). Indeed, YB-1 may be a signature feature of aggressive forms of breast cancer. We have determined that YB-1 is associated with relapse and poor survival in all breast cancer subtypes, expressed in 60-70% of the most aggressive subtypes (TNBC and HER2) and is a stronger prognostic marker for breast cancer than those currently used in the clinic (Habibi et al., 2008; Stratford et al., 2010). Since YB-1 and P-YB-1\textsuperscript{S102} expression are tightly associated with cancer recurrence we explored the idea that this is because YB-1 regulates TIC survival. TICs are hypothesized to be at the root of cancer recurrence as they are resistant to chemotherapy and radiation (Creighton et al., 2009; Lee et al., 2011; Li et al., 2008; Phillips et al., 2006; To et al., 2010). TICs, by definition, have an increased capacity to initiate tumor formation when transplanted into immunocompromised mice (Al-Hajj et al., 2003). They make up a subset of the entire tumor ranging from 10%-60% and can be enriched through flow cytometry sorting for cells with CD44\textsuperscript{+}/CD24\textsuperscript{-}/ESA\textsuperscript{+}/CD49f\textsuperscript{-} surface marker phenotype and also through non-adherent mammosphere culture conditions (Al-Hajj et al., 2003; Fillmore and Kuperwasser, 2008; Harrison et al., 2010; Meyer et al., 2010). TIC expression correlates with high-grade tumors, is associated with distant metastases and TICs have been detected in circulating tumor cells from women with breast cancer (Balic et al., 2006). Further, the CD44-associated gene signature is predictive of poor survival (Shipitsin et al., 2007). To support the role of YB-1 in regulating a TIC phenotype, we previously determined that YB-1 binds to the promoter of CD44 and CD49f and induces their expression (To et al., 2010).
Consequently, there is an enhancement of self-renewal and mammosphere growth, as well as an increase in drug resistance in TNBC cells (To et al., 2010). Conversely, silencing YB-1 decreases CD44 expression and sensitizes cells to chemotherapeutics such as paclitaxel (To et al., 2010). Collectively, these data point towards YB-1 as a promising molecular target for the treatment of aggressive forms of breast cancer.

TICs exploit many of the same canonical stem cell signaling networks that regulate normal tissue-specific stem cells. In the mammary gland, the Notch signaling pathway plays an important role in development and cell fate determination (Dontu et al., 2004). The Notch4 isoform in particular has been implicated in mammary stem cells. Notch4 mRNA levels are highest in undifferentiated bipotent human mammary progenitor cells and decrease upon differentiation (Raouf et al., 2008). Aberrant expression of the active intracellular domain of Notch4 (N4ICD) prevents differentiation and ultimately induces mammary carcinomas in mice (Gallahan et al., 1996). In breast cancer cell lines and patient samples, CD44+/CD24- /ESA+-sorted TICs express higher levels of activated N4ICD than their non-TIC counterparts (Harrison et al., 2010). Conversely, expression of Notch1 intracellular domain (N1ICD) is lowest in TICs indicating differential activation of Notch isoforms between TIC and non-TIC populations. Blocking Notch4 specifically using RNA interference reduces the number of CD44+/CD24-/ESA+ cells, suppresses mammosphere formation and completely inhibits tumor initiation whereas inhibiting Notch 1 has only a modest effect (Farnie et al., 2007; Harrison et al., 2010). Interestingly, YB-1 binds to the promoters of several stem-cell-associated genes including Notch4, yet, YB-1’s role in regulating TICs through Notch4 signaling remains to be explored (Finkbeiner et al., 2009).

However, with no small molecules or drugs to directly inhibit YB-1, we instead sought to block RSK kinase activity and thereby prevent phosphorylation of YB-1. We have demonstrated that inhibiting YB-1 using this approach is effective at eliminating TICs (Stratford et al., 2012). Further, since translating the use of RSK/YB-1 inhibitors into the clinic would be costly and time consuming, we questioned whether existing drugs had RSK-inhibitory activity. As the underlying mechanisms driving carcinogenesis become better understood, repositioning currently approved drugs for the treatment of cancer has become an area of interest (Ashburn and Thor, 2004; Duenas-Gonzalez et al., 2008; Ekins and
Williams, 2011). One of the best examples is the identification of salinomycin, which was derived from a screen of 16,000 off-patent compounds in a search to find new opportunities to inhibit breast TICs (Gupta et al., 2009). In another example of drug repositioning, the anti-diabetic drug metformin was shown to inhibit the growth of breast TICs (Cufi et al., 2012; Vazquez-Martin et al., 2011) and prevent relapse in xenograft models of prostate and lung cancer (Iliopoulos et al., 2011). Disulfiram, a drug used to manage alcoholism, has also been described as being able to kill CD44+ cells in models of breast cancer (Yip et al., 2011). We therefore hypothesized that there may currently be existing compounds that would inhibit the RSK/YB-1 pathway. To this end, we screened the Prestwick Chemical Library of 1120 off-patent drugs in RSK kinase assays and molecular docking. Two major advantages of this drug collection are that 85% of these chemicals are FDA approved and the safety, bioavailability and dosing schedules are established, making the transition from initial screening to drug application more efficient. Herein, we identified luteolin as having novel RSK inhibitory activity with the ability block YB-1/Notch4 signaling and suppress growth in TIC-enriched populations.

3.3 Results

3.3.1 Screening of Prestwick Chemical Library identified potential novel RSK inhibitors

We screened the Prestwick Library consisting of 1120 chemicals at 10 µM in an *in vitro* RSK1 kinase assay against a YB-1 peptide containing the Ser102 site. The YB-1 peptide was selected because it was previously characterized for binding to RSK1 using *in vitro* kinase assays (Stratford et al., 2008) and through molecular docking (Law et al., 2010). Thirty-two compounds were identified that inhibited RSK1 kinase activity >20% at 10 µM (Table S3.1). When compared to the short list from the *in silico* screen (including the 25 strongest predicted binders), 3 compounds were indicated in both screens: kaempferol, luteolin and apigenin (Table 3.1 and Table S3.1). The molecular docking screen theoretically identifies compounds that would inhibit RSK kinase activity using Glide and ICM docking software which consistently rank the highest in terms of docking scoring and accuracy (Chen et al.,
A crystal structure of RSK1 bound to ATP in the N-terminal kinase domain (2Z7Q.pdb) was used to predict that kaempferol, apigenin, luteolin bind to the kinase in its active conformation. Importantly, using this RSK1/ATP structure, kaempferol, apigenin and luteolin were predicted to bind to RSK1 at Leu$^{144}$ and Asp$^{142}$, both of which are the major sites for ATP binding in the NTKD (Table 3.1) (Gussio et al., 2010). Apigenin and luteolin were also predicted to bind to Gln$^{70}$. Relative to all of the drugs in the Prestwick Library, apigenin and luteolin ranked in the top ~1%, scoring higher than kaempferol (Table 3.1). The docking results were independently confirmed against two additional RSK1 structures in active conformations, RSK1 co-crystallized to staurosporine, and purvalanol A (Table S3.2). Taken together, we used biochemical screens and computational docking to short-list three agents that inhibited RSK at the NTKD. Kaempferol, apigenin and luteolin are all flavonoid analogues with remarkably similar structure, sharing a common backbone and differing only in hydroxy group location (Table 3.1). Kaempferol has known RSK inhibitory activity (Xu et al., 2006) and therefore it served as an unbiased internal control.
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$^1$the ranking of the compound among the 1120 Prestwick Chemical Library

$^2$the percentage of the compound among the 1120 Prestwick Chemical Library

Note: that the agents identified ranked in the top 1% of potential binders

**Table 3.1 Molecular docking supports ability of drugs to block RSK1 activity.**

Binding models for the lead compounds in relationship to the RSK1 NTKD. The RSK1 structure was obtained by co-crystallization with ATP. The major binding sites for ATP are Leu$^{144}$ and Asp$^{142}$. Notably kaempferol, apigenin and luteolin all bind to these sites. Luteolin and apigenin also bind to Gln$^{70}$ and Thr$^{204}$ while kaempferol binds to Asp$^{205}$. The binding mode and theoretical H-bonds are shown as well as the Glidescore and rank of the lead compounds in the Prestwick Library.
Following the RSK1 screen, a broad dose-response study (0.001-100 µM) was conducted against RSK2 using the YB-1 peptide as a substrate in cell-free assays (Table 3.2). Each of the agents inhibited its activity with similar IC₅₀ values ranging from 1.71-4.77 µM. BI-D1870 was included as a positive control as it is known to inhibit RSK1 and RSK2 (Sapkota et al., 2007) (Table 3.2). To further validate these data, the same range of concentrations was assessed using a secondary RSK substrate, the S6K peptide, in both the RSK1 and RSK2 kinase assays (Table S3.3A-B). Similarly, all three compounds inhibited the kinase activity of both isoforms in the low micromolar range. While the flavonoids were less potent than BI-D1870, they are favored because they are commercially available as dietary supplements and their safety/toxicity profiles are established (Ross and Kasum, 2002). Conversely, BI-D1870, while it is a potent RSK inhibitor, has never been tested in animals or humans to our knowledge.
### Table 3.2 Kaempferol, apigenin and luteolin block RSK2 kinase activity.

The lead candidates (0.001 μM-100 μM) were analyzed in an *in vitro* RSK2 kinase assay against the YB-1 peptide as the substrate (n=3) performed by SignalChem. The IC₅₀ for each was determined. Chemical structures for these candidates are shown. BI-D1870 was used as a positive control.
3.3.2 Luteolin prevents activation of YB-1 and subsequently inhibits TNBC proliferation, anchorage-independent growth and mammosphere formation

In the secondary screens, we investigated the lead compounds for their ability to block P-YB-1\(^{S102}\) in the TNBC cell line SUM149. In the absence of the inhibitors, activated YB-1 was present in the nucleus (Figure 3.1A). When cells were treated with the lead compounds, P-YB-1\(^{S102}\) immunofluorescence was diminished compared to the DMSO control (Figure 3.1A). Additional images are provided to illustrate that this was a general effect of the inhibitors (Figure S3.1). Further, immunoblotting confirmed that P-YB-1\(^{S102}\) was decreased with drug treatment (Figure 3.1B). The blots were scanned, normalized to αβ-tubulin and quantified. Luteolin inhibited P-YB-1\(^{S102}\) by ~80% at 24 hrs (Figure 3.1C). We next evaluated the YB-1 downstream target, CD44, by qRT-PCR. This target is particularly important, as it has been shown to be associated with a TIC signature ([Stratford et al., 2010]; plus references therein). All of the lead compounds decreased CD44 transcript levels (Figure 3.1D). These results paralleled those of an established RSK inhibitor, BI-D1870.
**Figure 3.1 Lead compounds block activation of YB-1.**

**A)** Drug treatments (10 µM) reduced nuclear P-YB-1S102 based on changes in immunofluorescence after 24 hrs. Scale bar represents 15 µm. **B)** Following drug treatment (10 µM/24 hrs), cell lysates were analyzed by immunoblotting for P-YB-1S102. BI-D1870 was used as a positive control. **C)** Immunoblot was scanned and the P-YB-1S102 band intensities were normalized to αβ-tubulin. **D)** The YB-1 downstream target and TIC marker, CD44, transcript levels were reduced with drug treatments (10 µM/48 hrs) (n=3; *P<0.05).
We recently published that inhibiting RSK and thereby blocking the activation of YB-1, leads to decreased growth in TNBC (Stratford et al., 2008; Stratford et al., 2012). We therefore assessed lead compounds for growth effects in models of TNBC (SUM149 and MDA-MB-231 cells). In these cells, monolayer growth was significantly decreased with 10 µM luteolin (Figure 3.2A). At 100 µM, kaempferol and apigenin were added to this list. No growth effect was observed in normal immortalized mammary epithelial cells (184hterTs) at 10 µM (Figure S3.2). We then tested kaempferol, apigenin and luteolin in a soft agar assay at 10 µM. These three compounds all showed significant inhibition of colony formation under anchorage-independent conditions in at least one cell line with luteolin significantly inhibiting colony formation in both (Figure 3.2B). We next assessed the compounds in mammosphere assays. Anoikis-resistant cells have increased tumor-initiating capacity in vivo validating this culture technique as a method of enriching for TICs (Harrison et al., 2010). SUM149 mammosphere formation was significantly inhibited in the presence of 10 µM, apigenin or luteolin (Figure 3.2C). Kaempferol reduced the number of mammospheres formed by about 50% in the MDA-MB-231 cells but had limited effect on SUM149 mammospheres. To address this seemingly discordant result, we questioned whether kaempferol would inhibit mammosphere formation upon serial passaging, which it did (~50%) by the tertiary passage when compared to the number of DMSO-treated primary mammospheres (Figure S3.3A). Alternatively, we tested whether daily dosing would improve kaempferol’s ability to inhibit mammosphere formation, as this drug may be less stable in this cell culture assay and found that this protocol also inhibited mammosphere formation (Figure S3.3B). We next asked whether these compounds could inhibit growth of mammospheres once they were already established. Apigenin and luteolin showed a marked reduction in the number of mammospheres in SUM149 cells and MDA-MB-231 cells (Figure S3.3C-D). Kaempferol however, had little effect on mammospheres once established in both cell lines supporting the idea that although they share similar backbones, differences in hydroxy groups between the compounds alters the structure-activity relationship regarding RSK inhibitory potential (Table 3.2, Figure S3.3C-D). Likewise, the positive control (BI-D1870) inhibited mammosphere formation and colony growth in soft agar (Figure 3.2B-C and Figure S3.3C-D).
Figure 3.2 Lead compounds inhibit growth in TNBC models and CD44+/CD24- cells.

A) SUM149 (n=5) and MDA-MB-231 (n=5) cells were treated with 10 µM or 100 µM of drug. After 72 hrs, the number of cells was counted and normalized to the DMSO control.
B) The soft agar assay was established with drugs (10 µM) added to the top layer at the time of seeding (n=3). Colonies were counted at 28 days. C) Lead compounds (10 µM) were assayed in mammosphere conditions, which enrich for tumor-initiating cells. Mammospheres formed (n=3) were counted after 7 days and expressed as a percent relative to DMSO control. D) CD44+/CD24−-sorted SUM149 cells were treated with lead compounds (10 µM or 100 µM) in monolayer (n=5) and E) mammosphere (n=3) conditions as described above. BI-D1870 (10 µM), a known RSK inhibitor, was used as a positive control in all assays. Student’s t-test was used to determine statistical significance (*P<0.05, **P<0.005).
3.3.3 Luteolin blocks growth of TIC-enriched populations and primary relapsed TNBC cells

SUM149 cells were sorted for CD44+/CD24− TIC-enriched fractions as described (Al-Hajj et al., 2003; Fillmore and Kuperwasser, 2008) and subsequently treated with the lead drugs and evaluated by immunofluorescence for P-YB-1S102 (Figure S3.4). Consistent with unsorted cells, P-YB-1S102 was predominantly expressed in the nucleus of the DMSO treated cells, yet each of the drugs causes marked elimination of it from the nuclear compartment (for additional images see Figure S3.5). Further, the lead agents significantly inhibited monolayer growth of CD44+/CD24− cells after 72 hrs (Figure 3.2D). The number of mammospheres formed by CD44+/CD24− cells was reduced by treatment with luteolin and apigenin but not kaempferol (Figure 3.2E). Since differentiation of CD44+/CD24− sorted populations occurs rapidly, cells were seeded into growth assays immediately after sorting. The control agent BI-D1870 also suppressed P-YB-1S102 and growth of CD44+/CD24− cells in monolayer and in mammosphere cultures (Figure 3.2D-E, Figure S3.4-S3.5).

When the results from all levels of screening were taken collectively, luteolin was identified as the lead candidate as it: 1) ranked in the top ~1% out of 1120 chemicals in the in silico RSK1 docking, 2) interacted with critical ATP binding residues in the RSK1 NTKD, 3) demonstrated ~80% knockdown of P-YB-1S102 protein at 24 hrs, 4) suppressed growth of both TNBC cell lines in monolayer, soft agar and mammosphere culture conditions and 5) inhibited growth of CD44+/CD24− cells in monolayer and in mammospheres. As such, luteolin underwent further evaluation. Two additional RSK substrates, P-GSK3βS9 and P-S6S236, were also confirmed reduced after treatment with luteolin at 24 hrs (Figure 3.3A). Additionally, luteolin’s inhibition of P-YB-1S102 was dose-dependant (Figure S3.6). Since luteolin has been shown to interact with proteins across several biological pathways, we compared the predicted binding of luteolin and RSK to other potential targets (Lin et al., 2008b; Lopez-Lazaro, 2009). When luteolin was docked against 252 known drug targets, RSK ranked highest among the list (Table S3.4). Although luteolin was predicted to bind to other targets in addition to RSK, some of these “off-target” proteins may have added benefit for cancer therapy as they have also been implicated in cancer survival. For example, KIT was identified as a putative luteolin target. KIT is a cytokine cell-surface receptor that binds
to stem cell factor and has been indicated as an emerging therapeutic target for breast cancer therefore may itself be effective at treating this disease (Stratford et al., 2010).

We next wanted to test the effect of combining luteolin with a front-line chemotherapeutic, paclitaxel. An undesirable effect of paclitaxel is that it activates RSK/YB-1 signaling and subsequently up-regulates CD44 expression (To et al., 2010). Moreover, clinical observation and studies in cell lines have demonstrated that taxanes enrich for CD44⁺/CD24⁻ cells indicating ineffective targeting of this population (Fillmore and Kuperwasser, 2008; Li et al., 2008). Conversely, our data suggest that luteolin actually suppresses growth of CD44⁺/CD24⁻ cells. Luteolin has also been reported to be a chemosensitizing agent (Du et al., 2008; Wu et al., 2008). We therefore hypothesized that the addition of the RSK inhibitor luteolin would not only increase the sensitivity of cells to paclitaxel but also eliminate the CD44⁺ cells. Indeed, we found that the combination of luteolin (50 µM) with paclitaxel (10 nM) significantly increased cell death compared to either drug alone as indicated by PI uptake (Figure 3.3B). Moreover, including luteolin in the regimen prevented activation of P-YB-1 S102 and enrichment of CD44⁺ cells by paclitaxel (Figure 3.3C-D). These data suggest that the addition of a RSK inhibitor such as luteolin to paclitaxel is an effective strategy to improve cell death and reduce the residual CD44⁺ cell burden.
Figure 3.3 Luteolin prevents enrichment for CD44<sup>+</sup> cells by paclitaxel.

A) Luteolin (10 µM) inhibited phosphorylation of RSK downstream targets GSK3β and S6 at 24 hrs as demonstrated by immunoblotting. B) Combining luteolin (10 µM or 50 µM) with paclitaxel (PTX) (10 nM) increased PI uptake (n=5). SUM149 cells were treated for 72 hrs and PI uptake was assessed using Cellomics ArrayScan VTI. C) Adding luteolin (10 µM or 25 µM) to paclitaxel (PTX) (1-10 nM) treatment prevented the induction of P-YB-1<sup>S102</sup> (n=5) and D) CD44 (n=5) by paclitaxel in SUM149 cells. Cells were treated for 72 hrs. (**P<0.005)
Extending these findings to primary TNBC, we tested the efficacy of luteolin in the x43 cell line, derived from a patient who suffered relapse thus suggesting this may be an aggressive case. Subtype classification was confirmed by NanoString (Figure S3.7). The x43 cells had low levels of ER, PR and HER2 mRNA when compared to HER2 over-expressing (HR6) cells and had marker expression that was similar to two TNBC cell lines (HCC1143 and MDA-MB-231). Moreover, the x43 cells may represent a basal-like breast cancer as they express EGFR, Keratin 5 and Keratin 6A (Figure S3.7). Treating x43 cells with luteolin suppressed growth by up to ~90% at 50 µM (Figure 3.4A). A similar effect was seen with positive control BI-D1870. Growth inhibition corresponded to a reduction in P-YB-1S102 beginning at 24 hrs at 10 µM (Figure S3.8). Luteolin induced cell death at these concentrations as indicated by PI uptake (Figure 3.4B). Moreover, x43 cells were exquisitely sensitive to RSK inhibition in non-adherent conditions, as luteolin completely blocked the ability to form mammospheres at 10 µM (Figure 3.4C-D).
Figure 3.4 Luteolin inhibits growth and induces apoptosis in primary human TNBC.

A) Treating the primary relapsed human TNBC cell line x43 with luteolin (10 µM or 50 µM) suppressed growth by ~90% at 72 hrs. BI-D1870 (10 µM) was used as a positive control (n=5; **P<0.005). B) Treating x43 cells with either luteolin (10 µM or 50 µM) or BI-D1870 (10 µM) for 72 hrs increased PI uptake (n=5; **P<0.005). C) Luteolin (10 µM or 50 µM) and BI-D1870 (10 µM) inhibited mammosphere formation counted at 7 days (n=3; **P<0.005). D) Representative pictures of mammospheres. Scale bar = 200 µm.
3.3.4 YB-1 regulates Notch4 expression and can be abrogated through RSK inhibition using luteolin

To begin to understand the mechanism by which RSK inhibitors elicited an effect on TIC-enriched populations we identified putative YB-1 target genes using ChIP-on-chip assays. These studies revealed that YB-1 binds to the promoters of several TIC-associated genes. Most notably, we found a 12-fold enrichment of YB-1 binding to the Notch4 promoter (Finkbeiner et al., 2009). Reports from others demonstrate that Notch4 signaling is elevated in CD44⁺/CD24⁻ cells and that inhibiting this pathway reduces mammosphere formation and prevents tumor initiation in vivo, identifying Notch4 as a critical regulator of breast cancer TICs (Farnie et al., 2007; Harrison et al., 2010). The prominent role of Notch4 in TICs hinted that luteolin’s efficacy against CD44⁺/CD24⁻ cells may be through inhibition of YB-1 and thereby suppression of Notch4. To confirm our ChIP-on-chip results, we tested the effect of YB-1 knockdown on all of the Notch isoforms (Notch1-Notch4). Knockdown of YB-1 using three different siRNAs increased Notch1 mRNA and decreased Notch4 mRNA with no effect on the Notch2 or Notch3 isoforms in SUM149 cells (Figure 3.5A). Conversely, over-expression of either wild-type YB-1 (Flag:YB-1WT) or a constitutively active mutant YB-1 (Flag:YB-1D102) in SUM149 cells increased levels of Notch4 mRNA (Figure 3.5B, Figure S3.9 for control blot). Interestingly, when comparing a panel of TNBC cell lines (SUM149, MDA-MB-231 and x43) the level of Notch4 mRNA and cleaved, activated, intracellular domain (N4ICD) correlated with the levels of P-YB-1S102 and P-RSK1/2S221/7 (Figure 3.5C-D).
Figure 3.5 Notch4 transcript levels are reduced by blocking YB-1 signaling and correlate with P-YB-1S102 and P-RSK1/2S221/7.

A) Inhibiting YB-1 using siRNA reduced Notch4 mRNA levels. SUM149 cells were transfected with three distinct siRNA’s against YB-1 and all four Notch isoforms were assessed using quantitative real-time PCR 96 hrs after transfection (n=3). B) Transient transfection of SUM149 cells with either Flag:YB-1WT or Flag:YB-1D102 plasmids for 96 hrs increased Notch4 levels compared to control empty vector (n=3; **P<0.005). C) Expression of Notch4 mRNA (n=3; **P<0.005) and D) active intracellular domain (N4ICD) correlates with P-YB-1S102 and P-RSK1/2S221/7 in a panel of TNBC cell lines (SUM149, MDA-MB-231, primary x43).
Building on the idea that the RSK/YB-1 pathway regulates Notch4 signaling we investigated the levels of Notch4 after YB-1 knockdown in the MDA-MB-231 cells (since this cell line expresses more Notch4 than the SUM149, Figure 3.5C-D). Reducing YB-1 using siRNA decreased Notch4 mRNA and correspondingly decreased N4ICD levels (Figure 3.6A). Similarly, knockdown of either RSK1 or RSK2 also reduced Notch4 mRNA and N4ICD (Figure 3.6B). This effect was further demonstrated in a second cell line (x43) where knockdown of either RSK1, RSK2 or YB-1 decreased Notch4 transcript (Figure S3.10). The RSK inhibitors BI-D1870 and luteolin paralleled these results and significantly reduced Notch4 mRNA in both the MDA-MB-231 and the x43 cell lines (Figure 3.6C-D). Thus, we conclude that RSK inhibition decreases Notch4 signaling by suppressing P-YB-1S102 (Figure 3.6E).
Figure 3.6 Inhibiting YB-1, RSK1 or RSK2 or blocking activation of YB-1 with RSK inhibitors repressed Notch4 mRNA and N4ICD.

A) YB-1 knockdown using siRNA reduced Notch4 transcript levels (n=3; **P<0.005) in MDA-MB-231 cells at 48 hrs and decreased N4ICD protein levels by 72 hrs. B) Knockdown of RSK1 or RSK2 reduced Notch4 transcript levels (n=3; **P<0.005) in MDA-MB-231 cells at 48 hrs and decreased N4ICD protein levels by 72 hrs. C) RSK inhibitors luteolin (10 µM or 50 µM) and BI-D1870 (10 µM) also decreased expression of Notch4 transcript in MDA-MB-231 cells (n=3; **P<0.005) and D) primary x43 cells at 48 hrs (n=3; **P<0.005). E) A schematic diagram of luteolin and BI-D1870 inhibiting the RSK/YB-1/Notch4 pathway.
3.4 Discussion

Herein, we identified the off-patent compound luteolin, has the novel ability to block RSK/YB-1/Notch4 signaling and thereby inhibit TNBC growth including TIC-enriched populations. Since RSK has recently been identified as a TNBC-specific target, we focused on screening for compounds that have the ability to block RSK activity. We used a dual approach of high-throughput and virtual screening, as these are complementary methods that can be integrated to improve inhibitor discovery (Bajorath, 2002). Notably, both screening techniques identified kaempferol, luteolin and apigenin that inhibited RSK1 and RSK2 at micromolar concentrations. Subsequent experiments identified luteolin as the lead compound as it suppressed growth in TNBC and inhibited RSK in cells. Consequently, it reduced phosphorylation of YB-1 and decreased Notch4 signaling, both of which are key pathways in sustaining TICs.

RSK2 is an emerging therapeutic target for developing treatments for TNBC, for which there are currently no targeted therapies available (Brough et al., 2011). Our group identified that RSK2 specifically has the most potent inhibitory effect on growth in TNBC (Stratford et al., 2012). Furthermore, we propose that RSK inhibitors could have applications beyond breast cancer to include other tumors that express high RSK2 such as those that develop in the lung, head and neck, prostate and hematopoietic system (Stratford and Dunn, 2011). Several other groups have demonstrated that the RSK2 isoform appears to be the most relevant in cancers (Kang and Chen, 2011). In an effort to identify new RSK2 specific inhibitors, Liu et al. identified eriodictyol through molecular docking (Liu et al., 2011). Interestingly, eriodictyol is a flavonoid that is structurally very similar to, luteolin, apigenin and kaempferol. Similarly, Berghe et al. found the flavanone, lavandulyl, to attenuate the ERK/RSK2 pathway suggesting that there is a structural basis for flavonoids in inhibiting RSK2 (Berghe et al., 2011). Flavonoids have also been indicated as promising anti-cancer agents in recent years. Additionally, they have shown evidence of having antioxidant, anti-bacterial, anti-viral, anti-inflammatory, and anti-cancer beneficial health effects (Wang, 2000). These naturally occurring compounds are therefore attractive starting points for synthesizing analogues that have higher selectivity and potency. Herein, we identified the flavones luteolin and apigenin with remarkably similar structure had
significant growth effects on TNBC. Kaempferol, which is also a flavonoid, served as the basis for making SL0101, an agent that is now utilized as a RSK inhibitor for research purposes (Smith et al., 2007b). Thus, kaempferol served as an excellent unbiased control in this study. The similarity in structure of all the compounds and the fact that they were identified in both in vitro kinase assays and in silico docking to an ATP-bound RSK crystal structure is indicative of a structure-activity relationship for these compounds as ATP competitive inhibitors against RSK. Accordingly, we demonstrate that luteolin blocks phosphorylation of a synthetic YB-1 peptide by RSK1 and RSK2 as well as phosphorylation of YB-1 in cancer cells and its nuclear localization. It also suppresses growth in TNBC models. Luteolin has previously been shown to have anti-cancer properties such as suppressing cell survival pathways while promoting apoptosis (Lin et al., 2008b; Lopez-Lazaro, 2009). This compound is found naturally in many fruits and vegetables and thus is considered safe for consumption (Lin et al., 2008b; Lopez-Lazaro, 2009). In vivo experiments have also demonstrated low toxicity with long-term treatment (Lin et al., 2008b). Moreover, it is sold commercially as LutiMax, a nutraceutical that has reported benefits not only for cancer but also for other disorders ranging from inflammation to neurologic conditions such as autism. The recommended dosing of LutiMax is 400-600 mg/day with no reported toxicities. It is not surprising that LutiMax is well tolerated because of the abundance of luteolin in fruits and vegetables. The commercial availability of luteolin sold as LutiMax potentially provides a means of translating our research to patients.

Luteolin is documented to alter several biological pathways (Lin et al., 2008b; Lopez-Lazaro, 2009). To attempt to identify other putative binding proteins, luteolin was docked to a library of 252 known drug targets. RSK scored highest within this library with the strongest predicted binding to luteolin. While we do not disregard that some of luteolin’s anti-cancer effects may be through alternate signaling pathways, we do posit that its activity as a RSK inhibitor could have a particularly significant effect in the context of TNBC given that RSK signaling is critical to the survival of this breast cancer subtype (Stratford et al., 2012). Moreover, some of the other signaling pathways that luteolin affects could be linked to RSK inhibition. Several studies show that luteolin inhibits NF-κB signaling and sensitizes cells to tumor necrosis factor (TNF)-induced apoptosis (Chen et al., 2007; Ju et
Interestingly, RSK regulates NF-κB signaling through IκB kinase (IKK), which phosphorylates IκB, targeting it for degradation and thereby allowing NF-κB to translocate to the nucleus and transcribe anti-apoptotic genes (Romeo et al., 2012). Therefore, inhibition of RSK kinase activity presents one feasible mechanism by which luteolin could inhibit NF-κB signaling. Furthermore, some of the putative targets we identified for luteolin also play a role in cancer, particularly in TNBC. KIT is co-expressed with EGFR and is associated with BRCA1-mutation carriers and in sporadic basal-like breast cancer (Lim et al., 2009; Nielsen et al., 2004). Perhaps, by targeting several biological pathways, luteolin could prevent the development of acquired resistance in cancer cells that can occur when cells circumvent the requirement for single pathways targeted by highly specific inhibitors.

TICs present a major obstacle in developing effective cancer treatments as many conventional therapies actually enrich for CD44+ cells (Creighton et al., 2009; Li et al., 2008; Phillips et al., 2006). However, reducing YB-1 expression or preventing its activation via RSK inhibition, are both effective strategies for reducing the TIC burden (Stratford et al., 2012; To et al., 2010). As such we investigated whether luteolin could suppress growth in CD44+/CD24- cells through inhibition of the RSK/YB-1 pathway. Additionally, since luteolin suppresses cell survival mechanisms and induces apoptosis in cancer cells we speculated that it might work as a chemosensitizing agent in conjunction with chemotherapeutics. It has demonstrated this property in other cancers including in gastric cancer when used in combination with cisplatin (Wu et al., 2008). Herein, we demonstrate that the addition of luteolin to paclitaxel increased cytotoxicity in TNBC. Importantly, unlike paclitaxel alone the combination of the two compounds did not enrich for CD44+ cells. Moreover, luteolin suppresses growth, induces apoptosis and inhibits mammosphere formation in primary human TNBC cells obtained from a patient that relapsed demonstrating that luteolin has activity against an aggressive cancer cell line. TICs also play an important role in mediating drug resistance in other breast cancer subtypes. In mice, the addition of luteolin reversed doxorubicin resistance in MCF-7 and 4T1 cells. It also remarkably enhanced the effect of doxorubicin on tumor suppression (Du et al., 2008). In this study, luteolin was actually safer as a single agent and more effective than doxorubicin (Du et al., 2008). We have shown that RSK and YB-1 are up-regulated in trastuzumab-resistant cell lines HR5 and HR6 when compared to their sensitive counterpart BT474.
Expression of an active mutant YB-1D102 induced expression of CD44 and conveyed trastuzumab insensitivity to BT474 cells. Conversely, reducing CD44 in HR5 and HR6 cells restored sensitivity to trastuzumab. Thus, combining luteolin with currently used chemotherapeutics may present an effective strategy for eliminating TICs across several breast cancer subtypes.

The Notch family of transmembrane receptors, particularly Notch4, has been implicated in mammary stem/progenitor cell self-renewal and expansion (Dontu et al., 2004). In both the normal mammary gland and breast cancer, Notch4 signaling maintains an undifferentiated stem/progenitor-like state (Farnie et al., 2007; Gallahan et al., 1996; Harrison et al., 2010; Raouf et al., 2008). Previous experiments revealed that YB-1 binds to the promoter of Notch4 and increases its expression (Finkbeiner et al., 2009). Herein, we show that inhibiting RSK/YB-1 signaling with siRNA or small molecules reduces Notch4 levels and activation. Harrison et al. demonstrate that Notch4 signaling is highest in TICs whereas Notch1 signaling is highest in non-TIC fractions (Harrison et al., 2010). Interestingly, YB-1 knockdown mimics this pattern of expression and reduces Notch4 mRNA while increasing Notch1 mRNA levels. Constitutive Notch4 signaling promotes an aggressive malignant phenotype in MDA-MB-231 cells increasing vascularization and growth of xenograft models (O'Neill et al., 2007). Conversely, inhibiting Notch4 using antibodies specific to this isoform is more effective at suppressing mammosphere formation than γ-secretase inhibitors which inhibit all Notch isoforms (Notch1-Notch4) indicating that the Notch4 isoform specifically, is important for mammosphere forming ability (Farnie et al., 2007). Furthermore, the γ-secretase inhibitor, MRK003, was recently shown to inhibit tumor initiation in mice using an ERBB2 model of mammary tumorigenesis and mice treated with MRK003 had durable long-term relapse free survival (Kondratyev et al., 2012). Collectively, these data point to an essential role for Notch4 in cancer recurrence through the maintenance of TICs.

We conclude that drug repositioning can be used to identify agents for molecular targets such as RSK. We identify luteolin as having the novel ability to inhibit RSK/YB-1 activation and suppress Notch4 signaling. The discovery of RSK-specific inhibitors that can be fast-tracked into clinic may have significant implications for treating TNBC, where the
disease is aggressive and targeted therapies are unavailable. This is an important advance because luteolin inhibits RSK activity and is commercially available as LutiMax. This preclinical study provides the rationale for addressing the potential for luteolin for the treatment of TNBC in a clinical trial setting.

3.5 Experimental Procedures

i) Initial RSK1 kinase screens and chemicals. For RSK1, the entire Prestwick Chemical Library (1120 chemicals; Canadian Chemical Biology Network at the University of British Columbia) was screened by SignalChem (Richmond, BC) in a kinase assay at 10 µM against a YB-1 peptide, PRKYLRSVG, (Law et al., 2010) as previously described (Stratford et al., 2008). This peptide contains the YB-1 S102 site. Results were compared to a staurosporine control, a broad-spectrum kinase inhibitor that has 100% activity at 10 µM. Compounds with >20% inhibitory activity were considered to be significant RSK1 inhibitors. Kaempferol, apigenin and luteolin were purchased from Sigma-Aldrich Chemical (Oakville, ON) and were dissolved in DMSO (Sigma) to stock concentrations of 100 mM. BI-D1870, a known RSK inhibitor (Sapkota et al., 2007), was synthesized by the Center for Drug Research and Development (Vancouver, BC).

ii) In silico RSK1 screens. In silico analysis was performed on lead compounds using the molecular docking program Glide (Friesner et al., 2004; Halgren et al., 2004). The docked poses were ranked based on docking score. The Glide docking was performed as follows: For the three crystal structures of N-terminal domain, the cognate ligands were used to define the active sites and generate the grid. Both Glide standard precision (SP) and extra precision (XP) modes were used for the docking, and for each ligand, the highest scored pose was written out. We used three different resolved crystal structures of RSK1, all of which are in the active conformation of the N-terminal domain (2Z7Q.pdb, 2Z7R.pdb, 2Z7S.pdb) co-crystallized with different ligands (ATP, staurosporine, and puravalnol A). Other parameters in Glide were kept at the default setting.

iii) RSK2 kinase. Kinase profiling services for RSK2 were provided by SignalChem, as previously described (Stratford et al., 2008). Briefly, the compounds kaempferol, apigenin, luteolin and BI-D1870 were screened in a RSK2 kinase assay at 0.001, 0.01, 0.1, 1.0, 10,
and 100 µM against a YB-1 peptide containing the Ser\textsuperscript{102} site (Law et al., 2010). Results were compared to a staurosporine control. For each compound, a graph of log concentration (µM) versus % inhibition of RSK2 activity was generated and IC\textsubscript{50} values were determined. To confirm inhibition of RSK2 activity, we also repeated the kinase assay with a secondary RSK substrate, S6K.

\textbf{iv) Cell culture.} The triple-negative breast cancer cell lines SUM149 (Asterand, Ann Arbor, MI) and MDA-MB-231 (American Tissue Culture Collection, Manassas, VA) were grown as previously described (Stratford et al., 2008). Primary relapsed TNBC cells, x43, were a generous gift from Dr. John Hassell (McMaster University, Hamilton, ON) and were cultured in RPMI containing 10% (v/v) fetal bovine serum plus 100 units/ml penicillin, 100 units/ml streptomycin and 0.5µg/ml fungizone amphotericin B. All experimentation involving human cells were done in accordance with the Helsinki guidelines and approved through McMaster University ethics committee.

\textbf{v) Immunofluorescence and immunoblotting.} SUM149 cells were plated on 8-well multi-chamber slides (40,000 cells/well) and treated with 10 µM of each lead compound for 24 hrs. Immunofluorescence was conducted as previously described (To et al., 2010) using P-YB-1\textsuperscript{S102} and YB-1 antibodies (Cell Signaling, Danvers, MA) with Alexa-Fluor 488 (Invitrogen, Burlington, ON) secondary antibody. Images were acquired on an Olympus BX61 microscope and analyzed using ImageJ (NIH, Bethesda, MD). For immunoblotting, cell lysates were collected after 24-72 hrs drug treatments or 48-96 hrs siRNA treatment and immunoblotting was performed as described previously (Law et al., 2010; Stratford et al., 2012) using RSK1; 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), RSK2; 1:500 (Santa Cruz Biotechnology), YB-1; 1:2000 (Cell Signaling Technology, Boston, MA), YB-1; 1:1000 (Epitomics, Burlingame, CA), CD44; 1:1000 (Abcam, Cambridge, MA), Flag; 1:2000 (Sigma, Oakville, ON), P-YB-1\textsuperscript{S102}; 1:1000 (Cell Signaling Technology), P-GSK3\beta\textsuperscript{S9}; 1:1000 (Cell Signaling Technology), P-S6\textsuperscript{S236}; 1:1000 (Cell Signaling Technology), Notch4, 1:500 (Santa Cruz Biotechnology), α/β-tubulin; 1:1000 (Cell Signaling Technology), Vinculin; 1:1000 (Upstate, MA) and Pan-actin; 1:1000 (Cell Signaling Technology).

\textbf{vi) Transfections.} To investigate the effect of altering YB-1 on expression of Notch isoforms, SUM149 cells were transfected with three distinct siRNAs against YB-1 or
scramble control (20 nM) for 96 hrs as described in (To et al., 2010). SUM 149 cells were transiently transfected with 4 µg of Flag:EV, Flag:YB-1-WT or Flag:YB-1-D102 plasmids and subsequently harvested after 96 hrs (To et al., 2010). MDA-MB-231 and x43 cells were treated with (20 nM) siRSK1 or siRSK2, (Qiagen, Mississauga, ON) or siYB-1#1 or siYB-1#3 or control scramble (Darmacon, Chicago, Illinis) for 72 hrs. Both RSK1 and RSK2 siRNA’s have been compared to alternate sequences targeting each isoform and found to have comparable knockdown and phenotypic effects (Stratford et al., 2012).

vii) Real-time quantitative reverse transcription PCR. RNA was isolated using RNeasy mini kit (Qiagen, Mississauga, ON) SUM149 cells treated with DMSO or 10 µM of kaempferol, apigenin or luteolin for 48 hrs. BI-D1870 (10 µM) was used as a control. The RNA was reverse transcribed and amplified using CD44 specific primers and probes (Applied Biosystems, Foster City, CA) as previously described (To et al., 2010). Ribosomal mRNA was quantified as a housekeeping gene (Applied Biosystems). Taqman Gene Expression Assays designed for Notch1, Notch2, Notch3 and Notch4 specific primers and probes (Applied Biosystems, Foster City, CA) were used with PPIA (Applied Biosystems, Foster City, CA) as the internal control.

viii) Monolayer, mammosphere and soft agar growth assays. Monolayer growth assays were performed with 5,000 (SUM149) or 3,000 (MDA-MB-231) cells/well/96 well plate. Cells were treated with DMSO, 10 or 100 µM of the drugs and counted by high-content screening as previously described (Law et al., 2008) after 72 hrs. For combination monolayer drug treatments; 5,000 SUM149 cells/well/96 well plate were seeded and treated at 24 hrs with various combinations of luteolin (0, 10 or 25 µM) and paclitaxel (0,1, 5 or 10 nM). Cells were fixed at 72 hrs and stained for P-YB-1S102 (Cell Signaling, Danvers, MA) with Alexa-Fluor 488 (Invitrogen, Burlington, ON) secondary and CD44-PE conjugated (BD Pharmingen, Mississauga, ON) and signal was quantified using Cellomics ArrayScan VTI as previously described (Stratford et al., 2012). Soft agar assays were performed as previously described (Sutherland et al., 2005). Compounds (10 µM) were added at seeding into the top layer and colonies were counted after 28-30 d. Percent change in growth was compared to DMSO control. Mammosphere assays were performed as previously described (To et al., 2010) in MammoCult® media (StemCell Technologies, Vancouver, BC).
Additionally, serial passaging of mammospheres (with fresh kaempferol added with each passage) and daily repeated dosing was conducted with 10 µM kaempferol and spheres were counted after 7 d. Growth and mammosphere assays were performed as described above for primary x43 cells. Cells were seeded at 5,000 cells/well/96 well plate and analyzed at 72 hrs for monolayer growth. For mammosphere assays x43 cells were seeded at 20,000 cells/well and treated with DMSO, 10 or 50 µM or BI-D1870 (10 µM) as a positive control.

ix) **FACS sorting for CD44⁺/CD24⁻ SUM149 cells.** SUM149 cells were sorted for the top 10% CD44⁺/CD24⁻ TICs as previously described (To et al., 2010) using anti-CD44 conjugated to PE (BD Biosciences, Mississauga, ON) and anti-CD24 conjugated to FITC (StemCell Technologies, Vancouver, BC). Immunofluorescence of P-YB-1S102 as well as monolayer and mammosphere assays were performed using the CD44⁺/CD24⁻ TIC-enriched population as described above.

x) **Apoptosis assays.** SUM149 and x43 cells (5,000 cells/well/96 well plate) were treated with DMSO, 10 or 50 µM luteolin, BI-D1870 (10 µM) or combined with paclitaxel (10 nM) for 72 hrs. PI-uptake was quantified using the Cellomics ArrayScan VTI as described in (Stratford et al., 2012).

xi) **NanoString gene expression profiling.** RNA (100-250 ng) from breast cancer cell lines was analyzed using the nCounter Gene Expression Analysis system at the Centre for Translational and Applied Genomics (CTAG) at the BC Cancer Agency (Vancouver, BC). A custom CodeSet containing probes for ER (RefSeq NM_000125.2), PR (RefSeq NM_000926.4), HER2 (RefSeq NM_004448.2), EGFR (RefSeq NM_005228.3), KRT5 (RefSeq NM_000424.2) and KRT6A (RefSeq NM_005554.3) was synthesized by NanoString Technologies (Seattle, WA, USA). All procedures related to mRNA quantification including sample preparation, hybridization, detection, scanning and data normalization were carried out as recommended by NanoString Technologies.

xii) **Statistical analysis.** Unless indicated otherwise, all data is presented as mean ± SD of at least three repeated individual experiments. Significance was evaluated using a paired Student’s t-test, and difference to be considered statistically significant when *P*<0.05 and
**$P<0.005$.**
3.6 Supplementary Data

3.6.1 Supplemental figures

Figure S3.1 Lead compounds block nuclear translocation of YB-1.
Additional immunofluorescence images of SUM149 cells treated with 10 µM of each lead compound for 24 hrs and stained for P-YB-1S102. Scale bar represents 15 µm.
Figure S3.2  The effect of lead compounds on normal immortalized epithelial cells (184hterts).

Growth of 184htert cells was not inhibited by 10 μM of drug candidates at 72 hrs (n=5).
Figure S3.3 The effect of lead compounds on mammosphere formation.

A) The effect of kaempferol (10 µM) on serially passaged mammospheres. SUM149 cells were passaged every 7 days in fresh MammoCult media containing kaempferol (10 µM) (n=3; *P<0.05). The percent change was calculated relative to primary DMSO control. B) The effect of kaempferol on SUM149 mammosphere formation when added daily (10 µM for 7 d) (n=3; **P<0.005). C) The effect of lead compounds on regression of formed mammospheres in the SUM149 and D) MDA-MB-231 cell lines. For this assay, mammospheres were grown for 3 days prior to drug treatment (10 µM) and were counted after a further 4 d (n=3; *P<0.05).
**Figure S3.4 Lead compounds block nuclear translocation of YB-1 in TIC-enriched populations.**

Immunofluorescence images of CD44+/CD24−-sorted SUM149 cells treated with 10 µM of each compound for 24 hrs and subsequently stained for P-YB-1S102 and DAPI. Scale bar represents 15 µm.
Figure S3.5 Lead compounds block nuclear translocation of YB-1 in TIC-enriched populations: Additional immunofluorescence images. CD44<sup>+</sup>/CD24<sup>-</sup>-sorted SUM149 cells treated with 10 µM of each compound for 24 hrs and subsequently stained for P-YB-1<sup>S102</sup>. Scale bar represents 15 µm.
Figure S3.6 Luteolin decreases P-YB-1\textsuperscript{S102} in a dose dependent manner.

P-YB-1\textsuperscript{S102} decreases in a dose-dependant manner upon treatment with luteolin for 72 hrs in SUM149 cells.
Figure S3.7 Classification of x43 primary human TNBC.

Transcript expression of cell surface markers was compared between the x43 cells, HER2 over-expressing cells (HR6) and TNBC cells (HCC1143 and MDA-MB-231) cell lines by NanoString technology.
Figure S3.8 Luteolin and BI-D1870 decrease P-YB-1$^{S102}$ in x43 cells.

Treatment with luteolin (10 µM) or BI-D1870 (10 µM) reduced P-YB-1$^{S102}$ levels. Cell lysates were collected after 24 hrs drug treatment.
Figure S3.9 Control blot validating Flag transgene expression.
SUM149 cells transfected with EV, Flag:YB-1<sup>WT</sup> or Flag:YB-1<sup>D102</sup> demonstrated expression of the transgene at 96 hrs.
Figure S3.10 Knockdown of YB-1, RSK1 or RSK2 reduces Notch4 mRNA in x43 cells.
Primary x43 cells were treated with siYB-1, siRSK1 or siRSK2 for 96 hrs. Notch4 mRNA expression was quantified using qRT-PCR (n=3; *P<0.05).
### 3.6.2 Supplemental tables

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<th>Compound</th>
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* also identified in molecular docking screen
Table S3.1 Results of primary screens determined compounds that had significant inhibition (>20%) of RSK1 kinase activity.

Shaded agents were also identified through ICM molecular docking of the Prestwick compounds to a homology model of RSK1 and were selected for validation in subsequent assays.
Docking using RSK1 N-terminal domain conformation co-crystallized with:

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<td>-7.45  39  3.25</td>
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\(^1\) the ranking of the compound among the 1120 Prestwick Chemical Library
\(^2\) the percentage of the compound among the 1120 Prestwick Chemical Library

Table S3.2 Molecular docking supports the ability of drugs to block RSK1 in additional crystal structures.

Binding models for the lead compounds in relationship to the RSK1 NTKD co-crystallized to staurosporine, and purvalanol A. The Glidescore and rank of the lead compounds in the Prestwick Library are also shown.
### A

<table>
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<th>0.01µM</th>
<th>0.1µM</th>
<th>1.0µM</th>
<th>10µM</th>
<th>100µM</th>
<th>IC50</th>
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Table S3.3 Kaempferol, apigenin and luteolin block RSK1 and RSK2 kinase activity. Lead candidates were screened in the A) RSK1 and the B) RSK2 kinase assay using an alternate RSK substrate, S6K (n=3).
Predicted binding of luteolin to 252 known drug targets

<table>
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<th>Protein</th>
<th>Score</th>
<th>pmfscore</th>
<th>drug rank</th>
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<td>-130</td>
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<tr>
<td>SYK <strong>Spleen tyrosine kinase</strong></td>
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<td>-121.7</td>
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<td>PNMT <strong>phenylethanolamine N-methyltransferase</strong></td>
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**Table S3.4 Predicted binding of luteolin to 252 known drug targets.**

3D structures from protein data bank (PDB) were used to dock luteolin using Glide and ICM. Targets were ranked based on pfm filters and ICM score.
CHAPTER 4. P90 RIBOSOMAL S6 KINASE (RSK)/Y-BOX BINDING PROTEIN-1 (YB-1) SIGNALING IS ELEVATED IN RESIDUAL CELLS AFTER CHEMOTHERAPY AND INHIBITING THIS PATHWAY CAN OVERCOME BROAD-SPECTRUM CHEMORESISTANCE

4.1 Overview

Recurrence and the development of refractory disease continue to present major challenges in treating breast cancer. The main modalities of treating advanced breast cancer include anthracycline and taxane-based chemotherapeutics. Cells that survive chemotherapy are enriched for a CD44⁺/CD24⁻ surface marker phenotype and characterization of this population may provide insight into critical pathways mediating drug resistance. Despite initial response to chemotherapeutics, triple-negative breast cancers (TNBC) paradoxically have the highest relapse rates. Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor abundantly expressed in TNBC (~70% of patients). It is activated predominantly by phosphorylation via p90 ribosomal S6 kinase (RSK) which itself is critical for TNBC survival. Once activated it up-regulates CD44 expression and enhances drug-resistance, implicating P-YB-1S102 may be important for residual cells’ survival.

We observe that P-YB-1S102 is associated with poor overall survival ($P<0.001$) and relapse ($P<0.001$) in a cohort of 1057 patients with invasive breast cancer. We therefore asked whether YB-1 is associated with resistance to taxanes or anthracyclines in breast cancer cell lines. Characterization of residual cells after paclitaxel or epirubicin treatment revealed elevated P-YB-1S102 and CD44, which, was increased via P-RSK1/2S221/7. Moreover, P-YB-1S102 identified cells with a high proliferative index based on co-staining for Ki67. Inhibiting YB-1 suppressed growth in residual cells while pre-emptive suppression of YB-1 sensitized cells to chemotherapeutic treatment with paclitaxel and epirubicin. Through
selective pressure via paclitaxel, we developed a chemoresistant cell line (SUM149-PTX\textsuperscript{R}), which also exhibited increased RSK/YB-1 signaling. Interestingly, SUM149-PTX\textsuperscript{R} cells were not only resistant to paclitaxel, but also exhibited cross-resistance to other chemotherapies, yet inhibiting either YB-1 or RSK induced cell death. Finally, activation of RSK and YB-1 is inversely correlated with response to chemotherapy. This pathway was highly up-regulated in a primary TNBC cell line (x43) derived from a patient that failed chemotherapy. We found the x43 cells were the least sensitive to paclitaxel and epirubicin compared to MDA-MB-231 and SUM149 cells, however, they remained exquisitely sensitive to YB-1 suppression. Collectively, these data indicate that YB-1 conveys broad-spectrum resistance to clinically relevant chemotherapeutic agents and that inhibiting RSK/YB-1 signaling can sensitize cells to chemotherapy, potentially reducing relapse in TNBC.

4.2 Introduction

Despite advances in treating breast cancer, disease recurrence continues to present an ongoing obstacle to curing this disease. Residual cells after treatment are available to drive recurrence and metastatic spread, often generating disease that is refractory to treatment. Thus, characterization of residual populations may provide insight into the cells that are surviving treatment and driving relapse. Triple-negative breast cancer (TNBC) is notorious for having an increased risk of distant recurrence and death within the first 5 years after diagnosis when compared to other breast cancer subtypes (Dent \textit{et al.}, 2009; Dent \textit{et al.}, 2007). Moreover, it is associated with shorter periods of relapse-free and overall survival (Bauer \textit{et al.}, 2007; Nishimura and Arima, 2008). Chemotherapy is the standard-of-care for TNBC since, by definition, this subtype does not express estrogen receptor (ER), progesterone receptor (PR) or HER2 receptor. These patients are therefore ineligible for therapies targeting these receptors such as anti-hormonal therapies and trastuzumab. In recent years there has been increasing \textit{in vitro} and clinical evidence that cancer stem cells also referred to as tumor-initiating cells (TICs) are resistant to chemotherapy. Cells that preferentially survive chemotherapeutic regimens are enriched for a CD44\textsuperscript{+}/CD24\textsuperscript{-} surface marker phenotype, mammosphere forming ability, an increased capacity to initiate tumors
in immunocompromised mice as well as a “stem-cell-associated” gene signature (Calcagno et al., 2010; Creighton et al., 2009; Li et al., 2008; Yu et al., 2007). Additional studies found that patients who displayed an increase in CD44+/CD24− content after chemotherapy also exhibited a high Ki67 index and shorter disease-free survival times (Lee et al., 2011). TICs therefore represent probable candidate drivers of relapse.

Y-box binding protein-1 (YB-1) is an oncogenic transcription/translation factor abundantly expressed in the most aggressive breast cancer subtypes: HER2-overexpressing and TNBC (~70% of cases) (Habibi et al., 2008). The phosphorylated, nuclear version of the protein has also been associated with drug resistance (Bargou et al., 1997; Dhillon et al., 2010; Habibi et al., 2008; Janz et al., 2002; To et al., 2010). YB-1 is phosphorylated predominantly by p90 ribosomal S6 kinase (RSK), which is a downstream effector in the Raf/MEK/ERK pathway. Upon phosphorylation at Ser102 YB-1 translocates to the nucleus to transcriptionally up-regulate expression of a variety of genes involved in cell growth and proliferation including EGFR (Stratford et al., 2007), HER2 (Wu et al., 2006) and MET (Finkbeiner et al., 2009) as well as cyclin A and B1 (Yu et al., 2010). Furthermore, phosphorylated YB-1 can promote drug resistance through a variety of mechanisms including increased drug efflux (Bargou et al., 1997; Fujita et al., 2005), enhanced DNA repair (Gaudreault et al., 2004; Shibahara et al., 2004) and also by mediating a TIC-like phenotype (To et al., 2010). Previous studies from our group demonstrated that P-YB-1Ser102 increases expression of the TIC-associated markers, CD44 and CD49f and enhances mammosphere-forming capacity. Transfection of wild-type YB-1 into TNBC cells (MDA-MB-231) increases mammosphere growth in the presence of paclitaxel demonstrating YB-1’s capacity to promote drug resistance within this subtype (To et al., 2010). These data position YB-1 as a critical mediator of resistance.

Interestingly, Raf/MEK/ERK pathway activation is associated with basal-like breast cancer (BLBC) (Balko et al., 2012; Hoeflich et al., 2009; Mirzoeva et al., 2009) as is RSK2 mRNA expression (Balko et al., 2012; Stratford et al., 2012). Although TNBC is defined based on cell surface marker expression, 70-90% of TNBC cases are also intrinsically classified as BLBCs (Foulkes et al., 2010). The RSK2 isoform is essential for TNBC survival as suppressing it induces apoptosis and inhibits tumor initiation in mice (Stratford
et al., 2012) while the RSK1 isoform has been indicated to promote invasion (Doehn et al., 2009; Larrea et al., 2009). Up-regulation of Raf/MEK/ERK signaling as well as increased RSK expression in TNBC provide a means for enhanced activation of YB-1 within this breast cancer subtype. YB-1’s role in mediating drug-resistance through a variety of mechanisms positions it as a promising target for overcoming this challenge and potentially reducing relapse. Thus, we hypothesized that YB-1 conveys broad-spectrum resistance to front-line chemotherapies with distinct mechanisms of action, which are currently used to treat TNBC.

4.3 Results

4.3.1 P-YB-1\textsuperscript{S102} is associated with relapse and poor overall survival in invasive breast cancer and is enriched in cells that survive treatment with paclitaxel and epirubicin

Utilizing quantitative reverse phase protein array (qRPPA) we interrogated a cohort of 1057 invasive breast cancer cases and found that P-YB-1\textsuperscript{S102} was significantly associated with poor overall survival (P<0.001, Spearman’s Rho correlation test) and relapse (P<0.001, Spearman’s Rho correlation test) when comparing patients with the highest expression (P-YB-1\textsuperscript{S102}; score = 2) to those with the lowest expression (P-YB-1\textsuperscript{S102}; score = 0)(Figure 4.1A-B). Disease relapse is driven by residual cells, which survive therapy and remain available to re-establish the tumor both locally and at distant metastatic sites. Given the correlation of P-YB-1\textsuperscript{S102} with relapse we investigated whether it was present in cells that survived treatment with front-line chemotherapies, paclitaxel and epirubicin, in cell line models of triple-negative breast cancer SUM149 and MDA-MB-231. Despite a dose-dependent decrease in cell number, a significant enrichment for cells with high P-YB-1\textsuperscript{S102} and YB-1 downstream target CD44 was observed upon treatment with either chemotherapeutic (Figure 4.2A-D). This effect was similarly observed in a second TNBC cell line, MDA-MB-231 (Figure S4.1A-D). Moreover, phosphorylation of YB-1 was functionally significant as nuclear translocation was evident in both cell line models (Figure
4.2E). The signaling in residual cells was further dissected using immunoblotting. In addition to confirming up-regulation of P-YB-1$^{S102}$ and CD44, we also noted that YB-1’s activating kinase, P-RSK1/2$^{S221/7}$ was increased after treatment with either paclitaxel (10 nM) or epirubicin (1 µM)(Figure 4.2F). Interestingly, the total levels of YB-1 were unaffected indicating the response to chemotherapy was mediated through activation of the pathway rather than alterations in protein levels. These data demonstrate that P-RSK1/2$^{S221/7}$ and P-YB-1$^{S102}$ signaling is elevated in residual cells surviving paclitaxel or epirubicin.
Figure 4.1 P-YB-1$^{S102}$ prognosticates relapse and poor overall survival in breast cancer patients.

A) P-YB-1$^{S102}$ is associated with relapse and B) poor overall survival in a cohort of 1057 patients with invasive breast cancer based on quantitative reverse phase protein array (qRPPA) staining for P-YB-1$^{S102}$. P-values are derived from a comparison of low P-YB-1$^{S102}$ scores (0) to high P-YB-1$^{S102}$ scores (2).
Figure 4.2 Paclitaxel and epirubicin enrich for cells with high P-RSK1/2\textsuperscript{S221/7}, P-YB-1\textsuperscript{S102} and CD44.

\textbf{A-B)} SUM149 cells were treated with doses of paclitaxel ranging from 1 nM to 10 nM or with \textbf{C-D)} epirubicin 100 nM to 1 µM for 72 hrs. Cells were fixed and stained with Hoechst33342, P-YB-1\textsuperscript{S102} and CD44. Fluorescence and cell number were quantified using
Cellomics, ArrayScan VTI (n=5; **P<0.005). E) SUM149 and MDA-MB-231 cells were seeded on glass cover slips over night then treated with either DMSO, paclitaxel (PTX) 10 nM or epirubicin (EPI) 1 µM for 72 hrs. Cells were fixed and stained with P-YB-1\textsuperscript{S102} (green) or CD44 (red) and mounted using Prolong Gold antifade containing DAPI. Images were taken using the DeltaVision DV deconvolution microscope. Scale bar is 15 µm. F) Immunoblotting of SUM149 cell lysate after 72 hrs treatment with either DMSO, PTX (10 nM) or EPI (1 µM).
4.3.2 Residual cells after paclitaxel or epirubicin are sensitive to YB-1 inhibition

A high proliferative index (determined by Ki67 staining) following neoadjuvant chemotherapy in patients who do not achieve pathological complete response (pCR) is indicative of increased risk of recurrence and worse overall survival (Guarneri et al., 2009; Jones et al., 2009). YB-1 promotes proliferation in breast cancer cells through transcriptional up-regulation of cell cycle regulators including cyclin A and B1 and by altering cell signaling to favor progression through G1/S checkpoint (Davies et al., 2011a; Yu et al., 2010). Herein, we find that P-YB-1S102 high cells exclusively proliferate in the presence of paclitaxel or epirubicin based on co-staining for Ki67 (Figure 4.3A-B). Likewise, high P-YB-1S102 correlated with the presence of mitotic figures indicating active cell division throughout drug treatment (Figure S4.2A-B). Thus, YB-1 is a marker for cells with the ability to proliferate throughout chemotherapeutic exposure. Next, we assessed the effect of inhibiting YB-1 in cells that survived treatment with chemotherapy. SUM149 cells were treated with either paclitaxel (2 nM – 5 nM) or epirubicin (100 nM – 50 nM) for 72 hrs resulting in elimination of >90% of cells (Figure 4.2A and C). The remaining cells were allowed to recover for an additional 72 hrs in drug-free media prior to experimentation (Figure 4.3C). Elevated levels of P-RSK1/2S221/7 and P-YB-1S102 induced by paclitaxel or epirubicin were maintained in residual cells at 72 hrs post-treatment in a dose-dependant manner (Figure 4.3D). Residual cells from 2 nM paclitaxel and 100 nM epirubicin treatments were viable; however, suppressing YB-1 using two unique oligonucleotide sequences significantly reduced growth of these cells (Figure 4.3E-F). These data indicate that YB-1 inhibition is effective at suppressing growth cells that have survived standard front-line chemotherapeutics.
Figure 4.3 YB-1 knockdown induces suppresses growth in residual cells after paclitaxel and epirubicin.

A) SUM149 cells were treated with paclitaxel (0.1 nM to 10 nM) or B) epirubicin (100 nM to 1 µM) for 72 hrs. Fixed cells were concurrently stained for P-YB-1S102 and Ki67 and quantified using the Cellomics, ArrayScan VTI. Differences in Ki67 expression between P-YB-1S102 high and low cells was evaluated (n=5; *P<0.05 **P<0.005). C) Schematic representation of methodology to obtain residual cell populations. D) Immunoblotting of residual cells after 72 hrs recovery from treatment with paclitaxel (PTX) 2 nM and 5 nM,
epirubicin (EPI) 100 nM and 500 nM or DMSO control. E) YB-1 knockdown in residual cells after paclitaxel (2 nM) or epirubicin (100 nM). Growth assays were extended out to 72 hrs (n=5; **P<0.005). F) Control immunoblots demonstrating YB-1 knockdown after 72 hrs.
4.3.3 Pre-emptive knockdown of YB-1 sensitizes cells to paclitaxel and epirubicin and eliminates CD44\(^+\)/CD24\(^-\) cells

The enrichment of P-YB-1\(^{S102}\) in residual cells led us to question whether pre-emptive inhibition of YB-1 would sensitize cells to treatment with paclitaxel and/or epirubicin. To this end, we suppressed YB-1 using siRNA for 72 hrs prior to treating with paclitaxel or epirubicin. Reducing YB-1 levels prior to treatment with paclitaxel prevented its phosphorylation and function as indicated by a reduction in YB-1 downstream target, CD44 (Figure 4.4A, Figure S4.3). Moreover, pre-emptive YB-1 suppression sensitized cells to paclitaxel and the combination of siYB-1 and paclitaxel suppressed growth to a greater extent than either treatment alone (Figure 4.4B). A similar effect was seen with YB-1 knockdown prior to treatment with epirubicin (Figure 4.4C-D, Figure S4.3). Alternatively, we prospectively isolated the drug-resistant, CD44\(^+\)/CD24\(^-\) TIC-enriched populations of SUM149 cells then treated them with siYB-1 where we found it significantly decreased growth of this population (Figure 4.4E)(Fillmore and Kuperwasser, 2008; To et al., 2010). We further confirmed the ability of YB-1 to sensitize cells to chemotherapeutics in an additional TNBC cell line model, HTRY-LT. This model of YB-1 driven, TNBC exhibits resistance to paclitaxel, doxorubicin, 5-FU and gefitinib (Davies, 2013); however, these cells were sensitized to paclitaxel and epirubicin with pre-emptive siYB-1 suppression (Figure 4.4F, Figure S4.4).
Figure 4.4 Pre-emptive knockdown of YB-1 sensitizes cells to paclitaxel and epirubicin and eliminates CD44+/CD24- cells.

A) Immunoblotting of SUM149 cells treated with paclitaxel (PTX; 10 nM) and SUM149 cells treated with YB-1 knockdown for 72 hrs prior to paclitaxel (10 nM). B) SUM149 cells...
were transfected with siYB-1, control scramble (Ctrl) or no treatment (No Tx) for 72 hrs then seeded into growth assays. The sensitivity of these cells to paclitaxel (0.5 nM, 1 nM and DMSO control) was compared. Cells were fixed at 72 hrs and stained for Hoechst33342 and cell number quantified using the Cellomics, ArrayScan VTI (n=5; **P<0.005). C) Immunoblotting of SUM149 cells treated with epirubicin (EPI; 1 µM) and SUM149 cells treated with YB-1 knockdown for 72 hrs prior to epirubicin (1 µM). D) The sensitivity of SUM149 cells with or without YB-1 knockdown to epirubicin (25 nM, 50 nM and DMSO control) was assessed as described above (n=5; **P<0.005). E) Growth assay of CD44+/CD24−-sorted cells treated with siYB-1 or control scramble. Cells were fixed at 96 hrs and quantified using the Cellomics, ArrayScan VTI (n=5; *P<0.05). F) An additional TNBC cell line model (HTRY-LT) with YB-1 induction (Dox+), with YB-1 suppression (Dox- + siYB-1#3) and control (Dox- + Ctrl) was also assessed for differences in sensitivity to paclitaxel (0.5 nM and 1 nM) and epirubicin (25 nM and 50 nM). A 72 hr knockdown was performed prior to seeding cells in growth assays, which extended for an additional 72 hrs (n=5; **P<0.005).
4.3.4 Elevated RSK/YB-1 signaling is present in chemoresistant cells and blocking this pathway induces cell death

To ascertain whether YB-1 could overcome acquired chemoresistance we created a *bona fide* paclitaxel-resistant SUM149 cell line (SUM149-PTX\(^R\)). Cells were cultured in increasing concentrations of paclitaxel (up to 1.5 nM) over the course of ~20 passages allowing for selection and expansion of resistant cell populations. SUM149-PTX\(^R\) cells did not experience any suppression in growth at 1.5 nM paclitaxel whereas this concentration reduced growth by ~80% in the control DMSO-treated counterparts at 72 hrs (Figure 4.5A). Importantly, their resistance was not due to a decreased proliferative rate since the SUM149-PTX\(^R\) cells and the control SUM149-DMSO cells had nearly identical doubling times (Figure S4.5). Similar to residual cells after chemotherapy, the SUM149-PTX\(^R\) cells had elevated P-RSK1/2\(^{S221/7}\), P-YB-1\(^{S102}\) and CD44 with no change in T-YB-1 (Figure 4.5B). Interestingly, P-AKT\(^{S473}\) levels remained similar in both the paclitaxel-resistant and the drug-naive cells suggesting that the increase in P-YB-1\(^{S102}\) was mediated through RSK signaling rather than the PI3K/Akt pathway (Figure 4.5B). Since most patients are treated with a cocktail of chemotherapeutics in the clinical setting, we also investigated whether the SUM149-PTX\(^R\) cells exhibited cross-resistance to additional compounds. Interestingly, when paclitaxel (1.5 nM) was used concurrently with either epirubicin (20 nM), 5-fluorouracil (5-FU)(1 \(\mu\)M) or a combination of all three drugs, the SUM149-PTX\(^R\) cells consistently displayed significantly less cell death as indicated by PI-uptake (Figure 4.5C). Yet, despite their broad-spectrum resistance to conventional chemotherapeutics, SUM149-PTX\(^R\) cells were responsive to YB-1 inhibition exhibiting ~90% reduction in growth after 7 days (Figure 4.5D). Similarly, blocking activation of YB-1 using the RSK inhibitor BI-D1870 was equally effective at suppressing growth and inducing cell death in the drug-resistant SUM149-PTX\(^R\) cells as in the SUM149-DMSO cells (Figure 4.5E, Figure S4.6). Moreover, the addition of BI-D1870 to paclitaxel increased apoptosis in drug-resistant SUM149-PTX\(^R\) cells as indicated by caspase-3 cleavage (Figure 4.5F).
Figure 4.5 Blocking RSK/YB-1 signaling suppresses growth and induces apoptosis in chemoresistant cells.

A) The response of paclitaxel-resistant cells (SUM149-PTXR) and drug-naïve cells (SUM149-DMSO) was compared in a 72 hr growth assay. Cell number was quantified
using the Cellomics, ArrayScan VTI (n=5; *P<0.05, **P<0.005). B) Immunoblotting characterization of the SUM149-DMSO and the SUM149-PTX\textsuperscript{R} cell lines. C) A comparison of the sensitivity of the SUM149-PTX\textsuperscript{R} cells and SUM149-DMSO cells to various combinations of paclitaxel (PTX; 1.5 nM), epirubicin (EPI; 20 nM) and 5-fluorouracil (5-FU; 1 µM). Cell death was quantified based on PI-uptake using the Cellomics ArrayScan VTI (n=5; **P<0.005). D) Growth assay of the SUM149-PTX\textsuperscript{R} cells with or without YB-1 knockdown. Assay was stopped after 7 days and cell number was quantified (n=5; **P<0.005). E) Growth assay comparing the response of the SUM149-PTX\textsuperscript{R} cells and the SUM149-DMSO cells to BI-D1870 (0.5 µM – 5 µM) (n=5; **P<0.005). F) Immunoblotting for cleaved caspase-3 with paclitaxel (PTX) alone or in combination with BI-D1870 (1 µM, 2 µM or 5 µM) after 72 hrs.
4.3.5 RSK/YB-1 signaling activation correlates with drug resistance

Finally we were fortunate to obtain a primary TNBC cell line (x43) (a kind gift from Dr. John Hassell) derived from a patient that eventually relapsed. This cell line exhibited high P-RSK1/2$^{S221/7}$ and P-YB-1$^{S102}$ levels when compared to MDA-MB-231 and SUM149 cells (Chapter 3, Figure 3.5D)(Reipas et al., 2013). Further, there was an inverse correlation between the level of RSK/YB-1 pathway activation and response to therapy, whereby the x43 cells were the least responsive compared to the other TNBC cell lines (Figure 4.6A). Despite the apparent aggressiveness of the x43 cells, reducing YB-1 utilizing RNA interference significantly inhibited growth (>90% at 7 d) and induced cell death as indicated by PI uptake (Figure 4.6B-C). YB-1 knockdown was further demonstrated to suppress growth by up to ~90% at 7 days in additional TNBC models SUM149, MDA-MB-231 and HCC1143 (Figure S4.7).
**Figure 4.6** Suppressing YB-1 decreases growth and induces cell death in primary TNBC.

**A)** Growth assay comparing the response of three TNBC cell lines (SUM149, MDA-MB-231 and x43) to paclitaxel and epirubicin. Assay was stopped at 72 hrs and cells were quantified using the Cellomics, ArrayScan VTI (n=5; ***P<0.0001).

**B)** YB-1 knockdown reduces growth and **C)** induces cell death in primary x43 cells at 7 days (n=5; **P<0.005). Control blot demonstrating knockdown in Figure B, inset.
4.4 Discussion

Elucidation of signaling pathways in cells that survive conventional chemotherapeutics can provide insight into the mechanisms that govern drug resistance. Further, functional studies of these pathways can determine their biological relevance and be used to guide mechanism-based design of targeted therapies with the potential to eliminate the residual cell burden and prevent metastatic recurrence. We observe that P-YB-1S\textsuperscript{102} predicts relapse and poor survival in patients with invasive breast cancer across all subtypes. Moreover, cells that survive treatment with paclitaxel or epirubicin exhibit elevated P-RSK1/2S\textsuperscript{221/7} and P-YB-1S\textsuperscript{102} as well as the YB-1 downstream target CD44. Thus, RSK/YB-1 is highly activated in residual cell populations. In support of our data, a recent study by Balko et al. (2012) identified DUSP4 deficiency (an ERK phosphatase) in residual tumor specimens from breast cancer patients after neoadjuvant chemotherapy. The authors report that DUSP4 is associated with ERK pathway activation and that knockdown of DUSP4 enhances resistance to docetaxel in breast cancer cell line models (Balko et al., 2012). Low expression of DUSP4 is also associated with significantly shorter time to relapse in an independent study interrogating the Wang et al. microarray dataset consisting of node-negative primary breast tumors (Martin et al., 2008). Collectively these data indicate the ERK/RSK/YB-1 signaling axis is activated in cells after chemotherapy and several components of this pathway are predictive of relapse. Additionally, our data show that cells dividing throughout chemotherapy can be identified based on P-YB-1S\textsuperscript{102} expression. Taken together, P-YB-1S\textsuperscript{102} is a marker for highly proliferative cells that survive conventional chemotherapeutic regimens.

Drug cocktails consisting of various chemotherapies acting via distinct mechanisms demonstrate superior efficacy over single agent regimens. This type of therapy reduces the development of acquired resistance since cancer cells then need to circumvent each drug's mechanism of action to survive and this may involve alterations in several pathways. We find that P-YB-1S\textsuperscript{102} is elevated after treatment with chemotherapeutics functioning through two unique mechanisms of action - paclitaxel being a microtubule stabilizing agent and epirubicin an anthracycline - indicating YB-1-mediated resistance is not limited to one class of chemotherapeutic. Moreover, pre-emptive suppression of YB-1 sensitizes cells to these
chemotherapeutics. Interestingly, we found that drug selection of SUM149 cells with paclitaxel leads to the development of cross-resistance to three of the most clinically relevant classes of chemotherapeutics (paclitaxel, epirubicin, and 5-FU) in the established SUM149-PTX8 cell line. Importantly, suppressing YB-1 or preventing its activation with BI-D1870 was effective at reducing growth and inducing apoptosis in these multidrug resistant cells. This suggests that inhibiting YB-1 may be an effective strategy for treating patients who have developed refractory disease to standard-of-care chemotherapeutic regimens. Finally, we show that P-RSK1/2\textsuperscript{S221/7} and P-YB-1\textsuperscript{S102} expression are increased in cancer cell lines that exhibit resistance to chemotherapy. Our primary breast cancer cell line (x43) containing the highest levels of activated RSK/YB-1 signaling were the least sensitive to paclitaxel or epirubicin yet these cells remained exquisitely sensitive to YB-1 suppression. Collectively, these data demonstrate the ability of YB-1 inhibition to sensitize TNBC to a variety of chemotherapeutics and highlight its promise as a molecular target for attenuating the development of broad-spectrum chemoresistance.

While we do not address the mechanism by which RSK and YB-1 become activated, the fact that several distinct classes of chemotherapeutics can cause their up-regulation suggests this is a stress inducible pathway. YB-1 itself contains an evolutionarily conserved, Cold-Shock domain (CSD) which is characteristic of proteins that promote survival throughout hypothermic stress (Goldstein et al., 1990). YB-1’s nuclear translocation after exposure to a variety of cellular stresses such as UV (Koike et al., 1997), DNA damaging agents such as cisplatin (Ise et al., 1999), oxidative stress (Das et al., 2007) and hyperthermia (Stein et al., 2001) is well documented. Thus, YB-1 may be commonly activated through several pathways induced by cellular stresses and studies designed to identify the precise mechanism of RSK/YB-1 activation in response to chemotherapy warrant further investigation. Once in the nucleus, YB-1 transcriptionally activates many genes with protective functions including those involved in drug efflux (Bargou et al., 1997; Stein et al., 2005; Vaiman et al., 2006) and DNA repair (Eliseeva et al., 2011) (Gaudreault et al., 2004; Hasegawa et al., 1991; Lenz et al., 1990) (Ise et al., 1999). When taken together, YB-1 is positioned to become activated and confer resistance to a wide range of chemotherapeutics. It’s established role as a marker of malignant cells and low expression
in normal cells make inhibiting this protein a promising opportunity to overcome resistance to a wide range of chemotherapies.

It is important to note that chemotherapy is given to any patient where disseminated or residual disease is present. Thus, patients with ER/PR-positive or HER2-positive breast cancers are also often indicated for a regimen that includes chemotherapy. It is possible that YB-1-mediated chemoresistance may be contributing to relapse within these subtypes as well. Indeed, our data herein indicate P-YB-1\({\text{S}102}\) is predictive of disease relapse across all breast cancer subtypes. Furthermore, YB-1 is predictive of relapse and poor survival in many other cancers including B-cell lymphoma (Xu et al., 2009), ovarian cancer (Kamura et al., 1999) and non-small cell lung carcinoma (Shibahara et al., 2001) (Gessner et al., 2004), where again the probability of achieving a cure without relapse after chemotherapy is significantly lower if P-YB-1\(^{\text{S102}}\) is elevated or localized to the nucleus. Thus, inhibiting YB-1 in conjunction with conventional chemotherapeutics may be an effective strategy to enhance the efficacy of treatment in a variety of cancers. In summary, we identified RSK/YB-1 signaling is critical for mediating cell survival in response to chemotherapy and that inhibiting this pathway can overcome resistance to a diverse spectrum of chemotherapeutics which may help prevent both local and distant disease recurrence.

4.5 Experimental Procedures

i) Quantitative reverse phase protein array. A quantitative reverse phase protein array of tumor lysates from invasive breast cancers was performed as previously described (Tibes et al., 2006). The expression of P-YB-1\(^{\text{S102}}\) was evaluated in 1057 breast cancer cases. Kaplan-Meier analyses for relapse-free survival and overall survival were performed using SPSS version 19.0 software (SPSS Inc., Chicago, IL, USA) with tertile cut-offs for P-YB-1\(^{\text{S102}}\) to denote low, intermediate and high groups; significance was evaluated at the \(P<0.001\) level by log-rank testing between the high (score=2) and the low (score = 0) groups (Habibi et al., 2008).
**ii) Cell lines and reagents.** SUM149 (Asterand, Detroit, MI) and MDA-MB-231 (ATCC) cells were used as models of TNBC and cultured as previously described (Stratford et al., 2008). Primary human TNBC cells (x43) were a kind gift from Dr. John Hassell (McMaster University, Hamilton, ON). All experimentation involving human cells were done in accordance with the Helsinki guidelines and approved through McMaster University ethics committee. The x43 cells were cultured as described in (Reipas et al., 2013). An additional model of TNBC (HTRY-LT) was also utilized. H16N2 HMECs with tetracycline-inducible YB-1 (HTRY) were generated using the T-Rex system (Invitrogen, Burlington, ON)(Berquin et al., 2005). Long-term induction (>30 days) with 1 µg/ml doxycycline (Calbiochem, Gibbstown, NJ, USA) established the transformed HTRY-LT cell line, which was classified as a TNBC (Davies, 2013). BI-D1870 was synthesized by the Center for Drug research and Development (CDRD) (Vancouver, BC) and was dissolved in DMSO. Paclitaxel and epirubicin and 5-FU (Sigma-Alderich, Oakville, ON) and were also dissolved in DMSO.

**iii) Growth and Immunofluorescence Quantification Assays.** Cells were seeded (3,000 SUM149 and SUM149-PTXR; 5,000 MDA-MB-231; 5,000 x43; 5,000 HTRY-LT) per well in 96-well plates and treated the next day with chemotherapeutics or BI-D1870. The exception to this was in experimentation comparing drug response between the three TNBC cell lines (SUM149, MDA-MB-231 and x43), where all cells were seeded at 4,000 cells/well. Cells were fixed in 2% paraformaldehyde, permeabilized and stained with the antibodies as follows: P-YB-1SI02 (1:100; Cell Signaling Technologies) followed by Alexafluor 488 anti-rabbit (1:1000), CD44-PE conjugated (1:200, BD Pharmingen, Mississauga, ON), Ki67 (1:200; Abcam, Cambridge, MA) and Hoechst33342 dye (1µg/ml). Representative images were obtained using identical staining protocols for P-YB-1SI02 and CD44 but mounted with Prolong Gold antifade with DAPI (Invitrogen, Burlington, ON) and images were analyzed using the DeltaVision DV deconvolution microscope (Applied Precision). Propidium iodide (1µg/ml) was added to the medium of unfixed cells and incubated at 37°C for 1h to assess cellular death. Immunofluorescence and mitotic figures was quantified using the Cellomics, ArrayScan VTI, as described in (Law et al., 2008).
iv) siRNA Transfections. Cells were transfected with RNAiMAX (Invitrogen; Burlington, ON). All siRNA knockdowns were performed at 20 nM and the siRNA target sequences given (Table S4.1). All experiments were performed once the cells had been transfected for 72 hrs unless otherwise stated. For growth assays extending out to 7 days, a second transfection was performed on day 3 of the growth assay in the 96-well plate.

v) Immunoblotting. Immunoblotting was performed as previously described (Wu et al., 2006). Antibodies were used as follows; RSK1; 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), RSK2; 1:500 (Santa Cruz Biotechnology), YB-1; 1:2000 (Cell Signaling Technology, Boston, MA), YB-1; 1:1000 (Epitomics, Burlingame, CA), CD44; 1:1000 (Abcam, Cambridge, MA), P-RSK1/2S221/27; 1:1000 (Invitrogen, Burlington, ON), P-YB-1S102; 1:1000 (Cell Signaling Technology), P-AKT*S473; 1:1000 (Cell Signaling Technology), cleaved caspase-3; 1:500 (Cell Signaling Technology), α/β-Tubulin; 1:1000 (Cell Signaling Technology) and Pan-actin; 1:1000 (Cell Signaling Technology).

vi) Residual Cell Experiments. SUM149 cells (500,000 cells/dish) were seeded in a 60 mm dish overnight and treated the next day with either paclitaxel (2 nM and 5 nM) or epirubicin (100 nM and 500 nM). After 72 hrs drug-containing media was removed, cells were washed 3 times with PBS and drug-free media was added. Residual cells were allowed to recover for an additional 72 hrs prior to immunoblotting. Only the 2 nM paclitaxel or the 100 nM epirubicin residual cells were utilized in subsequent growth assays.

vii) Fluorescence-Activated Cell Sorting (FACS). A single cell suspension of SUM149 cells was obtained as previously described (To et al., 2010). Cells were stained with CD44-PE conjugated (BD Pharmingen, Mississauga, ON), CD24-FITC conjugated (StemCell Technologies, Vancouver, BC) and 7-AAD viability dye (BD Pharmingen) and sorted for the top 10% CD44+/CD24− population. Experimentation on sorted populations was performed immediately after sorting.

viii) Creation of drug-resistant SUM149 cells. SUM149 cells were cultured in 60 mm dishes and treated with gradually increasing incremental concentrations of paclitaxel or equivalent DMSO for control cells. The starting concentration was 0.1 nM paclitaxel and cells were cultured for ~3 passages at each concentration (or as long as it took for them to
adapt and begin growing normally) until a final maximum concentration of 1.5 nM was reached over the course of ~20 passages. SUM149-PTX\textsuperscript{R} cells were consistently cultured in 1.5 nM paclitaxel-containing media, which was refreshed every 3-4 days.

\textit{ix) Mammosphere Assay.} Cells were seeded (SUM149: 20,000 cells/well) in ultra-low adherent 6 well plates (StemCell Technologies Inc., Vancouver, BC) and grown in MammoCult\textsuperscript{®} (StemCell Technologies Inc.) supplemented with hydrocortisone and heparin. Spheres with a minimum diameter of 50 μm (or >15 cells) were counted after 7 days.

\textit{x) Statistical analysis.} All data is presented as mean ± SD of at least three repeated individual experiments. Significance was evaluated using a paired Student’s \(t\)-test, and difference to be considered statistically significant when \(*P<0.05\) and \(**P<0.005\). Normally distributed data as assessed by Kolmogorov-Smirinov and Shapiro-Wilk tests of normality. One-way ANOVA with post-hoc testing was used to compare the response of TNBC cell lines to chemotherapy and differences were considered statistically significant when \(***P<0.0001\).
4.6 Supplementary Data

4.6.1 Supplemental figures

Figure S4.1 Paclitaxel and epirubicin enrich for P-YB-1S102 and CD44 in MDA-MB-231 cells.

A-B) MDA-MB-231 cells were treated with doses of paclitaxel (1 nM-10nM) or with C-D) epirubicin (100 nM-1 μM) for 72 hrs. Cells were fixed and stained with Hoechst33342, P-YB-1S102 and CD44. Fluorescence and cell number were quantified using the Cellomics, ArrayScan VTI (n=5; **P<0.005).
Figure S4.2 Percent P-YB-1S102 high and P-YB-1S102 low cells undergoing mitosis in the presence of paclitaxel or epirubicin.

A) SUM149 cells were treated with paclitaxel (1 nM-10 nM) or B) epirubicin (100 nM-1 µM) stained for P-YB-1S102 and Hoechst33342. The number of mitotic figures was compared between P-YB-1S102 high and P-YB-1S102 low cells using the Cellomics, ArrayScan VTI (n=5; **P<0.005).
**Figure S4.3 Control immunoblot validating YB-1 knockdown in SUM149 cells.**

SUM149 cells were treated with siYB-1, control scramble (Ctrl) or no siRNA (No Tx) for 72 hrs prior to experimentation with combinations of paclitaxel and epirubicin.
**Figure S4.4 Control immunoblot validating YB-1 knockdown in HTRY-LT cells.**

HTRY-LT cells were treated with siYB-1, control scramble (Ctrl) or no siRNA.
Figure S4.5 Comparison of the growth rates between SUM149-PTX$^R$ cells and the control SUM149-DMSO cells.

A time course growth assay comparing the growth rate of SUM149-PTX$^R$ cells and SUM149-DMSO cells. Quantification of cells every 24 hrs was performed using the Cellomics, ArrayScan VTI (n=5).
Figure S4.6 BI-D1870 induces cell death in drug-resistant SUM149-PTX<sup>R</sup> cells and drug-naïve SUM149-DMSO cells to a similar extent.

A comparison of the sensitivity of the SUM149-PTX<sup>R</sup> cells and SUM149-DMSO cells to RSK inhibitor, BI-D1870 across a range of concentrations (1µM – 5 µM). Cell death was quantified based on PI-uptake (n=5).
Figure S4.7 YB-1 inhibition suppresses growth in additional TNBC cell lines.

A) Growth assay demonstrating the effect of YB-1 knockdown using three distinct oligonucleotide sequences in the SUM149, B) MDA-MB-231 and C) HCC1143 TNBC cell line models after 7 days (n=5; *P<0.05, **P<0.005).
### 4.6.2 Supplemental tables

<table>
<thead>
<tr>
<th>siRNA</th>
<th>siRNA target sequence (5’ – 3’)</th>
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<td>Dharmaco</td>
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<tr>
<td>YB-1#2</td>
<td>CCACGCAATTACCAGCAAA</td>
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</tr>
<tr>
<td>YB-1#3</td>
<td>Hs_YBX1_1_HP Validated siRNA</td>
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**Table S4.1** List of siRNA target sequences.
CHAPTER 5. CONCLUDING REMARKS

5.1 Summary and Discussion

Developing curative treatments for breast cancer requires a thorough understanding of the processes that allow cells to survive current treatments and subsequently drive relapse. Patients with TNBC have a high risk of relapse within the initial years after diagnosis when compared to other breast cancer subtypes (Dent et al., 2009; Dent et al., 2007). Moreover, therapeutic options for patients with this subtype are limited to chemotherapy, making the need for discovery of novel targeted therapies with the ability to prevent relapse imperative. Residual populations of cells that survive conventional chemotherapy are enriched for a TIC phenotype (Li et al., 2008). They express a CD44+/CD24- cell surface marker phenotype and demonstrate a higher mammosphere-forming capacity as well as an increased ability to initiate tumors in mice relative to non-TICs (Al-Hajj et al., 2003; Li et al., 2008; Ponti et al., 2005). Enhanced drug efflux through over-expression of ABC transporters (Calcagno et al., 2010; Katayama et al., 2009), increased DNA repair (Karimi-Busheri et al., 2010) and elevated levels of anti-apoptotic proteins (Lee et al., 2010; Madjd et al., 2009) are just some of the documented mechanisms by which TICs promote drug resistance. Their ability to preferentially survive various chemotherapeutics makes them likely candidate drivers of relapse and as such future initiatives should be aimed at ensuring effective elimination of this population. Interestingly, TNBCs exhibit a high TIC content, providing a possible explanation for this subtype’s relatively higher relapse rates (Honeth et al., 2008).

YB-1 is a signature feature of malignancies in a variety of tissues. It is associated with relapse and poor overall survival across all breast cancer subtypes and is highly expressed in the most aggressive subtypes including, TNBC (Habibi et al., 2008). YB-1 transcriptionally regulates expression of several RTKs, which are highly relevant in breast cancer such as EGFR, HER2 and MET (Finkbeiner et al., 2009; Stratford et al., 2007; Wu et al., 2006). Importantly, YB-1 regulates drug resistance through a variety of mechanisms. This multifunctional protein mediates nearly every type of DNA repair (Eliseeva et al., 2011; Gaudreault et al., 2004; Ise et al., 1999; Sengupta et al., 2011) and is also associated with
elevated drug efflux (Bargou et al., 1997; Stein et al., 2005; Vaiman et al., 2006). Moreover, YB-1 regulates a TIC phenotype by inducing CD44 and CD49f and enhancing resistance to paclitaxel (To et al., 2010). YB-1 is activated predominantly by RSK and is a convergence point between two of the most commonly over-activated pathways in breast cancer (Raf/MEK/ERK and PI3K/Akt) both of which are activated through RTK signaling. As such YB-1 is positioned as an ideal candidate for mitigating drug resistance endowed through a variety of mechanisms.

The studies presented in this thesis identify RSK2 is critical to the survival of TNBC and demonstrate that inhibiting RSK activation of YB-1 can overcome resistance to current standard-of-care chemotherapies utilized to treat TNBC. Moreover, preventing activation of YB-1 through pharmacological inhibition of RSK has the unique ability to eliminate TIC-enriched populations of cells. The significance of this work lies in the identification of a novel, druggable target critical to the survival of TNBC. To our knowledge it is one of the only targeted therapies for TNBC that demonstrates activity across several TNBC subtypes. Therefore a broader spectrum of patients is likely to receive therapeutic benefit from RSK inhibitors than from many targeted therapies currently in clinical trials. Moreover, inhibiting RSK/YB-1 signaling specifically targets cells that survive clinically relevant chemotherapies and therefore may reduce relapse. The work herein provides strong pre-clinical evidence supporting the development of RSK inhibitors to treat TNBC.

RSK lies downstream of Raf/MEK/ERK signaling and therefore can be activated by most RTKs, including those most relevant to breast cancer such as EGFR and HER2. Many currently utilized targeted therapies bind to RTKs directly, inhibiting initiation of signaling cascades. However this strategy allows cancer cells the opportunity to circumvent RTK inhibition by activating kinases further downstream in signal transduction pathways thereby restoring pathway activation (Berns et al., 2007). Indeed, acquired resistance often preserves the original overall pathway addiction (Hoelder et al., 2012). Therefore targeting critical effector kinases that lie further downstream in signaling pathways may reduce the likelihood that cancer cells will circumvent pathway inhibition conferring resistance to specific targeted therapies. In chapter 2, we demonstrate that RSK2 is one of only three kinases that elicits a potent anti-proliferative effect in TNBC. We additionally demonstrate
that RSK2 mRNA is associated with basal-like breast cancer and worse overall survival. A recent publication from Dr. Arteaga’s group identified that ERK pathway activation is also associated with BLBC in primary breast cancer (Balko et al., 2012). Additionally, gene expression profiles of TNBC cell lines are similar to those of KRAS-mutant cancers and in vitro experiments demonstrate that these cells are sensitive to MEK inhibition (Hoeflich et al., 2009; Mirzoeva et al., 2009). Moreover, BLBC cell lines are more sensitive to EGFR inhibition than luminal cell lines and an EGFR-associated, poor prognostic signature is highly expressed in primary basal-like tumors (Hoadley et al., 2007). These data further indicate that Raf/MEK/ERK signaling is critical for TNBC survival. RSK2 promotes cell proliferation (Fujita et al., 2003) and cell survival (Anjum et al., 2005; Bonni et al., 1999; Romeo et al., 2012; Xing et al., 1996) therefore inhibition of RSK signaling can directly block these processes which are critical for sustaining carcinomas. Excitingly, RSK2 inhibition demonstrates efficacy across various TNBC subtypes in functional viability screens (Chapter 2) and further validated in cell line models (SUM149, MDA-MB-231) as well as a primary TNBC cell line. Based on gene expression profiling, Lehmann et al. classified the MDA-MB-231 cells as mesenchymal stem-cell like (not harboring BRCA1 mutations), while the SUM149s fall into the basal-like 2 (BL2) category (and do carry BRCA1 mutations) (Lehmann et al., 2011). RSK inhibition by either BI-D1870 or luteolin (Chapter 2 and 3) demonstrates efficacy at suppressing growth in both of these cell lines. Many of the targeted therapies currently in clinical trial only confer therapeutic benefit to patients with particular subsets of TNBC. For example, although PARP inhibitors improve outcome in BRCA-related cancers, their clinical utility beyond this select population remains uncertain (Gelmon et al., 2012). Given the diversity of TNBC, it is most beneficial to develop therapies indicated for most if not all patients rather than a subset. Additionally, utilizing combinations of targeted therapies that inhibit kinases at various levels of signaling cascades may hinder the development of resistance (for example an EGFR inhibitor with a RSK inhibitor). Of course one consideration to targeting intracellular kinases is that sufficiently lipophilic, small molecules with the ability to cross cell membranes and reach their targets need to be utilized. An ever-growing body of knowledge exists on techniques to make molecules more permeable through the addition of various side groups and this
method could be employed to modify identified RSK inhibitors to ensure they reach their target.

Although our RNA interference data indicates the RSK2 isoform in TNBC, the pan-RSK inhibitors BI-D1870 and luteolin (Chapter 2 and 3) also demonstrate activity against TNBC despite the fact that they target all RSK isoforms. As the development of RSK inhibitors progresses it will be necessary to distinguish the isoform-specific effects of RSK suppression to determine whether inhibitors that target select RSK isoforms or pan-RSK activity is the most advantageous strategy. While we demonstrate herein that RSK2 is critical for sustaining survival of TNBC, inhibiting RSK1 may have additional therapeutic benefits. Several studies identified that RSK is a principle effector of Raf/MEK/ERK-mediated motility and invasion in both non-transformed epithelial and carcinoma cells (Doehn et al., 2009). Moreover, RSK1 promotes cancer cell invasion and metastasis (Larrea et al., 2009; Smolen et al., 2010). Exploring whether inhibiting RSK1 can reduce migration in breast cancer warrants further investigation as it may assist in preventing metastatic dissemination of this disease, particularly in a subtype prone to distant recurrence such as TNBC (Dent et al., 2009; Dent et al., 2007). RSK3 and RSK4 are often found in lower abundance in tumor tissue than in normal tissue and therefore their activity may play a less significant role in sustaining neoplasms (Bignone et al., 2007; Thakur et al., 2008). However, activation of RSK3 and RSK4 has recently been shown to promote resistance to PI3K inhibitors therefore preventing their up-regulation may have added therapeutic benefit (Serra et al., 2013).

Although the studies herein focus on RSK activation of YB-1, RSK has a plethora of additional targets that are also critical for sustaining cancer. RSK plays essential roles in several pathways regulating proliferation, cell survival and drug resistance, all of which likely contribute to the potent anti-tumor effect of RSK inhibitors. RSK1 and RSK2 directly promote cell cycle progression via p27kip1 phosphorylation and release of CDK2 inhibition (Fujita et al., 2003). Moreover, RSK activates transcription factors that drive cell proliferation such a c-Fos (David et al., 2005). RSK promotes cell survival through activation of the anti-apoptotic transcription factors, CREB and NF-κB (Bonni et al., 1999; Escarcega et al., 2007; Ghoda et al., 1997; Schouten et al., 1997; Xing et al., 1996) as well
as inhibition of the pro-apoptotic proteins BAD (Bonni et al., 1999) and DAPK (Anjum et al., 2005). Finally, RSK regulates a TIC phenotype through phosphorylation and activation of YB-1, which contributes to resistance to paclitaxel (To et al., 2010). The ability of RSK to promote drug resistance has also been demonstrated in melanoma where it enhances DNA damage checkpoint silencing by inhibiting Chk1 (Ray-David et al., 2012). The capacity of RSK inhibitors to simultaneously reduce proliferation, enhance apoptosis and attenuate drug resistance in cancer make targeting this kinase an ideal strategy to blocking all of these processes.

A critical consideration when developing any type of therapy is the effect it may have on normal tissues since toxicity to normal cells leads to unwanted and often dose-limiting side effects. Ideal targets for therapeutic development should be highly expressed in cancerous tissue but low or non-essential in normal tissue. Importantly, we demonstrate that P-RSK1/2<sup>S221/7</sup> and P-YB-1<sup>S102</sup> are present in TNBC but not in normal breast tissue (chapter 2), suggesting that cancer cells could be selectively targeted utilizing anti-RSK therapy. Studies from Smith et al. also demonstrate that mean levels of total RSK1 and RSK2 are statistically higher in breast cancer than that in normal tissue (Smith et al., 2005). Furthermore, in chapter 2 and 3 we demonstrate that siRNA interference or pharmacological inhibition of RSK using BI-D1870, SL0101 or luteolin has minimal effect on growth of normal mammary epithelial cell lines. It is promising that both RSK2 knockout and RSK1/RSK2/RSK3 triple-knockout mice are viable (Dumont et al., 2005). While RSK2 knockout mice do exhibit cognitive impairment (Dufresne et al., 2001; Poirier et al., 2007) this phenotype may be most severe when RSK is absent during development and treating adults with RSK inhibitors could be less detrimental to fully developed brains. RSK2 knockout mice also exhibit osteopenia and delayed T-cell activation (Lin et al., 2008a) however, when compared to the severe myelosuppression induced by many chemotherapeutics these effects represent a substantial improvement. We attempt to address the effect of RSK inhibition on hematopoietic differentiation by testing BI-D1870 in hematopoietic progenitor clonogenic assays. We found indications that at low doses the RSK inhibitor BI-D1870 did not have any effect on hematopoietic differentiation suggesting there may be a therapeutic window for RSK inhibitors in the treatment of
TNBC. However, this assertion will need to be assessed in *in vivo* experiments and will be highly dependent on the ability of RSK inhibitors to be absorbed into tissues from the bloodstream.

In an attempt to fast-track RSK inhibitors into clinical use, we screened a library of off-patent compounds for compounds with novel RSK-inhibitory activity (chapter 3). These studies revealed that the flavonoid luteolin, could inhibit both RSK1 and RSK2 kinase activity in cell-free assays thus definitively demonstrating its activity against RSK. Importantly, the phenotypic effects of luteolin in TNBC mirrored those of BI-D1870, an established RSK inhibitor and provided further evidence for the efficacy of RSK inhibitors in TNBC using a second compound. Unfortunately however, luteolin was less potent and less specific than BI-D1870 and so may not represent the ideal candidate for further development into clinical use as a RSK inhibitor. That said, luteolin may have some advantages over BI-D1870. Its current application as a nutraceutical for the treatment of various neurological conditions and general overall health has so far revealed no associated toxicities even at relatively high doses. As such, this natural compound, which exhibits anti-tumor activity *in vitro* may prove to be a beneficial supplement for patients with TNBC pending results that demonstrate its *in vivo* anti-tumorigenic activity.

Throughout all three chapters, we specifically address the effect of inhibiting RSK/YB-1 signaling on TIC-enriched populations since these cells are increasingly indicated as drivers of disease relapse. We demonstrate that inhibiting RSK or YB-1 is capable of suppressing growth in CD44+/CD24−-sorted cells and reducing mammosphere formation (chapter 2-4). These populations are enriched for highly tumorigenic cells when transplanted into immunocompromised mice (Al-Hajj *et al.*, 2003; Ponti *et al.*, 2005). Fillmore *et al.* further demonstrated that the SUM149 cell line could be prospectively sorted based on cell surface marker expression to obtain populations with differential tumor-initiating capacity, lending credence to our use of this cell line to enrich for TICs (Fillmore and Kuperwasser, 2008). Our data demonstrating that inhibiting RSK/YB-1 signaling can eliminate the CD44+/CD24− cell population was of particular interest to us since many conventional chemotherapeutics enrich for TICs (Creighton *et al.*, 2009; Li *et al.*, 2008). Furthermore, we discover that inhibiting YB-1 can reduce Notch4 signaling. Notch4 is implicated by several other groups
as a transmembrane receptor essential for maintaining both normal and neoplastic cells in an undifferentiated state and for sustaining tumorigenic propensity (Dontu et al., 2004; Farnie et al., 2007; Harrison et al., 2010). Thus, in addition to regulating growth in TIC-enriched populations, YB-1 transcriptionally activates an ever-expanding list of TIC-associated genes. One caveat to this work is that we do not directly assess the ability of stable cell lines lacking RSK2 or YB-1 to initiate tumors in limiting dilution assays, which is considered the gold standard for measuring TICs. Depletion of either of these proteins reduces cell viability and long-term suppression of YB-1 or RSK2 would require an inducible knockdown system, which we currently do not have. However, we do demonstrate that transient knockdown of RSK2 is sufficient to delay tumor initiation in vivo suggesting that prolonged inhibition may have a more dramatic effect.

In chapter 4, rather than prospectively sorting for TIC-enriched populations, we functionally selected for cells that survived clinically relevant chemotherapeutics as we wanted to interrogate signaling within these cells. We found activated RSK and YB-1 to be highly up-regulated in residual cells after chemotherapy. Interestingly, chemotherapies that act by two distinct mechanisms (paclitaxel and epirubicin) similarly enrich for cells with a P-RSK1/2S\textsuperscript{221/7}/P-YB-1S\textsuperscript{102}/CD44\textsuperscript{+} phenotype. ERK pathway activation was also recently demonstrated in clinical samples after neoadjuvant chemotherapy providing a possible explanation for our observation that chemotherapy increases P-RSK1/2S\textsuperscript{221/7} and P-YB-1S\textsuperscript{102} (Balko et al., 2012). However the exact mechanism in which this pathway becomes activated remains elusive to us. Balko et al. indicate that promoter methylation and suppression of DUSP4 (an ERK phosphatase) leads to elevated ERK signaling in residual cells. Thus, DUSP4-mediated activation of RSK may also be occurring through ERK in clinical samples, however this hypothesis was not explored in their study. Clinically, breast cancer can present as refractory or may acquire resistance to a wide range of chemotherapeutics after treatment. Through prolonged exposure to paclitaxel, we generated a cell line that exhibited cross-resistance to several distinct classes of chemotherapy. Yet, despite their chemoresistance these cells remained sensitive to RSK/YB-1 signaling inhibition. This response suggests that RSK inhibitors could be useful in chemo-refractory disease. Moreover, our data suggest that when used as a front line treatment concurrently
with chemotherapeutics, this combination may be more effective at reducing bulk tumor and eliminating CD44+/CD24- cells than chemotherapy alone.

Finally, the use of RSK inhibitors may have clinical utility beyond TNBC. Although RSK2 mRNA was most highly expressed in BLBC, it was present across all breast cancer subtypes (chapter 2). YB-1 is also expressed across all breast cancer subtypes with high expression in the HER2-positive subtype (~70%) and work from our group has demonstrated that RSK/YB-1 signaling mediates resistance to trastuzumab (Dhillon et al., 2010; Habibi et al., 2008). As such, patients with HER2-positive breast cancer may also benefit from RSK inhibitors to block YB-1. Beyond breast cancer, YB-1 is highly up-regulated in malignant tissue relative to normal tissue in a variety of neoplasm’s including; glioblastoma multiforme (Faury et al., 2007), colorectal (Shibao et al., 1999), non-small cell lung cancer (Gu et al., 2001; Kashihara et al., 2009; Shibahara et al., 2001), ovarian (Kamura et al., 1999; Yahata et al., 2002) and prostate (Gimenez-Bonafe et al., 2004) to name a few. Moreover, its multifunctional role in DNA repair, drug-efflux and mediating a TIC phenotype make it an ideal candidate to prevent drug-resistance endowed through a variety of mechanisms. Furthermore, both RSK1 and RSK2 are over-expressed in several types of cancer including prostate (Clark et al., 2005), multiple myelomas (Cuadrado and Nebreda, 2007; Kang et al., 2007), T-cell lymphoma (Kang et al., 2007; Kang et al., 2009) and melanoma (Mirmohammadsadegh et al., 2007) as well as head and neck cancers (Kang et al., 2010). As is the case with TNBC, RSK2 may be the most relevant isoform in these cancers, driving oncogenesis and tumor progression (Kang and Chen, 2011). Therefore in addition to holding promise to improve the treatment of TNBC, RSK inhibitors may have extensive therapeutic applications in a multitude of cancers where the ability to block YB-1 may again prove effective at mitigating chemoresistance.
5.2 Future Directions

The evidence provided herein indicates that immediate future initiatives should focus on advancing RSK inhibitors into pre-clinical models of breast cancer, specifically TNBC. This could be achieved by either advancing current RSK inhibitors, such as BI-D1870, into in vivo models or through the identification of novel RSK inhibitors.

To our knowledge, there are currently no clinically available RSK inhibitors and even RSK inhibitors utilized for research purposes have yet to undergo pharmacokinetic (PK) evaluation in vivo. BI-D1870’s high specificity and potent RSK-inhibitory activity make it a promising candidate to advance as a clinical RSK inhibitor. Moreover, we have provided substantial in vitro evidence of this compound's efficacy. Initial evaluation of its PK profile in mice will be necessary to determine whether BI-D1870 is tolerated and whether it can be sufficiently absorbed into breast tissue to reach therapeutic concentrations. Even if BI-D1870 does not exhibit a favorable PK profile, modifications to the chemical structure through the addition of side groups that increase the molecule's lipophilicity may improve its absorption. The evaluation of RSK inhibitors in orthotopic xenograft models of TNBC is an essential next step to provide pre-clinical, in vivo evidence supporting RSK inhibition for the treatment of TNBC. It will be necessary to demonstrate that RSK inhibitors can suppress TNBC tumor growth in mice. If the PK analysis of BI-D1870 reveals that modification need to be made, it may still prove to be a useful tool compound for providing proof-of-principle for treating TNBC with RSK inhibitors. Different modes of administration may achieve sufficient levels to demonstrate therapeutic benefit at which point further investment in the development of RSK inhibitors will be founded.

An alternative strategy to advancing BI-D1870 would be to identify novel RSK inhibitors. This should be done with the intention of identifying inhibitors with at least equivalent specificity to RSK and potency as BI-D1870. However, prior to additional screens, the effect of RSK1, RSK3 and RSK4 inhibition should be further assessed to determine whether pan- or isoform-specific inhibitors should be sought. In vitro RNA knockdown of individual RSK isoforms (RSK1-4) in TNBC cell lines will elucidate the phenotypic effects of each RSK isoform and can be utilized to guide therapeutic development. In particular, we have not yet investigated the consequences of suppressing RSK3 and RSK4 in TNBC. These
RSK isoforms have tumor-suppressive activity and if this is the case in TNBC then the development of RSK2-specific inhibitors would be a more justified strategy (Roux et al., 2003). The recently established crystal structure of RSK2 identified unique aspects of RSK2 NTKD activation, which could be utilized in bioinformatics screening of drug libraries (Malakhova et al., 2009). There may be sufficient differences between RSK isoforms to allow for somewhat isoform-specific inhibition of RSK2 compared to other RSKs. Alternatively, high-throughput screening for molecules that reduce P-YB-1102 in TNBC after short (~24 h) exposures is another technique for identifying novel RSK inhibitors. This cell-based method would not only identify molecules with RSK inhibitory activity, but would also eliminate compounds that are not cell permeable from further screening. We have optimized the Cellomics ArrayScan VTI for precisely this type of antibody-based, fluorescence quantification in 96-well plates. Short-listed compounds would then be subjected to validation in cell-free kinases assays and immunoblotting for additional RSK targets in a process similar to our validation of luteolin.

Conversely, inhibiting RSK1 kinase activity as well as RSK2 could confer additional therapeutic benefit. We have preliminary data indicating RSK1 inhibition may reduce motility in a murine model of TNBC. This model consists of a non-metastatic cell line (67NR) as well as a highly metastatic, syngenic variant (4T1) that readily metastasizes to the lungs (Aslakson and Miller, 1992). Given our preliminary data, as well as the existing published data indicating that RSK is a principle effector of Ras/MAPK-mediated motility (Doehn et al., 2009; Smolen et al., 2010) it would be very interesting to test whether RSK inhibitors could reduce metastatic dissemination of TNBC. Orthotopic xenotransplant of 4T1 cells into the mammary fat pad of immunocompromised mice and subsequent treatment with BI-D1870 would provide an elegant model for assessing the effect inhibiting RSK has on breast cancer dissemination from its primary site. Positron emission tomography (PET) would be an ideal method for imaging whole-body, metastatic spread as tumor cells exhibit enhanced [(18)F]fluorodeoxyglucose (FDG) uptake due to over-active metabolic pathways.

Finally, in vivo assessment of combinations of RSK inhibitors with current standard-of-care chemotherapy could provide clues as to whether combined therapy could further suppress tumor growth than either therapy alone. Comparing tumor regression between orthotopic
xenograft tumors from TNBC cell lines treated with paclitaxel alone or BI-D1870 and paclitaxel will indicate whether combined therapy improves response. Subsequent ex vivo characterization of residual cells after therapy could then identify whether TICs have been differentially eliminated between either treatment arm. For example, residual cells collected after in vivo treatment could be assayed for TIC component through flow cytometry analysis for CD44+/CD24- marker expression, mammosphere-forming capacity and the ability to re-initiate tumors when serially transplanted. Our in vitro data provides evidence to suggest that the addition of a RSK inhibitor to paclitaxel would eliminate the residual TIC burden. If this proves to be the case in vivo, it would provide strong evidence that RSK inhibitors can eliminate TICs identifying such compounds as a type of TIC-directed therapy.

In summary, pharmacological inhibition of RSK and subsequent inactivation of YB-1 is a promising, novel therapeutic strategy to overcome drug resistance and the future development of RSK inhibitors holds great promise to improve the treatment of TNBC.
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