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Novel Cell Cycle Regulation in Breast Cancer Treatment Resistance

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By

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DECLARATION OF CO-AUTHORSHIP

I. Co-Authorship Declaration

I hereby declare that this dissertation incorporates material that is result of joint research, as follows:

Chapter 2 incorporates the outcome of a joint research undertaken in collaboration with Janice Tubman under the supervision of professor Porter. In all cases, the experimental design, primary ideas, and infection of the cells were performed by the author. The contribution of the co-authors was primarily through the provision of injecting the cells into the in vivo model, data analysis and interpretation.

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ABSTRACT

Classification of breast cancer relies on the presence or absence of estrogen receptor alpha (ERα) and progesterone receptor (PR) as well as the overexpression or amplification of the Her2/neu receptor. Targeted therapies against these proteins has increased the overall 5-year survival rate of breast cancer patients. However, a subset of breast cancer patients can acquire resistance or are initially unresponsive to these therapies. Understanding the molecular pathways that can cause resistance within the various types of breast cancer is of high priority. The cell cycle regulatory factor Speedy (Spy1) has been found to be upregulated in a variety of human cancers, including invasive mammary carcinomas, as well as being downstream of two important pathways in breast cancer initiation and progression; MAPK and c-Myc. My study sought to investigate the role of Spy1 downstream of ERα and to determine its role in regulating treatment response in the presence or absence of ERα. My work defines a novel positive feedback loop whereby Spy1 activates ERK1/2 in a MEK-independent fashion. This activation was further demonstrated to increase the ligand-independent activation of ERα, correlating with a decrease in tamoxifen sensitivity. We tested our findings using an *in vivo* zebrafish model, demonstrating elevated levels of Spy1 alter tamoxifen sensitivity. We further demonstrate significantly high levels of Spy1 within the triple negative group of breast cancers; which correlates with decreased sensitivity to chemotherapy as well as CDK inhibitor treatment. These data could define an efficient mechanism driving proliferation and resistance in select cancers and may represent a potent drugable target.

DEDICATION

This work is dedicated to my loving family. To all of my aunts, uncles and cousins who have encouraged me my entire life and have been a great support system, I can't thank you all enough! To my parents who have always supported me in my quest for knowledge and who taught me that in life you must always keep your integrity. To my aunt Mary and uncle Courtney who have taken the time to help me through every struggle and every problem that has arisen since my day of birth. You were my second set of parents and without you life would be hard and life would be much less enjoyable. I appreciate all of your support and love. To my loving grandparents; Nonna and Pa, who were my biggest fans through the entirety of my educational career, but sadly passed away shortly before they were able to see me finish. Without them I would not be the person I am today. I know from Heaven they are still supporting me and are proud of me pursuing my goal to become a "Dottore." Lastly, I would like to dedicate my work to my fiancée Matt, without whom I would have been too afraid to even enter the Ph.D. program. He is my rock and biggest source of motivation from the beginning to the end.

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LIST OF ABBREVIATIONS/SYMBOLS

AF...*activating function*

AI..*aromatase inhibitor*

ALDH..*aldehyde dehydrogenase*

ARF...*alternate reading frame*

ATP..*adenosine triphosphate*

BRCA1...*breast cancer 1*

CAK..*cyclin activating kinase*

CDK..*cyclin dependent kinase*

CKI..*cyclin dependent kinase inhibitor*

CSC..*cancer stem cell*

DBD...*DNA binding domain*

DCIS..*ductal carcinoma in situ*

DDR...*DNA damage response*

DMEM..*Dulbecco's modified Eagle's medium*

E2..*17β-estradiol*

EGFR..*epidermal growth factor receptor*

EGTA..*ethylene glycol tetraacetic acid*

EMT...*epithelial mesenchymal transition*

ER..*estrogen receptor*

ERE..*estrogen response element*

ERK..*extracellular-signal regulated kinase*

FBS..*foetal bovine serum*

G0...*Gap0*

G1...*Gap1*

PI3K...phosphatidylinositide 3-kinase

PR...progesterone receptor

QRT-PCR...quantitative real time polymerase chain reaction

RB...Retinoblastoma tumour suppressor

RINGO...Rapid Inducer of G2/M progression in Oocytes

RQ..relative quantification

SAC...spindle assembly checkpoint

SCF...Skp1-Cullin1-F-box

SDS...sodium dodecyl sulfate

SERM...selective estrogen receptor modulator

shRNA..small hairpin RNA

Spy1A...Spy1/RINGO A

S/R...Speedy/RINGO

TBST...Tris buffered saline and Tween 20

TMA...tissue microarray

TNBC...triple negative breast cancer

5'UTR...5' untranslated region

WHO..World Health Organization

X-Spy1...Xenopus Spy1

CHAPTER 1

GENERAL INTRODUCTION

Breast Cancer Heterogeneity

Breast cancer is a complex disease with no single cause, affecting 1 in 9 Canadian women (CBCF, 2014). It is a heterogenous disease that can be classified under histological or genetic/molecular classifications. Mammalian female breasts, or mammary glands, undergo growth and development postnatally and can continue to undergo cycles of regeneration and development throughout the life of the organism. Development and regeneration of the female breast is tightly regulated by cascades of hormones and growth factors. While 1% of breast cancer cases occur in males, 99% of the disease occurs in females supporting that these cycles of regulation are an important component in the initiation and progression of a large subset of the disease (Medina, 1996; Silberstein et al., 1994; Visvader and Stingl, 2014). The mature female mammary gland is comprised of rings of epithelial cells called alveoli that are capable of producing milk during pregnancy (Malhotra et al., 2010; Visvader and Stingl, 2014). Several alveoli are grouped together into lobules and share one lactiferous duct, that transports milk from the lobules to the nipples (Malhotra et al., 2010; Visvader, 2009). The alveoli and ductal structures are organized as a 2 cell layer system, an inner luminal epithelial layer and an outer myoepithelial layer. In the alveoli the luminal cells are capable of differentiating into milk producing alveolar cells. Myoepithelial cells are contractile cells which serve the purpose of forcing the milk proteins through the ductal network (Visvader, 2009). This entire network is encased in connective tissue, extracellular matrix and stroma containing adipocytes, fibroblasts and inflammatory cells. The majority of breast cancers arise in the epithelial cells of the lobules or ducts (Malhotra et al., 2010). Breast cancer metastasis requires transit from this organized network into other tissues, such as the

bones, lymphatic system, liver, lung, or the brain (Malhotra et al., 2010; Pestalozzi, 2009; Petrut et al., 2008; Selzner et al., 2000; Shayan et al., 2006). Understanding the cues regulating breast epithelial cells is critical in the successful treatment of this disease.

Classification of Breast Cancer

Histological Classification

Histological classification of breast cancer can be broadly separated into two groups; *in situ* carcinoma and invasive or infiltrative carcinoma. Breast cancer is then sub-categorized depending on where in the tissue it originated; ductal or lobular. Ductal carcinoma *in situ* (DCIS) is more common than lobular carcinoma *in situ* (LCIS) and is further sub-typed dependent upon the characteristics of the tumour (Malhotra et al., 2010). Invasive/infiltrative carcinomas can also be found in the duct or lobules, but there are more subdivisions of invasive tumour types including papillary, medullary, tubular and mucinous carcinomas. Infiltrating ductal carcinoma (IDC) is the most common and affects approximately 80% of invasive lesions (Malhotra et al., 2010; Weigelt et al., 2010). IDC can be further subtyped based on the differentiation status of the tumour; grade I-well-differentiated, grade II-moderately differentiated, and grade III-poorly differentiated (Malhotra et al., 2010).

The more differentiated the tumour cell, the less the cell resembles a stem cell and the greater the ability to specifically target and treat the cancer cells. The existence of cancer stem cells (CSCs) within a tumour has been established in multiple cancers, discovered first in leukemia studies. Clarkson and Fried (1971) found CSCs in leukemia lead to relapse and failure to treat the cancer with chemotherapy (Clarkson and Fried, 1971). CSCs have been correlated with prognosis in medulloblastoma, lung cancer, and

prostate cancer; however, whether CSCs have a role in treatment resistance is still being speculated in the breast (Al-Ejeh et al., 2011). CSCs have three characteristic functions, they can initiate tumourigenesis, are capable of self-renewal, and can differentiate into tumour cells that do not self-renew (Al-Ejeh et al., 2011; McDermott and Wicha, 2010). Breast cancer stem cells are characterized as tumour initiating cells. The CSC theory states that a tumour is composed of cells with tumour initiating and progression potential, while the remaining tumour cells have a low tumourigenic potential (Malhotra et al., 2010). Where CSCs arise and which cell is the cell of origin for the CSCs is still a subject of debate; there is data to support that CSCs can arise from naturally occurring stem cells that are protected in a quiescent state thereby evading apoptosis, enabling a mutated stem cell to pass dangerous mutations to its daughter cells through self-renewal (McDermott and Wicha, 2010; Wicha et al., 2006). There is also data to support the hypothesis that CSCs arise from progenitor cells that gain the capacity to self-renew and give rise to different sub-types (Malhotra et al., 2010; McDermott and Wicha, 2010).

Molecular Classification

The growth pattern and differentiation of a tumour results in a specific histology, classically used to diagnose the disease and guide treatment decisions (Malhotra et al., 2010; Weigelt et al., 2010). The World Health Organization (WHO) has classified at least 17 histological breast cancer subtypes, encompassing different types and grades of tumour (Weigelt et al., 2010). After basic histology, clinicians look at receptor and growth factor status of the cancer with a focus on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (Her)2/neu protein levels (Malhotra et al., 2010). The presence or absence of these pathways provides important

prognostic information and determines the course of treatment. While not currently used as standard practise in the clinic, it is now well established that patients can also be subclassified according to their gene expression signature. The basic molecular signatures can be divided into five main subtypes of breast cancer; luminal A, luminal B, Her2-enriched, basal-like, and claudin-low (Perou et al., 2000).

I) Luminal A

Luminal breast cancers (subtypes A and B) arise from the luminal cells, which line the alveoli and ducts. Luminal A breast cancer is one of the most prevalent subtypes of breast cancers, making up approximately 40% of breast cancer cases (Ethier et al., 1993; Malhotra et al., 2010; Ogba et al., 2014; Zubor et al., 2015). Patients grouped in this subtype are found to be ER positive, PR positive, and Her2 low or negative. Furthermore, this subtype has low expression of the proliferation marker, Ki67, and less than half of the tumours in this group have a mutated *p53* gene; a tumour suppressor gene involved in DNA damage response signalling (Malhotra et al., 2010; Perou et al., 2000). Luminal A patients have the best prognosis with low recurrence rates (Metzger-Filho et al., 2013; Paik et al., 2004).

II) Luminal B

Luminal B breast cancers have a worse prognosis among the luminal cancers. Luminal B tumours comprise approximately 20% of all breast cancer cases. They are characterized as having lower ER expression than luminal A, and are PR and Her2 positive (Ethier et al., 1993; Malhotra et al., 2010; Ogba et al., 2014; Zubor et al., 2015). Furthermore, they have high Ki67 expression and 30% of all luminal B breast cancer cases have a mutated *p53* gene (Malhotra et al., 2010). Survival rates for patients in this

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subtype are still high; however, not as high as luminal A breast cancer survival rates (Anders et al., 2011; Metzger-Filho et al., 2013).

III) Her2-enriched

Her2 is a transmembrane tyrosine kinase protein and is a part of the Her family of human growth factor receptors. Her2 can form homo- or heterodimers with other family members including Her1/epidermal growth factor receptor (EGFR), Her3, and Her4 (Lund et al., 2010; Malhotra et al., 2010; Weigelt et al., 2010). Following dimerization, phosphorylation occurs on the tyrosine residues of the cytoplasmic domain, which activates the downstream pathways, phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) (Lund et al., 2010). The Her2-enriched subtype comprise approximately 10% to 15% of all breast cancer cases. Clinically, 60% of cases labelled as Her2 positive will fall into this subtype. Most Her2-enriched patients have a lower expression of ER and PR; however, 30% to 40% present with ER positive tumours (Perou, 2010; Perou et al., 2000). Furthermore, the Her2-enriched subtype does not necessarily contain Her2 positive or Her2 amplified tumours, some patients are Her2 negative, but are a part of the Her2-enriched subtype due to the similarity of the gene expression profile of the tumour in comparison to those with Her2 positive or Her2 amplification. The Her2-enriched subtype is also characterized by a high expression of Ki67 and approximately 75% of these tumours have a mutated *p53* gene. Patients with this subtype of breast cancer have a poorer prognosis than luminal subtypes and are prone to early recurrence rates and increased metastasis (Lund et al., 2010; Perou, 2010; Perou et al., 2000). Her2-positive patients can be treated with the targeted therapy trastuzumab,

which binds to and inhibits the dimerization of Her2 transmembrane protein (Dean-Colomb and Esteva, 2008; Yaal-Hahoshen and Safra, 2006; Zhang et al., 2015).

IV) Basal-like

The term basal-like breast cancer is used because this subtype has similar features and cytokeratin expression, expressing cytokeratins 5, 6, or 17, as the basal epithelial cells of the skin and airways as well as the basal or outer layer of the mammary ducts (Perou, 2010; Perou et al., 2000; Prat and Perou, 2011). They are characterized as having no expression of ER, PR, or Her2/neu expression or amplification. They have, however, been found to have positive expression of EGFR. Basal-like breast cancer represents approximately 10% to 25% of all breast cancers. The majority of basal-like breast cancers are *p53* mutated and are found to be highly proliferative (Perou et al., 2000). These tumours lack Retinoblastoma protein (pRB) function, which is critical in cell cycle regulation. The loss of pRB and *p53* enhances cell growth and proliferation (Perou, 2010). Moreover, there is a high association of basal-like breast cancers with a mutation in breast cancer 1 type, early onset, susceptibility protein (BRCA1). *BRCA1* is a tumour suppressor gene responsible for DNA repair and when mutated, the DNA repair mechanism cannot fix damaged DNA (Hill et al., 2014). Basal-like breast cancer is labelled as the breast cancer with the poorest prognosis. Targeted therapies do not exist for this subtype and treatment relies solely on chemotherapy. Recurrence and metastasis rates are high in these patients, especially within the first 3 years of treatment, and overall survival of the patients is low (Perou et al., 2000).

V) Claudin-low

Claudin-low breast cancer is a newly categorized subtype. Previously, patients under this subtype were classified as basal-like; however, after DNA microarray studies were performed, it was found that a subset of tumours presented with low levels of the claudin genes. Claudins are needed for epithelial cell tight-tight junctions (Prat et al., 2010). Tumours in this subtype, which make up 5% to 10% of all breast cancers, show low expression for claudins 3, 4, and 7, as well as E-cadherin, a protein required for cell-cell junction (Perou, 2010; Perou et al., 2000; Prat and Perou, 2011). Furthermore, they are normally ER/PR/Her2 negative (Sabatier et al., 2014). Claudin-low tumours have shown an increase in immune cell infiltration, stem cell features, and features representing epithelial-mesenchymal transition (EMT). Some researchers believe claudin-low breast cancers derive from the lobules, mainly because they are associated with high grade tumours, have little differentiation and are able to infiltrate the immune cells (Prat et al., 2010; Sabatier et al., 2014). Similar to basal-like breast cancers, claudin-low breast cancers also have a poor prognosis and cannot benefit from targeted therapy, and, therefore, only chemotherapy is used as a form of treatment (Perou et al., 2000).

Characteristics of Triple Negative Breast Cancer

The Her2-enriched, basal-like, and claudin-low breast cancers share one common feature; all have the potential to encompass a special group of breast cancer, triple negative breast cancer (TNBC). TNBC are generally characterized as being negative for ER, PR, and Her2 (Perou, 2010; Perou et al., 2000). Basal-like breast cancer accounts for over 50% of all TNBC cases and all claudin-low breast cancers are triple negative. TNBCs are also found to be less differentiated, have increased proliferative capacity,

have a poor prognosis, and the rate of relapse is significantly increased within the first 3 years of chemotherapy treatment (Malhotra et al., 2010; Perou, 2010; Perou et al., 2000; Sabatier et al., 2014). Molecular and immunohistochemical profiles have been produced to investigate a possible molecular signature for TNBC. TNBC profiles show specific expression of myoepithelial and basal markers as well as *p53* gene mutations and gene amplification and overexpression of the transcription factor, c-Myc (Kreike et al., 2007).

ER Signalling; A Central Driver of Luminal Breast Cancers

ER Structure and Signalling

The ER is a part of the nuclear receptor protein superfamily that can act as a transcription factor by binding to estrogen response elements (EREs) on DNA either as a monomer or homodimer. There are two isoforms of ER, ER alpha (ERα) and ER beta (ERβ). The two isoforms have opposing roles in the proliferation and differentiation of breast cancer (Kampa et al., 2013). Biological functions mediated by mitogenic effects are governed by ERα (Brisken and Ataca, 2015; Kampa et al., 2013; Morani et al., 2008; Musgrove et al., 1993), whereas ERβ has more of a tumour suppressive role (Kampa et al., 2013; Rizza et al., 2014). ERβ can inhibit specific ERα gene expression targets, and has been considered a partial dominant negative receptor. When ERβ is co-expressed with ERα, patient prognosis is much more favourable and has a less aggressive phenotype (Rizza et al., 2014).

ERα can be bound by steroid hormones, thyroid hormones, retinoids, and vitamin D3. Most commonly, it is bound to and activated by 17β-estradiol (E2) (Musgrove et al., 1993). ERα is characterized by 3 principle domains; the N-terminal domain (NTD), a highly conserved DNA binding domain (DBD) and a ligand binding domain (LBD). ERα

also contains two activation function domains (AF1 and AF2), which reside in the NTD and LBD, respectively. ERα contains two other regions; the D-region or hinge region, found between the DBD and LBD, which contains the nuclear localization signal, and the F-region, which follows the LBD and is found to be important in receptor dimerization (Figure 1) (Kallen et al., 2007; Kumar et al., 2011). The AF1 and AF2 are responsible for the transcriptional activity of ERα. AF2 is ligand-dependent and promotes classical signalling of ERα through direct binding of EREs and activation or repression of specific genes (Figure 2A). When E2 binds to the hormone binding pocket of the LBD, helix 12 realigns and exposes a hydrophobic motif (LXXLL), which allows for the binding of cofactors. 'Non-classical' genomic signalling also exists where the E2-ERα complex interacts with co-activators Fos and Jun, to transcriptionally regulate genes like Cyclin D1 that do not contain EREs (Figure 2A) (Castro-Rivera et al., 2001; Gottlicher et al., 1998; Musgrove et al., 1993; Planas-Silva and Weinberg, 1997). The AF1 region within the NTD does not require the binding of E2 for activation (Kumar et al., 2011; Shiau et al., 1998; Tanenbaum et al., 1998). This mode of ligand independent activation is triggered when amino acid residues within the AF1 region are modified through phosphorylation (Benecke et al., 2000; Kumar et al., 2011; Tanenbaum et al., 1998). This post-translational modification results in ERα dimers that can complex directly with G-proteins, receptor tyrosine kinases, and non-receptor tyrosine kinases triggering downstream pathways, such as the Ras/MAPK and the PI3K/Akt pathways (Figure 2B) (Levin, 2005; Likhite et al., 2006). When fully phosphorylated, ERα dimers can activate both classical and non-classical transcriptional signalling.
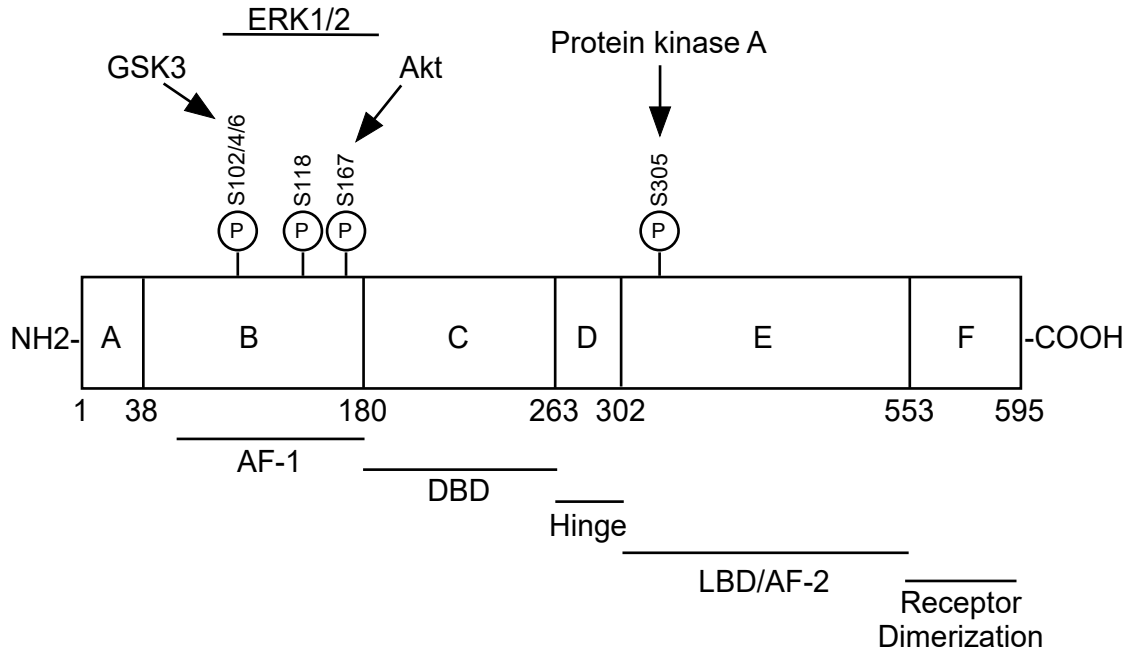
Figure 1. Schematic representation of estrogen receptor domains. The estrogen receptor structure contains domains A-F. A/B make up the N-terminal domain (NTD). Within domain B there is the activating function (AF)1, which regulates ligand-independent signalling. Phosphorylation sites within the NTD are dependent upon ERK1/2, GSK3, and Akt signalling. The C region contains the DNA binding domain (DBD). The D region or hinge region, contains the nuclear localization signal. The E region contains the ligand binding domain and AF-2 region. This domain promotes classical and non-classical genomic signalling. Finally, the F region promotes receptor dimerization.
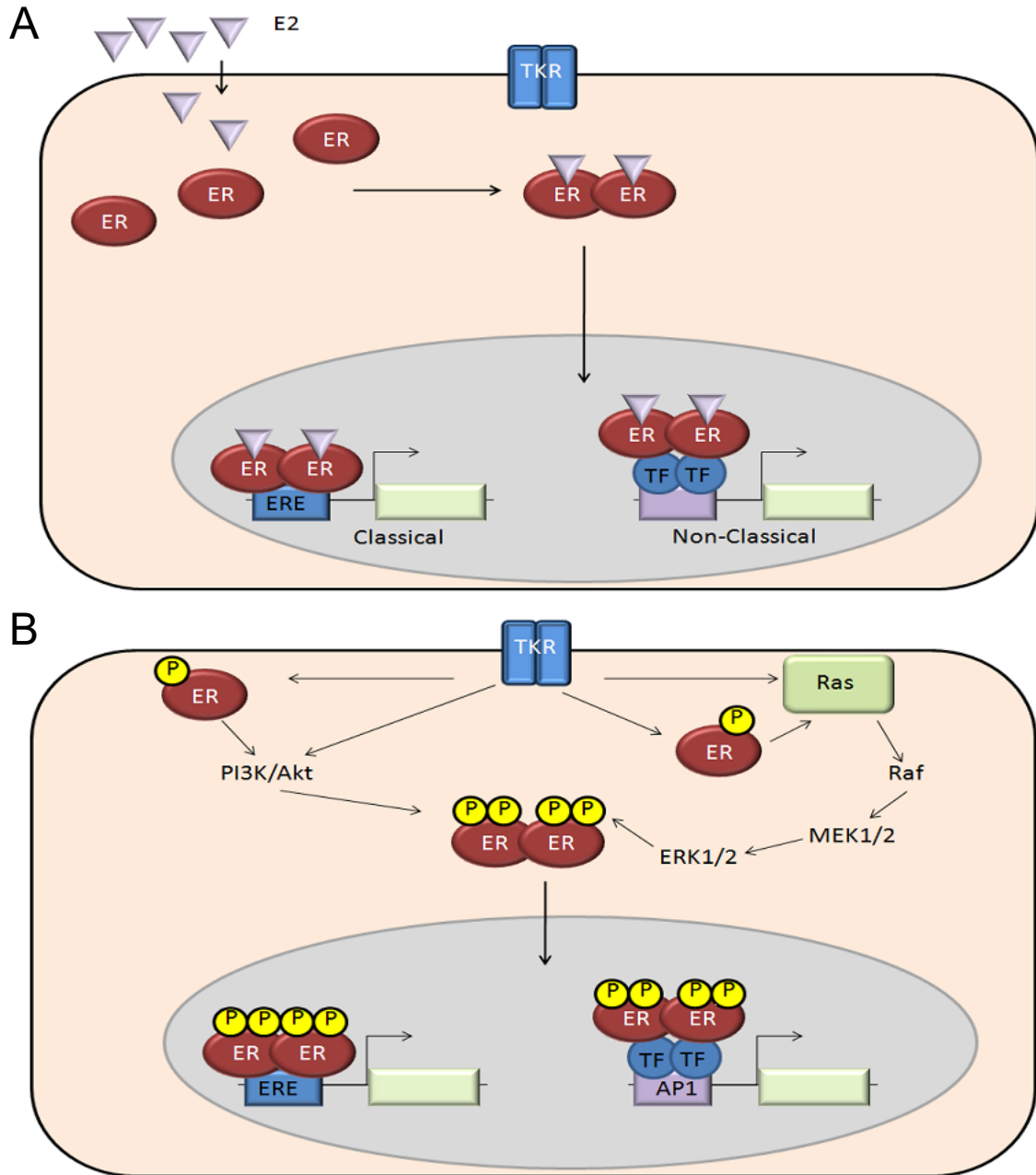
Figure 2. Schematic diagram of estrogen receptor signalling. (A) Classical activation of ER targets require E2 binding (E2-ER), E2-ER dimerization, and binding of estrogen response elements (ERE). Genes without EREs are activated by non-classical genomic signalling, where after dimerization, transcription factors such as Fos and Jun tether the E2-ER dimers to specific promoters to initiate transcription. (B) Non-genomic signalling. After ER alpha is stimulated by growth factors, cytoplasmic signalling cascades, such as the Ras-Raf-MEK-ERK pathway and the PI3K/Akt pathway, are activated. The ER can be activated through phosphorylation by tyrosine kinase receptor (TKR) or through the signalling cascades, promoting ligand-independent signalling.

Post-Translational Modification of ERα

ERα is modified on many different sites, all influencing ERα function. ERα modification can lead to stability as well as non-genomic signalling; acetylation can activate or inhibit transcription, depending on which site is modified, sumoylation can activate transcription and assist DNA binding, and palmitoylation promotes nuclear localization (Le Romancer et al., 2011). A primary and well-documented form of modification is phosphorylation (Bunone et al., 1996). Phosphorylation can facilitate ligand binding and classical activation of the ER, it can also alter the efficiency by which select ligands can bind, and it can promote ligand-independent signalling (Kumar et al., 2011).

One of the primary pathways to modify ERα is the MAPK pathway. Extracellular signal-related kinase (ERK)1/2 is the final kinase within the MAPK pathway; following Ras-Raf-MEK1/2 signalling. The MAPK pathway is an important mitogen-driven pathway found to be hyper-activated in 30% of human cancers (Giltnane and Balko, 2014; Huynh et al., 2003). In breast cancer, elevated levels of ERK1/2 are positively correlated with more aggressive tumour formation (Cui et al., 2006; Giltnane and Balko, 2014); partially through its activation of the proto-oncogene c-Myc, which high levels have been linked to lower ERα levels and a basal-like genomic subtype (Dimitrakakis et al., 2006; Musgrove et al., 2008a; Riggins et al., 2007). ERK1/2 can modify ERα through phosphorylation on multiple residues, with primary sties being serine (S)102/4/6, S118, S167, and S305 (Chen et al., 2013; Thomas et al., 2008). These sites can also be modified by glycogen synthase kinase (GSK)3 (S102/4/6, S118), Cyclin A-cyclin dependent kinase (CDK)2 complex (S104/S206), CDK7 (S118), mTOR (S118), IKKα (S118), and

PI3K/AKT (S167) (Chen et al., 2000; Chen et al., 2002; Cheng et al., 2007; Thomas et al., 2008). The resulting function of the ERα can differ when modified by a differential pathway, likely indicating that other changes/binding partners cooperate in the final response to treatment (Bunone et al., 1996).

Cancer Initiation and Progression Depends on Abnormal Cell Cycle Regulation

All cells are under the regulation of the cell cycle and at a pivotal level the cell cycle regulates growth, differentiation and decisions to undergo senescence and apoptosis. In many cancers, including breast cancer, there is a disruption of the core machinery driving the cell cycle (Collins et al., 1997). How the cell cycle is altered in specific cancers impacts the growth characteristics of that cancer and also determines how the cancer will respond to therapies that depend on a cell cycle arrest and apoptosis.

The cell cycle is made up of interphase; comprising Gap phase 1 (G1), DNA replication phase (S), and Gap phase 2 (G2), and mitosis (M). When conditions are not favourable for growth, the cell will enter a state of quiescence (G0). Quiescent cells do not enter S phase and stay metabolically active, awaiting cell cycle re-entry (Salomoni and Calegari, 2010). Each phase of the cell cycle is regulated by the oscillating accumulation of proteins referred to as cyclins, which are selectively expressed and degraded at different phases. Their catalytic partners, the CDKs, are expressed at a constant level although, enzymatically, they are inactive until bound by their cyclin binding partner (Solomon, 1993).

Activation of CDKs

The active site of the CDK, where ATP binds, is found deep within a cleft. CDK substrates interact with this active site; however, in an inactive CDK, a T-loop blocks this

site to suppress its activity (Gu et al., 1992; Jeffrey et al., 1995). Cyclin binding induces a conformational change, which exposes the catalytic cleft and presents the CDK with a domain essential for substrate selection and binding (Bourne et al., 1996; Brown et al., 1999; Holmes and Solomon, 2001; Schulman et al., 1998). Binding of cyclins does not fully activate the CDK. Full activation of the CDK requires posttranslational modifications. CDK activating kinase (CAK), which is composed of CDK7, Cyclin H, and Mat1, phosphorylates a threonine residue found on the T-loop of the CDK. Phosphorylation of this specific residue flattens the T-loop, moving it near the cyclin. This creates a binding site for substrates that contain a consensus sequence ((S/T)PX(K/R)) (Bourne et al., 1996; Gould et al., 1991; Holmes and Solomon, 2001; Jeffrey et al., 1995; Solomon and Kaldis, 1998). Cyclins also have a hydrophobic patch, which is characterized by an MRAIL motif. This patch has the ability to bind to a CDK substrate with moderate affinity if the substrate has the complementary RXL sequence. This extra interaction increases the affinity of the kinase for its substrate (Brown et al., 1999; Horton and Templeton, 1997; Loog and Morgan, 2005; Parker et al., 1992; Schulman et al., 1998; Solomon and Kaldis, 1998; Watanabe et al., 1995; Welburn et al., 2007). Furthermore, inhibitory phosphorylation of threonine (T)-14 and tyrosine (Y)-15 by Wee1 and Myt1 kinases must be removed by the Cdc25 phosphatases (Parker et al., 1992; Solomon and Kaldis, 1998; Watanabe et al., 1995; Welburn et al., 2007). Three isoforms of Cdc25 exist, each regulating specific cyclin-CDK complexes (Donzelli and Draetta, 2003; Karlsson-Rosenthal and Millar, 2006). Cdc25A dephosphorylates Cyclin E-CDK2, Cyclin A-CDK2 and Cyclin B-CDK1, promoting entry into S phase and progression into G2/M transition. Cdc25B and Cdc25C only dephosphorylate Cyclin B-

CDK1 and, therefore, promote entry into M phase (Donzelli and Draetta, 2003; Karlsson-Rosenthal and Millar, 2006). Specific formation of cyclin-CDK complexes and their subsequent activation govern each phase of the cell cycle.

G1 Phase Regulation

G1 phase prepares the cell for replication. This phase is controlled by D-type cyclins bound to CDK4/6 and E-type cyclins bound to CDK2. From early to mid-G1 phase, Cyclin D-CDK4/6 forms a complex and controls the cell cycle. Three D-type cyclins, D1, D2, and D3, are expressed differently depending on cell lineage. Initially, growth factors stimulate the expression of Cyclin D1 (Lim and Kaldis, 2013; Salomoni and Calegari, 2010). The binding and activation of Cyclin D1-CDK4/6 triggers the phosphorylation of pRB (Smith and Nevins, 1995; White et al., 2005). The RB protein plays an important role as a checkpoint regulator in G1, known as the restriction point, to block entry into S-phase. pRB is normally bound to E2F, a family of transcription factors. When pRB is hyperphosphorylated, it releases E2F, which then promotes the transcription of a number of genes, including the two isoforms of E-type Cyclins, E1 and E2 (Nevins et al., 1991; Smith and Nevins, 1995; White et al., 2005). Cyclin E activation of CDK2 occurs in late G1 phase and promotes entry into S-phase. Cyclin E-CDK2 complex continues to phosphorylate pRB, inhibiting its function as a transcriptional repressor (Horton and Templeton, 1997; Hwang and Clurman, 2005; White et al., 2005).

S- and G2 Phase Regulation

S-phase allows for DNA replication. As stated previously, the complex controlling S-phase is Cyclin A-CDK2. Cyclin A is required for the progression through S-phase and also controls entry into G2 (Brown et al., 1995). Cyclin A is a unique cyclin,

such that it can bind to and activate CDK2 and CDK1 (in G2 phase). No other classical cyclin has the ability to activate two CDKs (Arellano and Moreno, 1997).

G2 phase prepares the cell for mitosis. This phase requires Cyclin A to bind to and activate CDK1, but it also requires Cyclin B to bind to and activate CDK1 (Lindqvist et al., 2009; Solomon et al., 1990). These complexes phosphorylate specific transcription factors, such as FoxM1; a member of the forkhead box (Fox) superfamily (Laoukili et al., 2008). Activation of transcription factors upregulates target genes/regulators required for mitosis and the spindle assembly checkpoint (Rattani et al., 2014). Prior to entry into mitosis, cells undergo another checkpoint to check for DNA damage. If damage is detected, CDK1 is inhibited and blocks entry into mitosis (Arellano and Moreno, 1997).

CDK Inhibitors (CKIs)

G1/S phase of the cell cycle can be transiently inhibited by a family of CKIs, the Cip/Kip family. This family includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip1}$. These CKIs can inhibit not only Cyclin E-CDK2, but also the S-phase complex, Cyclin A-CDK2. This family of CKIs inhibit the cyclin-CDK complexes by binding to both the hydrophobic MRAIL patch on the cyclin and a large domain on the CDK. This interaction alters the conformation of the complex, limiting access to the catalytic cleft (Brown et al., 1995; Nakayama, 1998). Cyclin D-CDK4/6 sequesters $p27^{Kip1}$, to hinder its inhibitory actions on the other cyclin-CDK complexes. The sequestered $p27^{Kip1}$ is released once mitogen signalling has ended. This, in turn, enables $p27^{Kip1}$ to bind to and inhibit Cyclin E-CDK2 activation and S-phase entry (Cheng et al., 1999; Nakayama, 1998; Promwikorn et al., 2000). Cyclin E-CDK2 also has the ability to phosphorylate $p27^{Kip1}$ on threonine (T)-187, which is a specific residue for ubiquitin mediated degradation of $p27^{Kip1}$ by SCF^{Skp2}

(Alessandrini et al., 1997). Another family of CKIs, specific only for CDK4/6 is the Ink4 family, consisting of p16^{Ink4a} or its alternate reading frame (ARF) p14^{ARF}, p15^{Ink4b}, p18^{Ink4c}, p19^{Ink4d} and p19^{ARF} (Bandoh et al., 2005; Nakayama, 1998; Sangfelt et al., 1997). This family of inhibitors specifically binds to monomeric CDK4 or 6, inducing a conformational change, effectively inhibiting the ability of Cyclin D to bind and activate the complex (Nakayama, 1998). Regulation of G1 to S phase by these cyclin-CDK complexes have an important role in the initiation and progression of breast cancer.

D-type Cyclins in Breast Cancer

In breast cancer, Cyclin D1 and D3 have been found to be upregulated in ERα positive breast cancers (Kenny et al., 1999; Peurala et al., 2013). E2 activation of ERα targets Cyclin D1 to promote its mitogenic effects. There have been positive correlations between Cyclin D1 levels and a positive ERα status (Barone et al., 2006; Castro-Rivera et al., 2001). Furthermore, upregulated Cyclin D1 is mainly present in low grade tumours that are well-differentiated and slow-growing. No specific correlation has been made with regard to PR or Her2/neu status. Cyclin D1 is amplified in 20% of all breast cancers, while over 50% of breast cancers have Cyclin D1 overexpressed (Kenny et al., 1999; Perez-Roger et al., 1999; Weroha et al., 2006). The other two isoforms of Cyclin D have not been as highly documented as Cyclin D1; however, it was discovered that breast cancer progression correlates with a loss of Cyclin D2 expression (Evron et al., 2001). Furthermore, in Her2-induced breast cancers, it was found that Cyclin D1 expression is downregulated, while Cyclin D3 levels were significantly elevated (Zhang et al., 2011).

E-type Cyclins in Breast Cancer

Both E-type cyclins have been associated with breast cancer in different ways; however, both are regulated by estrogen signalling. Both Cyclin E isoforms have been found to be overexpressed and amplified, but there are also cases of Cyclin E truncations which form constitutively active Cyclin E complexes (Dhillon and Mudryj, 2002; Hwang and Clurman, 2005; Keyomarsi et al., 2002). Using a database with 863 breast cancer patient transcripts, Caldon *et al.* (2012) showed both Cyclin E1 and Cyclin E2 genes, *CCNE1* and *CCNE2*, respectively, increased with progression from benign breast cancer to DCIS to IDC, as well as correlating with an increase in tumour grade (Caldon et al., 2012). However, both genes were found to be differentially expressed in different subtypes. *CCNE1* was found to be upregulated in basal-like breast cancers, and *CCNE2* was higher in both basal-like breast cancers and Her2 amplified (Caldon et al., 2012; Scaltriti et al., 2011). Cyclin E2 has been correlated with poor disease outcome in ERα positive breast cancers (Keyomarsi et al., 2002).

The Potent Mammary Oncogene c-Myc

The transcription factor c-Myc can regulate approximately 10% to 15% of the human genome, having potent effects on cell proliferation, differentiation, apoptosis, and senescence (Amati and Land, 1994; Amati et al., 1993; Evan et al., 1994; Evan et al., 1992), which has made c-Myc a desirable target for many different cancers. c-Myc activates transcription by forming a complex with its binding partner MYC-associated factor X (Max) (Amati et al., 1992; Littlewood et al., 1992). This complex binds to E-box elements containing the consensus sequence CACGTG (Amati, 2004). c-Myc not only transcriptionally activates other genes, it can also repress genes by binding to MYC-

interacting zinc finger protein-1 (Miz-1) (Adhikary and Eilers, 2005). c-Myc expression activates the cell cycle by activating Cyclin E bound to CDK2 as well as repressing the CDK inhibitor (CKI), p21[Cip1] (Amati et al., 1998). Repression of p21[Cip1] increases G1 to S phase entry into the cell cycle, as well as cell differentiation (Wu et al., 2003). c-Myc has also been shown to inhibit the transcription of another CKI, p27[Kip1], while activating Cyclin D1, CDK4, Cdc25A, and the E2F family of transcription factors (Xu et al., 2010). Comparatively, c-Myc can also trigger cell cycle arrest and apoptosis. c-Myc activates the tumour suppressor p19[ARF], which then antagonizes the regulation of Mdm2 on p53. This stabilizes p53 and induces the activation of pro-apoptotic genes (Tao and Levine, 1999; Weber et al., 2000; Wu et al., 2003; Xu et al., 2010). Furthermore, c-Myc can suppress Ras-induced senescence with aid from CDK2. CDK2 has been shown to phosphorylate c-Myc on Serine 62 (S62), which is a stabilization site (Hydbring et al., 2010; Hydbring and Larsson, 2010a; Hydbring and Larsson, 2010b; Sears et al., 1999). When CDK2 binds c-Myc and phosphorylates S62, it is correlated with low expression of genes which activate senescence, p21[Cip1] and p16[INK4A], and higher expression of hTERT and BMI1, which are genes that suppress senescence (Campaner et al., 2010; Hydbring et al., 2010; Hydbring and Larsson, 2010a). However, in CDK2 null cell lines, c-Myc was found to induce senescence when p53/p21 and p16-pRB pathways were intact, which are the same pathways that are normally upregulated by Ras (Campaner et al., 2010). The high complexity of c-Myc regulatory function creates a sensitive balance between regulation required for normal growth and development and the development of carcinogenesis. When the balance is shifted towards carcinogenesis, the high degree of c-

Myc regulatory control over the genome creates aggressive phenotypes, which are found to be difficult to treat.

Breast Cancer Therapies

Treatment Strategies for Luminal Breast Cancers

Luminal breast cancer therapies have a higher success rate over the other forms of breast cancer because of the presence of hormone receptors. Early stages of luminal breast cancers are treated with surgery followed by radiotherapy. To combat recurrence, adjuvant treatments are administered (Al-Ejeh et al., 2011; Prat et al., 2012). ERα positive cancers are usually targeted through the use of selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) (Dowsett and Haynes, 2003; Lumachi et al., 2013); both of which aim to inhibit estrogen signalling.

SERMs, such as tamoxifen, are nonsteroidal compounds that antagonize ERα by acting as a competitive inhibitor for E2 binding (Connor et al., 2001; de Leeuw et al., 2011). The structure of tamoxifen is similar to E2, but it lacks a second hydroxyl group and has a dimethylaminoethyl sidechain that extends from the C-ring of tamoxifen (Shiau et al., 1998). These differences cause conformational changes to ERα, such that co-activators no longer bind. For instance, the side-chain on tamoxifen will extend between helices 3 and 11 of ERα creating new hydrophobic interactions (Shiau et al., 1998). One of the most significant changes to ERα when tamoxifen binds is the positioning of helix 12. Helix 12 gets repositioned such that it covers the hydrophobic motif. Co-activators will no longer bind, ERα will remain inactivated, and the downstream genes will not be transcribed (de Leeuw et al., 2011; Ring and Dowsett, 2004; Shiau et al., 1998; Vendrell et al., 2005). Tamoxifen has been used alone or in combination with AIs.

AIs block the enzyme aromatase, which reduces the amount of estrogen circulating in the body. Aromatase, also known as cytochrome P450 aromatase, is an enzyme that metabolizes testosterone to E2. There are two types of AIs; type I are steroidal, which is a permanent inhibition of aromatase, type II are nonsteroidal, they competitively inhibit aromatase and are reversible. The intention of AIs is to inhibit the production of E2 to decrease the activity of ERα and its downstream targets (Dowsett and Haynes, 2003; Lumachi et al., 2013).

Treatment of ERα-Negative Breast Cancer

Breast cancer subtypes that cannot be treated with targeted therapies are treated through surgery, radiation, chemotherapy, or a combination of these treatments (Al-Ejeh et al., 2011; Raguz and Yague, 2008). Chemotherapy treatment has significantly increased overall survival of breast cancer patients. There are many forms of chemotherapy drugs with over 100 currently in use; most are designed to take advantage of a functioning and rapid cell division cycle and all intend to inhibit the cell cycle to promote cell death, or apoptosis. A disadvantage of this treatment is chemotherapy cannot distinguish between a cancer cell and a normal cell (Raguz and Yague, 2008). Understanding each phase of the cell cycle and its regulators is essential in ensuring proper administration of chemotherapy drugs to optimize their effects on cancer cells.

Function of Classes of Chemotherapy Agents

Alkylating Agents

Alkylating agents directly damage DNA by adding an alkyl group to the guanine base of DNA, forming a cross-link. Cross-linking holds the DNA in a coiled position unable to separate, thereby preventing DNA synthesis (Kennedy et al., 1995). A common

class of alkylating agents are the nitrogen mustards; one example of this is cyclophosphamide. Cyclophosphamide is rapidly absorbed and then metabolised by cytochrome P450 enzymes in the liver to its active metabolites. The main metabolites are 4-hydroxycyclophosphamide and aldophosphamide (Emadi et al., 2009; Hall and Tilby, 1992). Most aldophosphamide can be oxidised by aldehyde dehydrogenase (ALDH) to produce carboxycyclophosphamide; however, some aldophosphamide gets broken down into two compounds, phosphoramide mustard and acrolein. Most of the effects seen with cyclophosphamide administration are due to phosphoramide mustard. This metabolite is present only when cells have low levels of ALDH and is highly toxic to cancerous and normal cells. Alkylating agents have a grave disadvantage to their use (Hall and Tilby, 1992; Kohn and Sladek, 1985); causing long-term damage to the bone marrow of a patient, which often leads to acute leukemia (Kohn and Sladek, 1985; Lohrmann, 1984).

Platinum based drugs fall in with the alkylating agents because they have a similar mechanism for damaging the DNA (Rosenberg et al., 1969; Wang and Lippard, 2005). Platinum drugs, such as cisplatin, carboplatin and oxaliplatin, differ from other alkylating agents in that they do not contain an alkyl group. After administration, one of the chloride ligands in a platinum drug is displaced by water, allowing a platinum atom to bind to the bases of DNA, preferably guanine. This crosslink promotes the displacement of the second chloride ligand, followed by the binding of the second platinum atom, preferably with another guanine (Siddik, 2003). The formation of cross-links interferes with cell division, triggering the DNA repair machinery, which will, in turn, activate the apoptotic pathway if damage cannot be fixed. Platinum-DNA adducts do not get metabolized into harmful by-products and, hence, this class of drugs is less toxic to

normal cells and are less likely to lead to leukemia in the future (Pruefer et al., 2008; Rosenberg et al., 1969; Siddik, 2003; Wang and Lippard, 2005).

Anti-tumour Antibiotics

Anthracyclines, such as the drug doxorubicin, are classified as an anti-tumour antibiotic. They can work in all phases of the cell cycle; however, they have preference for interfering with the enzymes involved in DNA replication; therefore, most effects are seen in S-phase (Minotti et al., 2004; Pommier et al., 2010; Weiss, 1992). Anthracyclines have four mechanisms of action. They can inhibit DNA and RNA synthesis through intercalation with the DNA or RNA (Weiss, 1992), they can inhibit topoisomerase II, the enzyme responsible for the separation of the DNA strands so they can be transcribed (Pommier et al., 2010), they can generate free oxygen radicals, which then damage DNA, proteins and cell membranes (Weiss, 1992), and, lastly, they can provoke histone eviction from chromatin, which leads to activation of the DNA damage repair pathways or activation of apoptosis (Pang et al., 2013). Long-term use at high dosages of anthracyclines can permanently damage the heart and can increase the risk of a second cancer, such as acute myelogenous leukemia (Minotti et al., 2004; Weiss, 1992).

Mitotic Inhibitors

Most mitotic inhibitors are derived from plant alkaloids and other natural products (Jordan and Wilson, 1998; Jordan and Wilson, 2004). They inhibit M-phase of the cell cycle, but damage of cells in other phases has also been discovered (Bharadwaj and Yu, 2004). One common mitotic inhibitor is paclitaxel. When paclitaxel is administered, it is metabolized by isoenzymes, CYP2C8 and CYP3A4 (Marsh et al., 2007), to primarily produce the active metabolite 6-α-hydroxypaclitaxel. 6-α-hydroxypaclitaxel stabilizes

microtubules and shields the polymer from disassembly (Ganguly et al., 2010; Jordan and Wilson, 2004). This function leads to defects in spindle assembly, segregation of chromosomes, and, ultimately, cell division. This blocks mitosis and delays the spindle assembly checkpoint (SAC), which then activates apoptosis. A downfall of this class of inhibitors is that it has the potential to cause peripheral nerve damage (Bharadwaj and Yu, 2004; Brito et al., 2008; Lohrmann, 1984).

Breast Cancer Treatment Resistance

Resistance to Hormone Therapy

Breast cancer mortality rates have decreased 43% since 1986; this success can be attributed in part to the availability of targeted reagents (CBCF, 2014). However, despite high rates of initial response to treatment approximately 30% of hormone receptor positive patients fail to respond to tamoxifen treatment and have poor prognosis to endocrine treatment (Schiff et al., 2003). Tamoxifen resistance can occur either *de novo*, at the beginning of a patient's treatment, or the patient can acquire resistance after prolonged tamoxifen treatment (Osborne and Schiff, 2011; Schiff et al., 2003).

Lack of ERα expression and/or function is one main mechanism towards tamoxifen resistance. *De novo* resistance is driven by the lack of ERα expression when a histological sample is being characterized, and these negative tumours will receive alternate form(s) of therapy. Most patients who are ERα-positive prior to treatment remain so upon relapse (Ring and Dowsett, 2004; Schiff et al., 2003); however, Dowsett *et al*. (2003) found 17% of patients who were ERα-positive before tamoxifen treatment became ERα-negative upon relapse. This showed that ERα expression can be lost during tamoxifen treatment; likely due to a downregulation of ERα (Dowsett and Haynes, 2003).

Multiple splice variants of ERα have also been discovered. One ERα isoform, ERα46, lacks exon 1 and the AF1 domain (Flouriot et al., 2000). Another, ERα36, has an alternate transcription initiation site in intron 1. ERα36 lacks AF1, part of AF2, and has a unique amino acid sequence on the C-terminal end, within exon 9 (Kampa et al., 2013; Wang et al., 2005; Wang et al., 2006). ERα36 has been found in ERα positive and negative breast cancers (Lee et al., 2008). This isoform responds to E2 as well as anti-estrogens, inducing membrane-initiated signalling cascades. Furthermore, ERα36 can stimulate proliferation and can contribute to a more aggressive breast cancer phenotype (Lee et al., 2008; Wang et al., 2005; Wang et al., 2006). Other variants have been found in cancer cells, all of which lack the 5'UTR of the receptor. All of the isoforms can heterodimerize with the wild-type, full-length ERα and repress AF1 activity (Kampa et al., 2013). These characteristics are pertinent in the classification of breast cancer, especially when molecular signatures are analysed and may be important in assessing response to therapy.

Mutations within the ERα gene, *ESR1*, can lead to the development of a functionally inactive ERα, although expression of ERα will still be present. This could cause a false-positive during histological classification (Ring and Dowsett, 2004). Mutations occurring in *ESR1* can alter the binding of anti-estrogens, making ERα less sensitive to their inhibition and more sensitive to E2 signalling (Fuqua et al., 2000; Herynk et al., 2010). One such mutation could be an alteration in phospho-sites within the AF1 domain; this could result in a conformational change or by enhancing the binding of co-activators even in the presence of low E2 levels (Cheng et al., 2007; Connor et al., 2001; Fuqua et al., 2000; Thomas et al., 2008; Yamashita et al., 2008).

Alterations in post-translational modification of ERα is a common mechanism of endocrine resistance. Phosphorylation on (S)102/4/6 by ERK1/2 stabilizes ERα and controls transcriptional activity upon ligand binding (Thomas et al., 2008). S118 phosphorylation by ERK1/2 in the absence of a ligand can render ERα hypersensitive to E2 and insensitive to SERMs; however, phosphorylation of this same site by CDK7 occurs in the presence of a ligand and indicates that ERα is active, properly functioning and responsive to treatment (Bunone et al., 1996). S167 phosphorylation by PI3K/Akt and ERK1/2 is associated with conflicting clinical data; at times phosphorylation at this site has indicated an increased sensitivity to SERM treatment, while other patient data has been correlated with lack of response or future relapse (de Leeuw et al., 2011; Guo et al., 2010; Huderson et al., 2012; Weitsman et al., 2009; Yamnik and Holz, 2010). Lastly, S305, which is present in the hinge region of ERα, is phosphorylated by protein kinase A and is correlated with resistance to SERM treatment. This site is important for the control of ubiquitination and proteasomal degradation of ERα (Bostner et al., 2010; Kumar et al., 2011). There are many more phosphorylation sites in ERα, research unravelling the biology of the structure and function of ERα will continue to inform about the mechanisms by which SERM resistance can occur.

Resistance to Chemotherapy

Innate chemotherapy resistance refers to the total lack of patient response to a given therapy, whereas acquired resistance implies that a patient develops resistance after an initial response. Breast cancer patients treated with anthracycline and/or paclitaxel can develop acquired resistance to one or both of the drugs (Raguz and Yague, 2008). There are also examples where after prolonged exposure to one form of therapeutic,

development of acquired resistance to multiple drugs occurs, this is termed cross-resistance. Innate and acquired chemotherapy resistance occurs via multiple mechanisms, including drug metabolism, changes in drug target expression or function, DNA damage repair modifications, or altered apoptotic signalling pathways (Raguz and Yague, 2008; Rivera and Gomez, 2010).

The cytochrome P450 enzymes are a family of enzymes found in the liver that induce metabolism of chemotherapeutic drugs. In cancer, they are often overexpressed and this overexpression contributes to drug resistance through alteration of metabolic pathways (Marsh et al., 2007; Raguz and Yague, 2008). Chemotherapy drugs require appropriate activation of metabolic pathways to ensure adequate delivery and access to the tumour tissue. Mutations in topoisomerase II or altered activity prevents anthracyclines from binding and, thus, leads to the repair and transcription of the damaged DNA strands. DNA damage response pathways are regulated by the tumour suppressor gene, *p53*; however, *p53* is one of the most common molecular mutations in breast cancer. Alterations in the DNA damage response pathways can lead to the evasion of apoptosis or senescence (Pommier et al., 2010; Raguz and Yague, 2008; Rivera and Gomez, 2010).

Paclitaxel resistance occurs widely in breast cancer patients with altered or overexpressed tubulin, mutations in tubulin that affect the stabilization of the microtubules, or post-translational modifications on tubulin (Jordan and Wilson, 1998; Jordan and Wilson, 2004). An important goal in the breast cancer field is to develop therapies that can work alone or in combination with standard of care chemotherapy to override known causes of resistance. Chemotherapy agents require an active cell cycle;

therefore, understanding the role of the cell cycle in cancer progression can increase the likelihood of developing novel treatment options.

Cell Cycle Alterations in Breast Cancer Resistance

Gene signatures for cancer progression in tamoxifen-resistant cancers or metastatic cancers have shown Cyclin E2 to be elevated, whereas Cyclin E1 is absent (Caldon et al., 2012; Muller-Tidow et al., 2001). Furthermore, higher levels of the Cyclin E2 gene, *CCNE2,* were shown to have a shorter distant metastasis-free survival after endocrine therapy (Muller-Tidow et al., 2001). Inhibitors of G1/S phase of the cell cycle are also altered. Increased phosphorylation and degradation of p27^{Kip1} has been seen in tumourigenesis (Abukhdeir and Park, 2008; Sheaff et al., 1997) and low levels of both p27^{Kip1} and p21^{Cip1} correlates with various resistant phenotypes (Abukhdeir and Park, 2008). Abukhdeir *et al.* (2008) discovered that loss in p21^{Cip1} expression in ERα positive breast cancers increased tamoxifen resistance (Abukhdeir et al., 2008). Furthermore, a loss of p21^{Cip1} expression promoted tamoxifen-mediated proliferation. p21^{Cip1} null cells significantly increased phosphorylation of ERα on S118, a site correlated with tamoxifen-resistance; however, when p21^{Cip1} wild-type cells were treated with tamoxifen, this phosphorylation of ERα was not seen (Abukhdeir et al., 2008). Similarly, loss of p21^{Cip1} and p27^{Kip1} in ERα positive cells reduced cell cycle arrest when the cells were treated with anti-estrogens (Cariou et al., 2000). p27^{Kip1} was demonstrated to have an important role in response of Her2-enriched breast cancers to trastuzumab treatment. The presence of p27^{Kip1} indicates sensitivity to this therapy (Casalini et al., 2007; Okutur et al., 2015; Yang et al., 2006). This is in part because trastuzumab arrests cells in the G1 phase of the cell cycle and there is a subsequent decrease in proliferation due to the increase in p27^{Kip1}

levels, which form complexes with CDK2 (Sheaff et al., 1997). Nahta *et al.* (2004) discovered cell lines that are resistant to trastuzumab had decreased levels of p27[Kip1] and significantly increased CDK2 activity. Furthermore, using this resistant Her2 expressing cell line, when p27[Kip1] was ectopically expressed in these cells they became highly sensitive to trastuzumab treatment (Nahta et al., 2004). p27[Kip1] has a similar structure to p21[Cip1] (Toyoshima and Hunter, 1994); however, their roles in the cell cycle are slightly different. Both inhibit the same CDKs, but it was found that p21[Cip1] inhibition of the cell cycle was preferentially in the G1 phase. Furthermore, p21[Cip1] can be upregulated by p53 to induce cell cycle arrest and apoptosis. p27[Kip1] is not upregulated by p53 and can lead to cell cycle arrest at any stage of interphase (Abukhdeir and Park, 2008). These small differences may indicate a unique role for each in resistance.

Synthetic CKIs as an Approach for Breast Cancer Resistance

When functional, CKIs trigger cellular response to many existing therapeutics by halting the cell cycle and directing cells toward apoptosis. However, many CKIs become deregulated during cancer progression and their cyclin targets subsequently become elevated, both contributing to resistance to chemotherapy and many forms of targeted therapy, including endocrine therapy (Abukhdeir et al., 2008; Bandoh et al., 2005; Caldon et al., 2012; Dhillon and Mudryj, 2002). Synthetic CKIs have been designed to mimic p21[Cip1] and p27[Kip1] by competitively binding to the ATP-binding pocket of CDKs to inhibit kinase activity (Nair et al., 2011). Recently, some pan-CKIs, such as flavopiridol and roscovitine, have entered clinical trials (Byrd et al., 2007; Meijer and Raymond, 2003). As single agents, these CKIs have had little success presented with significant toxic effects on normal cells (Byrd et al., 2007; Harrison et al., 2009). Some

clinical trials are introducing combinations of synthetic pan-CKIs with various chemotherapeutic agents (Deep and Agarwal, 2008; Johnson and Shapiro, 2010). The outcome thus far has not shown promising benefits for breast cancer patients, but has shown promising results in populations of prostate cancer cells (Deep and Agarwal, 2008; Flaig et al., 2007) and in phase II clinical trials in relapsed and refractory multiple myeloma (Kumar et al., 2015).

The downfalls of using synthetic CKIs as a therapeutic can somewhat be attributed to the extreme conservation of the CDK active site between different forms, leading to a great deal of nonspecific effects (Asghar et al., 2015). Second generation inhibitors have begun to selectively target specific CDKs. Among these studies very few have made any efforts to stratify patient populations. Only a current CDK4/6 CKI clinical trial specifically looking within ERα-positive breast cancer, has stratified its patient population based on the molecular signature of the patient (Turner et al., 2015); specifically looking for an amplification of Cyclin D1, loss of p16, or both of these characteristics (Finn et al., 2009; Turner et al., 2015). This is the first documented clinical trial to stratify patients, resulting in improved responses thus far (Turner et al., 2015). Furthermore, synthetic CKIs target proteins essential for proliferation, such as CDK1, and survival, such as CDK9. This form of therapy cannot differentiate between a normal proliferating cell and a cancerous cell; which causes greater toxicities to patients (Asghar et al., 2015). A better understanding of the role of select cyclin-bound complexes may lead to increased specificity of and response to CKIs. This may be particularly relevant for CDK1, which has shown synthetic lethality in aggressive TNBC patients with amplified c-Myc (Horiuchi et al., 2012; Kang et al., 2014). An additional point that has

not yet been considered in the clinic is the existence of cyclin-like proteins that can bind and activate CDKs in the presence of CKIs (Nebreda, 2006), the importance of these proteins in breast cancer has been the topic of this thesis.

Speedy/RINGO Family of Cell Cycle Regulators

The first member of the Speedy/RINGO (Rapid Inducer of G2/M progression in Oocytes) family of proteins, coined X-Spy1, was initially isolated from a *Xenopus laevis* ovarian cDNA library in a genetic screen to find genes that confer resistance to gamma irradiation in a *rad1* deficient strain of *S. pombe* (Lenormand et al., 1999). Microinjections of X-Spy1 mRNA into stage VI oocytes activated MAPK and CDK1 and induced rapid oocyte maturation in the absence of progesterone (Lenormand et al., 1999). X-Spy1 binds and activates CDK2, which is important for Spy1-mediated maturation, but structurally has no sequence homology to the classically defined cyclins (Lenormand et al., 1999).

The human homologue of X-Spy1, originally termed Spy1, has since been isolated from a human testis cDNA library and was found to share 40% homology with X-Spy1 (Porter et al., 2002). Full length Spy1, Spy1A2, appears to only be found in testis, but a smaller splice variant, Spy1A1, hereafter referred to as Spy1, is found in low levels across most adult human tissues. The family of Speedy/RINGO proteins is now known to consist of at least 6 proteins, all harbouring a highly conserved core of approximately 100 residues, called the Speedy/RINGO (S/R) box, predicted to have an α-helical structure and found to be crucial for its interaction with CDKs (Figure 3A) (Cheng et al., 2005a; Dinarina et al., 2005). Diversity among the family members occurs at both

the C- and N-termini; possibly representing the ability of the Spy1 family members to bind to different CDKs or their affinity for specific substrates (Figure 3B).

A

N-terminus	Speedy/RINGO Box	C-terminus
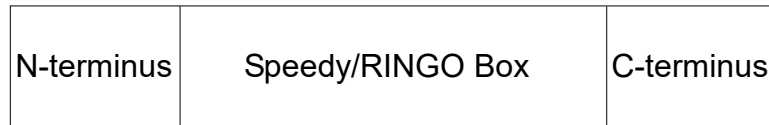
Degradation
of Spy1
Al Sorkhy, Craig et al. 2009

CDK1 and CDK2
Binding
Cheng, Xiong et al. 2005
Dinarina, Perez et al. 2005

CDK2
Activation
Cheng, Xiong et al. 2005
Dinarina, Perez et al. 2005

B

Name	Species	CDK Binding	Reference(s)
X-RINGO X-Spy1	*Xenopus laevis*	CDK1/CDK2	Ferby, Blazquez *et al.* 1999 Lenormand, Dellinger *et al.* 1999
Speedy/ RINGO A1 (Spy1A1)	*Homo sapiens Mus musculus Pan troglodytes Danio rerio Ciona intestinalis*	CDK1/CDK2	Porter, Dellinger *et al.* 2002 Dinarina, Perez *et al.* 2005 Chauhan, Zheng *et al.* 2012
Speedy/ RINGO A2 (Spy1A2)	*Homo sapiens Mus musculus Sus scrofa*	CDK1/CDK2	Kume, Endo *et al.* 2007 Cheng, Xiong *et al.* 2005
Speedy/ RINGO B	*Mus musculus*	CDK1	Dinarina, Perez *et al.* 2005 Cheng, Xiong *et al.* 2005
Speedy/ RINGO C	*Homo sapiens Sus scrofa*	CDK1/CDK2	Dinarina, Perez *et al.* 2005
Speedy/ RINGO D	*Mus musculus*	?	Cheng, Xiong *et al.* 2005
Speedy/ RINGO E	*Homo sapiens*	CDK1/CDK2/ CDK5	Cheng, Xiong *et al.* 2005 Dinarina, Perez *et al.* 2005

Figure 3. Schematic of Speedy/RINGO domain organization and Speedy/RINGO isoforms. (A) A schematic of Speedy/RINGO protein domains. (B) Table demonstrating Speedy/RINGO family members.

The *SPDYA* gene is ubiquitously expressed in many mammalian adult tissues. Specifically, high levels have been found in hormonally sensitive tissues, such as the testis and ovary (Cheng et al., 2005a; Porter et al., 2002). X-Spy1 and mammalian Spy1 protein bind to CDK1 and CDK2, initiating kinase activity (Al Sorkhy et al., 2012; Karaiskou et al., 2001). Consistent with its CDK binding ability, *SPDYA* expression and Spy1 protein levels are regulated in a cell cycle dependent fashion, with expression coming on in late M and accumulating through G1/S (Gutierrez et al., 2006; Porter et al., 2002). There appears to be at least 2 different mechanisms of protein degradation, one that functions at late G2 phase of the cell cycle and is driven by the E3 ligase, NEDD4 (Al Sorkhy et al., 2009), and another occurring at late G1 phase and is dependent on the E3 ubiquitin ligase, Skp2 (Gutierrez et al., 2006). Overexpression of Spy1 increases cell proliferation and shortens G1 phase of the cell cycle in a CDK2 dependent manner (Golipour et al., 2008; Porter et al., 2002). In contrast, a reduction of Spy1 levels decreases the rate of cell division and the population of cells in G1/S phase (Porter et al., 2002). While Spy1 binds to similar residues on the CDK as canonical cyclins, Spy1 activates the CDK in quite a unique fashion (Dinarina et al., 2005). Spy1-mediated activation of CDKs occurs independent of CAK-mediated phosphorylation of the residues T161 and T160 on CDK1 and CDK2, respectively, within the T-loop of the CDK (Cheng et al., 2005a; Dinarina et al., 2005). Furthermore, when Spy1 is bound to CDKs the complex is less sensitive to the inhibitory phosphorylation on T14 and Y15 mediated by Wee1 and Myt1 kinases and are less susceptible to the inhibitory action of p21[Cip1] (Karaiskou et al., 2001). Spy1 also indirectly activates CDKs via a direct

interaction with the CKI, p27^{Kip1} (McAndrew et al., 2007; Porter et al., 2003). A characteristic of G1/S phase transition is the degradation of p27^{Kip1} by SCF^{Skp2}, through its phosphorylation on T187 by Cyclin E-CDK2 (Pagano et al., 1995; Sheaff et al., 1997). Spy1-bound p27^{Kip1} promotes the phosphorylation of p27^{Kip1} and subsequent degradation, thereby, effectively overriding this important CKI (McAndrew et al., 2007). Spy1-bound CDKs also have an altered substrate specificity. A cyclin-CDK complex has a well-established ((S/T)PX(K/R)) consensus sequence and a strong affinity for a substrate with a lysine residue at the +3 position (Bourne et al., 1996; Cheng et al., 2005a; Jeffrey et al., 1995). Spy1 bound to CDK2 is able to phosphorylate canonical CDK substrates, such as histone H1; however, the CDK demonstrates a broader substrate specificity with preference for non-canonical CDK substrates lacking lysine residues at the +3 position (Cheng et al., 2005a). One example of a non-canonical substrate phosphorylated by Spy1-CDK2 is the Cdc25 proteins, which are classically inactivated during a checkpoint response, potentially indicating another mechanism by which Spy1 can override these protective cellular barriers.

Spy1 and DNA Damage Response (DDR)

The unique ability of Spy1 to activate CDKs in an environment indicative of a checkpoint response, and to override the effects of CKIs, such as p21^{Cip1} or p27^{Kip1}, speaks to the mechanism by which Spy1 may override cell cycle arrest seen in the original yeast screen. Indeed it was later demonstrated that Spy1 can also override the DDR in response to a host of DNA damaging agents including cisplatin and UV damage (Barnes et al., 2003; Karaiskou et al., 2001). The cell cycle is protected by several checkpoints with the most common being the restriction point (G1/S), the G2/M

checkpoint, and the spindle assembly checkpoint in M-phase. The G1/S and G2/M checkpoints are governed by the cell inhibiting the action of the relevant CDKs (Arellano and Moreno, 1997). In the presence of elevated levels of Spy1, the phosphorylation effect on Cdc25 by Spy1-CDK2, as well as decrease in sensitivity to p21[Cip1], allows for the bypass of the G1/S and G2/M checkpoints, increasing the proliferation of damaged cells (Gastwirt et al., 2006). Spy1 was demonstrated to have a regulatory role within the intrinsic DDR pathway (Barnes et al., 2003; Gastwirt et al., 2007) and confer resistance to p53- and p21[Cip1]-mediated apoptosis (Gastwirt et al., 2006). Elevated levels of Spy1-CDK2 leads to a resistant phenotype to genotoxic agents, radiation, and chemotherapeutic agents (Barnes et al., 2003). Spy1 overexpression has been shown to decrease sensitivity to agents used as cancer therapeutics (Barnes et al., 2003); hence, elevated levels of Spy1 may represent a targetable mechanism driving aspects of drug resistance.

Functional Roles for Spy1 in Normal Development and Carcinogenesis

The role of Spy1 in normal development in various tissues is still emerging. Under normal growth and developmental conditions, Spy1 levels are tightly regulated at select stages of development (Golipour et al., 2008; Lubanska and Porter, 2014b; Porter et al., 2002). In the mammary gland, Spy1 is expressed during the proliferative stages of the gland, such as puberty and pregnancy and reduced during terminal differentiation (Golipour et al., 2008). In the brain, Spy1 levels are elevated in embryonic tissues and decline with aging (Lubanska and Porter, 2014b). Spy1 levels are also upregulated during regeneration in the mammary gland (Golipour et al., 2008), peripheral nervous system (Huang et al., 2009) and stem cell populations in the adult brain (Lubanska et al., 2014;

Lubanska and Porter, 2014b). These data suggest that Spy1 has developmental roles and may play a role in regenerative processes. In the brain, Spy1 plays a role in regulating symmetric division of adult stem cell populations (Lubanska et al., 2014; Lubanska and Porter, 2014b). How Spy1 functions in development may provide important answers for its role in disease states, such as carcinogenesis.

Indeed, Spy1 levels are highly elevated in a number of human cancers including Non-Hodgkin's Lymphoma (NHL) (Hang et al., 2012), hepatocellular carcinoma (Ke et al., 2009), breast cancers (Al Sorkhy et al., 2012) and brain cancers (Lubanska et al., 2014; Lubanska and Porter, 2014b). In NHL and brain cancers, Spy1 levels correlated with an overall poor outcome for patients (Hang et al., 2012; Lubanska et al., 2014), indicating Spy1 as a potentially valuable prognostic marker. In NHL samples with elevated Spy1, a significant increase in phosphorylated p27^{Kip1} on T187, as well as increased proliferation, was observed (Hang et al., 2012). In breast, Spy1 levels are regulated downstream of both c-Myc and MAPK and forced Spy1 expression in orthotopic breast models drives tumour formation (Golipour et al., 2008). Knocking down Spy1 in leukemic, liver, brain and breast cancer cells significantly decreased cell proliferation (Al Sorkhy et al., 2012; Hang et al., 2012; Ke et al., 2009; Lubanska et al., 2014). Importantly, Spy1 levels appear to drive a more stem like population in breast (unpublished data) and brain (Lubanska et al., 2014; Lubanska and Porter, 2014b) and targeting of Spy1 may reduce the aggressiveness and stemness of the initiating cell population (Lubanska and Porter, 2014a; Lubanska and Porter, 2014b). Hence, further resolving the potential roles for Spy1 in different cancers and determining the

mechanisms that would result in the most effective targeting is an important next step for this work.

HYPOTHESIS AND OBJECTIVES

This work aims to determine the potential role of the atypical cell cycle regulator Spy1 as a prognostic marker and a novel, viable drug target in subsets of breast cancer.

This study will test the hypothesis that: Spy1 plays a pivotal role in fuelling proliferation downstream of ERα and promotes resistance to anti-estrogen therapy. We further hypothesize that Spy1 is of pivotal importance to those aggressive tumours driven by c-Myc and targeting Spy1 will represent a novel and important approach for this subset of patients. *We will address this hypothesis with the following objectives:*

- To determine the molecular mechanism by which Spy1 functions in the ERα signalling pathway.
- To determine the potential relevance of Spy1 as a target in Myc-driven tumours.
- To determine whether Spy1 levels influence the sensitivity of breast cancer cells to synthetic CKIs and/or chemotherapy.
- To determine whether CKIs can sensitize a host of chemotherapy treatments for TNBC.

The data obtained in this study will contribute to the advancement of understanding the role of the cell cycle regulators in breast cancer treatment. Furthermore, this study may reveal novel diagnostic and therapeutic strategies for patients with aggressive breast cancers.

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CHAPTER 2

THE CYCLN-LIKE PROTEIN, SPY1, REGULATES THE ERα AND ERK1/2 PATHWAYS PROMOTING TAMOXIFEN RESISTANCE

INTRODUCTION

Overall 5 year survival rates for breast cancer have increased by almost 20% since 1975, largely because of improved screening and drugs developed against estrogen signalling (ie. tamoxifen) and the Her2/neu receptor (ie. trastuzumab) (Siegel et al., 2012). Despite these advances, a subset of patients either progress to, or initially present with, cancers that are unresponsive to current targeted therapies (Hackshaw et al., 2011; Viani et al., 2007). As such, breast cancer remains the second leading cause of death from cancer among women (CBCF, 2014). Determining the mechanisms regulating the initiation and/or progression to a drug resistant status represents a current challenge in the breast cancer field.

Estrogen receptor alpha (ERα) is a steroidal receptor that changes into an active conformation upon binding to the ligand estradiol (E2) (Klinge et al., 2001). Classical ERα activation promotes receptor homodimerization, nuclear translocation and subsequent DNA binding to estrogen response elements (EREs) to regulate the expression of various genes (Barone et al., 2010). 'Non-classical' genomic signalling also exists where the E2-ERα complex bind transcription factors to regulate genes like Cyclin D1, which lack EREs (Castro-Rivera et al., 2001; Gottlicher et al., 1998; Musgrove et al., 1993; Planas-Silva and Weinberg, 1997). ERα dimers, activated by E2 or other growth factors, can also interact and form complexes directly with G-proteins, receptor tyrosine kinases, and non-receptor tyrosine kinases (Levin, 2005). ERα dimers are, therefore, able to activate signal transduction pathways, such as Ras/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K/Akt) (Likhite et al., 2006). Collectively,

ERα works via these diverse mechanisms to promote breast cell growth and survival (Fujita et al., 2003; Klinge et al., 2001).

Tamoxifen functions by competitively binding to the ligand binding domain (LBD) of ERα, altering its conformation such that it can no longer bind to E2, hence, preventing E2 proliferative signalling (Connor et al., 2001). The binding efficiency of tamoxifen can be altered by the phosphorylation status of residues within ERα capable of inducing ligand independent signalling (Chen et al., 2002; Kato et al., 1995). Phosphorylation on serine (S)-118 by extracellular signal-regulated kinases (ERK)1/2 is one prominent example of such a modification. S118 phosphorylation promotes hypersensitivity to E2 and decreases ERα affinity for tamoxifen (Bunone et al., 1996; Chen et al., 2002; Chen et al., 2013; Cheng et al., 2007; Yamashita et al., 2008). ERK1/2 is the final kinase at the end of the Ras/MAPK signalling cascade, succeeding Ras, Raf and MEK activation. Hence, ERK1/2 carries out non-classical signalling downstream of ERα, as well as providing a positive feedback to augment ERα signalling.

The Ras-Raf-MEK-ERK cascade is hyperactivated in approximately 30% of human cancers, a large percentage characterised by a mutation in either the Ras or Raf genes (Giltnane and Balko, 2014; Huynh et al., 2003). Constitutively activated MEK1/2 is frequently seen in cancer cell lines, contributing to increased cell survival, migration and transformation (Huynh et al., 2003). Overexpression and hyperphosphorylation of ERK1/2 has been seen in various cancers, including hepatocellular carcinoma and breast cancer (Giltnane and Balko, 2014; Huynh et al., 2003). Pharmacological intervention upstream of ERK1/2 has received considerable focus; however, to date clinical results are largely underwhelming, with pre-clinical and clinical documentation showing a

development of acquired resistance shortly after receiving treatment (Emery et al., 2009). Resistance is largely associated with re-activation of ERK1/2 signalling (Morris et al., 2013). As such, specific inhibitors of ERK1/2 have become a focus over the last 5 years and promising pre-clinical data are beginning to emerge (Morris et al., 2013). To this end, it has been shown in melanoma, breast, and colon cancer cell lines, that the use of an ERK1/2 inhibitor can overcome acquired resistance to both BRAF and MEK1/2 inhibitors (Hatzivassiliou et al., 2012; Morris et al., 2013). This exciting data has led to the introduction of the ERK1/2 inhibitor into phase I clinical trials for solid tumours (Morris et al., 2013). Understanding the activation of all components of this pathway influences the successful intervention of a large number of cancers, including breast cancer.

This work focused on the observation that a cell cycle protein coined Spy1 (Speedy, RINGO) (gene *SPDYA*) is capable of promoting the activation of the MAPK pathway when injected into unfertilized *Xenopus* oocytes (Lenormand et al., 1999). Spy1 is one member of a family of 'cyclin-like' proteins in that they are expressed and degraded in a cell cycle dependent manner and are able to directly bind and activate the cyclin dependent kinases (CDKs) (Dinarina et al., 2009; Ferby et al., 1999; Lenormand et al., 1999; Porter et al., 2002). Spy1 functions in an atypical manner to classical cyclins in that it binds to both the G1/S and G2/M CDKs and directs phosphorylation of non-canonical CDK substrates (Al Sorkhy et al., 2012; Cheng et al., 2005a; Karaiskou et al., 2001). Activation of the CDKs by Spy1 also occurs independent of phosphorylation by CAK within the T-loop and dephosphorylation on the defined inhibitory residues (Cheng et al., 2005a). Further, Spy1 directly binds and promotes the degradation of the CDK

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inhibitor, p27^{Kip1} (McAndrew et al., 2007; Porter et al., 2003). Indeed, Spy1 supports rapid progression through the cell cycle even in the face of senescence and apoptotic-inducing stimuli (Barnes et al., 2003; Gastwirt et al., 2006). This suggests a mechanism by which Spy1 overrides cell-cycle induced apoptosis caused by therapeutic agents, which could support drug resistance. Spy1 levels are elevated in a number of human cancers, including liver, brain, breast and blood (Hang et al., 2012; Ke et al., 2009; Lubanska and Porter, 2014a; Lubanska and Porter, 2014b; Zucchi et al., 2004). In the breast, Spy1 levels are elevated by MAPK/ERK and c-Myc signalling to promote proliferation and override differentiation stimuli (Golipour et al., 2008; Lenormand et al., 1999). In this work, we questioned whether Spy1 could activate aspects of the MAPK pathway in human somatic cells and if this played a role in the development of resistance to tamoxifen.

MATERIALS AND METHODS

Cell Culture. Human embryonic kidney (HEK)-293 and MCF7 cells were purchased from ATCC and were subcultured in DMEM media supplemented with 10% FBS and 30,000 units penicillin/30,000 μg streptomycin solution. LCC9 cells (Lombardi Comprehensive Cancer Center, Georgetown University) were routinely subcultured in DMEM phenol red free media supplemented with 1 mM L-glutamine, 30,000 units penicillin/30,000 μg streptomycin, and 10% charcoal treated FBS. All cells were maintained under normoxic conditions (5% CO_2) at 37°C.

Plasmids. Creation of the Myc-Spy1-pCS3 was described previously (Porter et al., 2002). Plasmids for Rc-CMV-Cyclin E (#8963), pEGFP-C1-ERα (#28230), HA-CDK1-DN (#1889) and pLKO-scrambled control (#8453) were purchased from Addgene. pLKO-shSpy1 and pLKO-shCyclin E were cloned to express a short hairpin previously described to knockdown Spy1 and Cyclin E, respectively, control pLKO contains a scrambled sequence previously described (Lubanska and Porter, 2014b). The CDK mutants D90 (Cheng et al., 2005c) and R170 vectors (Al Sorkhy et al., 2015, in review) have been previously described. pEIZ vector was generously donated from Dr. B. Welm. The creation of pEIZ-Spy1 was completed by inserting Spy1 oligo into the *Eco*RI and *Xba*I sites of pEIZ.

Immunoblotting (IB). Total protein was isolated from cell cultures by harvesting cells and lysing them in NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) containing protease inhibitors (100 μg/ml PMSF, 5 μg/ml aprotinin, and 2 μg/ml leupeptin) for 1 hour on ice. Bradford reagent was used to determine the concentration of protein following the manufacturer's instructions (Sigma).

80-100 µg of protein were subjected to electrophoresis on denaturing 10% SDS polyacrylamide gels and transferred to PVDF-Plus 0.45 micron transfer membrane (Osmonics Inc.) for 2 hours at 30 volts using a wet transfer method. Blots were blocked for 1 hour in 1% BSA solution at room temperature. Primary antibodies were reconstituted in blocker and incubated overnight at 4°C, secondary antibodies were used at 1:10000 dilution in blocker for 1 hour at room temperature. Blots were washed three times in TBST for three minutes following incubation with primary and secondary antibodies. Chemiluminescent Peroxidase Substrate was used for visualization following manufacturer's instruction (Pierce). Chemiluminescence was quantified on an AlphaInnotech HD2 (Fisher) using AlphaEase FC software. Antibodies were used at the following concentrations: Actin MAB150 1R (Chemicon-Millipore; 1:1000), Spy1 (ThermoScientific; 1:1000), c-Myc (Sigma; 1:1000), anti-phospho-ERα-S118 (Abcam; 1:1000), anti-ERα (Santa Cruz Biotechnology; 1:1000), Cyclin E1 (Abcam; 1:1000), anti-RIPK2 (Santa Cruz Biotechnology; 1:1000), anti-phospho-Raf1 (Abcam; 1:500), anti-Raf1 (Santa Cruz Biotechnolgy; 1:1500), anti-p-ERK 1/2 [Thr 202/Tyr 204] (Cell Signaling; 1:1000), anti-ERK1/2 (Santa Cruz Biotechnology; 1:1000), anti-MKP1 (Santa Cruz Biotechnology; 1:1000), anti-MKP2 (Santa Cruz Biotechnolgy; 1:1000), anti-MKP3 (Santa Cruz Biotechnology; (1:1500), and anti-PP2A (Santa Cruz Biotechnology; (1:1000).

Lentivirus Production. VSV-G pseudotyped lentivirus was produced by transient transfection of HEK-293 LentiX cells with transfer vector and the multi-deleted packaging plasmids (pMDG, pMDL2, pRSV) using polyethylenimine (PEI) (Sigma) reagent with 1:3 DNA to PEI ratio and incubation for 5 hours at 37°C, 5% CO_2. The virus

was collected the next day and concentrated for 3 hours at 4°C using an ultracentrifuge. The titer for pEIZ was determined by transducing 293T cells and analysis of eGFP protein expression by flowcytometry at 72 hours post transduction. The titer for pLKO lentivirus was assessed by puromycin selection followed by crystal violet staining and quantification of resistant colonies. The titered virus was filter sterilized and stored at -80°C.

Transfection/Infection. *Transfection*: Cells were transfected using PEI branched reagent (Sigma). In brief, 10 µg of DNA was mixed with 3 µl of 10 mg/ml PEI for 10 minutes then added to a 10 cm tissue culture plate. Transfection media was changed after 24 hours. *Lentiviral Infection*: 8000 cells were seeded in fully supplemented growth media in 96-well plates for 2 hours. Cells were starved by removing serum and penicillin/streptomycin from the media, followed by the use of 1 mg/ml polybrene (Santa Cruz Biotechnology) and MOI 3 of the specific vector used. Infected media was changed to fully supplemented media 24 hours after infection. For knockdown, cells were incubated with 1mg/ml puromycin (Sigma) 48 hours after infection for 72 hours to allow for puromycin selection. Media is thereafter changed every 48 hours with puromycin included.

Inhibition Treatments. HEK-293 cells were seeded equally in 10 cm dishes at a density of 5 x 10^5 cells. Upon 80% confluency, HEK-293 cells were incubated with either 10 µM SB202474 (control) or 10 µM U0126 (MEK 1/2) inhibitors (Calbiochem) for 1 hour. For Raf inhibition, 5 µM GW5074 (Sigma) was added to the cells for 24 hours. For Ras inhibition, 20 µM Farnesyl Thiosalicyclic Acid (Santa Cruz Biotechnology) was added to

the cells for 24 hours. For ERK1/2 inhibition, 10 μM SCH772984 (ApexBio) was added to the cells for 24 hours.

Estradiol/Tamoxifen Treatments. MCF7 or HEK-293 cells were seeded equally in 10 cm dishes at a density of 5 x 10^5 cells. Upon 70% confluency, the cells were treated with phenol red-free RPMI media, supplemented with 10% charcoal treated FBS and 30,000 units penicillin/streptomycin. After 48 hours, cells were incubated with either dimethyl sulfoxide (DMSO), 50 ng/ml E2 (Sigma), or 100 nM 4-Hydroxytamoxifen (Sigma) for specified time points, followed by cell harvesting for protein extraction and IB.

Animal Care and Handling. Wildtype Zebrafish (*Daniorerio)* were handled in compliance with local animal care regulations and standard protocols of Canada and following the University of Windsor animal care protocol AUPP#12-14. Adult fish were kept at 28.5°C and bred according to available protocols (Westerfield, 2000).

Implantation and Treatment. Eggs were collected after fertilization and kept in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33mM $MgSO_4$, 10^{-5}% Methylene Blue) at 32°C in an incubator until ready to inject. Before injection 200,000 cells were reconstituted in 200 μL of serum-free media containing 1 μL of DiO (green) (Vybrant, Invitrogen) at 37°C for 20 minutes. Cells were washed with 200 μL of serum free media twice and resuspended in 20 μL of serum free media, kept at 37°C for 20 minutes, and placed on ice until injection. 48 hours post-fertilization (hpf) the embryos were dechorionated with fine tip forceps and anesthetised with 0.168 mg/ml of Tricaine (Sigma). 50-100 labelled cells/ 9 nL were loaded into glass capillary needles and injected into the yolk sac of each embryo using a Nanoject II (Fisher Scientific). After injection, embryos were placed in E3 embryo media and 2 hours post-implantation (hpi) were

examined using a Leica fluorescence stereoscope to exclude any embryo with cells outside of the implantation area. 24 hpi (0 hours post-treatment (hpt)) the embryos were anesthetised, imaged and placed in a 96-well plate; one embryo per well. At 48 hpi the embryos were treated with either DMSO or 10 μM tamoxifen. The embryos were imaged again at 72 hpi (24 hpt) and the fold change in tumour burden calculated.

Image Analysis. All image analysis was completed using ImageJ software. The image for each embryo was imported into ImageJ, the image was converted to a 32-bit greyscale, and the threshold was adjusted to eliminate background pixels. Total area of fluorescence was measured as the area of tumour burden. All measured results were copied into Excel files and fold change in tumour burden calculated from 24 to 72 hpi.

RESULTS

Spy1 overexpression enhances ERK1/2 phosphorylation.

Overexpression of Spy1 in HEK-293 cells results in a significant increase in phosphorylated threonine (T)202- and tyrosine (Y)204-ERK (pERK) compared to total ERK protein levels (Figure 1A). To determine whether the activation of pERK was unique to Spy1 overexpression, or if there were redundancies among the cyclin proteins, Spy1 and Cyclin E were overexpressed in HEK-293 cells. Spy1 overexpression significantly increased overall phosphorylation of ERK protein, but Cyclin E overexpression did not result in any significant change (Figure 1B). To determine if Spy1 is a necessary mediator of ERK activation, cells were infected with shRNA lentivirus targeting two separate regions of the Spy1 mRNA (shSpy1.1, shSpy1.2). shRNA against Cyclin E1 was also used to address the essentiality of classical cyclin-CDK activation (shCyclinE) and a pLKO-shScrambled control (pLKO) was used. Both of the shSpy1 constructs significantly decreased endogenous activated ERK levels (Figure 1C); however, this effect was not noted with shCyclinE treatment despite successful knockdown (Figure 1C; left panel representative blot). To ensure that Spy1 knockdown effects are specific, we have also overexpressed two different rescue constructs unable to be recognized by the shSpy1 (resSpy1; Figure 1D). The rescue constructs reversed Spy1 knockdown effects on ERK phosphorylation. Collectively, these results support the hypothesis that Spy1 activates ERK1/2 in a manner unique from classical cyclin-CDK activation (i.e. Cyclin E-CDK2).
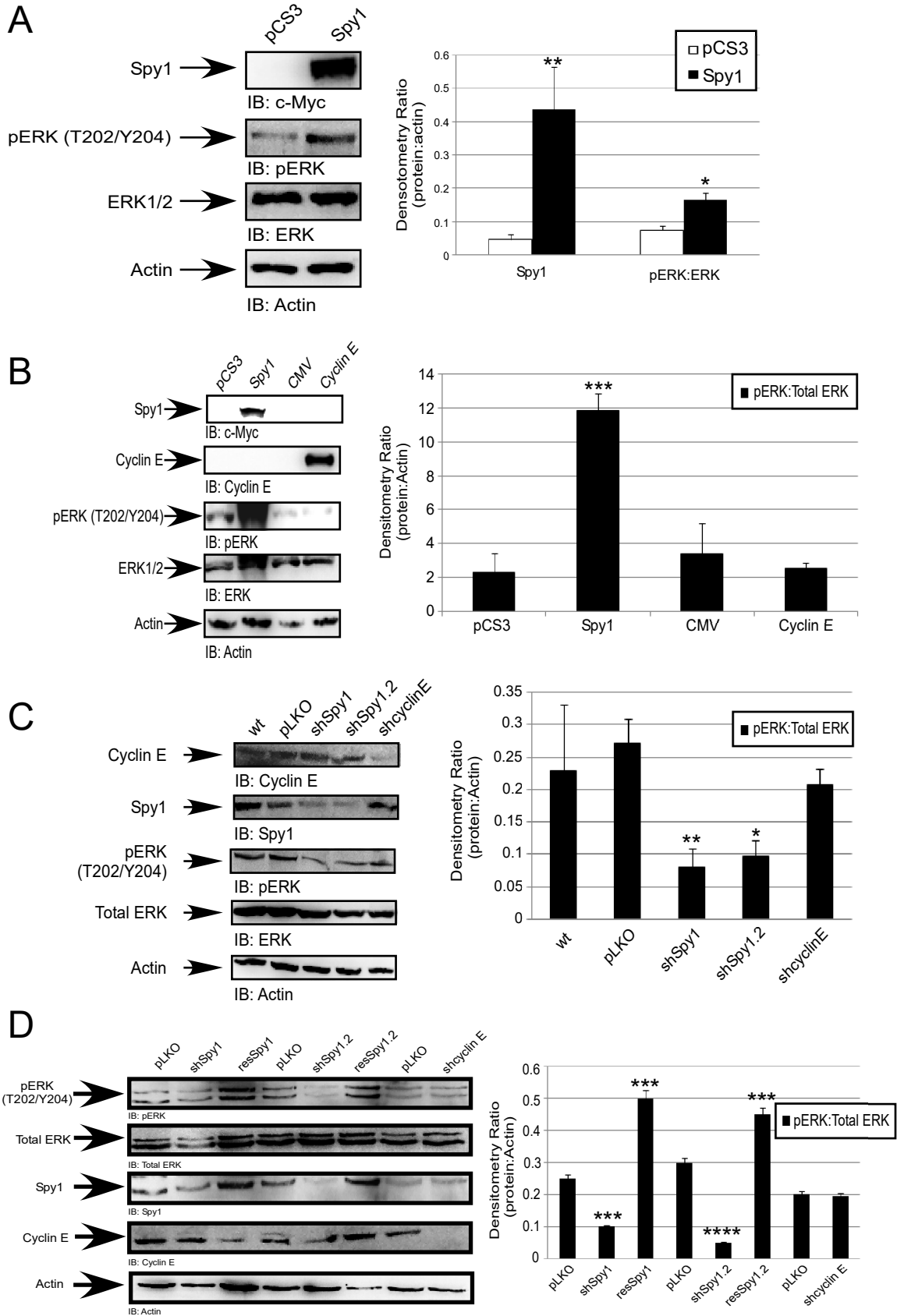
Figure 1. Spy1 overexpression enhances ERK1/2 phosphorylation. (A) Cells were transfected with pCS3 or Spy1 vectors, followed by SDS-PAGE and IB. n=3 (B) Cells were transfected with pCS3, Spy1, pCMV, and Cyclin E vectors, followed by SDS-PAGE and IB. n=9. (C) Cells were infected with shScrambled (denoted pLKO), 2 constructs of shSpy1 (shSpy1.1, shSpy1.2), and shCyclin E (shCyclin E), followed by SDS-PAGE and IB. (D) Cells were infected with shScrambled (pLKO), 2 constructs of shSpy1 (shSpy1.1, shSpy1.2), and shCyclin E (shCyclin E), or rescue vectors, followed by SDS-PAGE and IB. (A-D) A representative blot is shown (left panel) and the densitometry ratio of protein to loading control actin is shown (right panel). Error bars reflect SE between experiments. Student's t-test was performed;*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Spy1-mediated ERK phosphorylation is CDK dependent.

Using a previously characterized Spy1-CDK non-binding mutant (Spy1-D90A) (Cheng et al., 2005c), we sought to determine whether the direct binding between Spy1 and the CDK is essential for activation of ERK1/2. Transient transfection with wild-type Spy1 shows a significant increase in the activation of ERK1/2, while Spy1-D90A does not significantly affect phosphorylated ERK1/2 levels (Figure 2A). Furthermore, a significant increase in proliferation was seen in Spy1 overexpressing cells as compared to control and D90 transfected cells (Figure 2B). These data support the hypothesis that the activation of ERK1/2 is dependent on Spy1-mediated CDK activity. Spy1 can bind to both CDK1 and CDK2 (Al Sorkhy et al., 2012; Cheng et al., 2005a; Ke et al., 2009). To determine which CDK is most influential on Spy1-activated ERK, cells were transfected with Myc-tagged Spy1 and low levels of either an HA-tagged CDK1 or CDK2 dominant negative (DN) vector (CDK1 DN or CDK2 DN), or relevant controls. The concentration of DN vector used did not significantly impair growth alone; however, both CDK1 and CDK2 DN vectors significantly impaired the ability of Spy1 to activate ERK1/2 (Figure 2C). These data support that the mechanism by which Spy1 initiates the phosphorylation of ERK requires at least one of the CDKs to be present and bound.

Spy1-mediated ERK1/2 activation is MEK-independent.

In the presence of U0126, a MEK1/2 inhibitor, we see a decrease in pERK1/2, as well as a significant decrease in overall Spy1 protein levels. Interestingly, in cells overexpressing Spy1, U0126 does not significantly reduce the ability of Spy1 to activate ERK1/2 (Figure 2D). Another level of regulation of ERK1/2 phosphorylation is through the steady state removal of phosphorylation by the relevant phosphatases. Four major

phosphatases regulate ERK1/2; PP2A, MAPK Phosphatase (MKP)1, MKP2, and MKP3 (Raman et al., 2007). Neither Spy1 or Cyclin E overexpression significantly decreased the phosphatases, Spy1 actually significantly increased MKP2 protein levels (Figure 2E); possibly indicating that the cell is attempting to regulate the enhanced activation of ERK1/2 to maintain steady state activity.
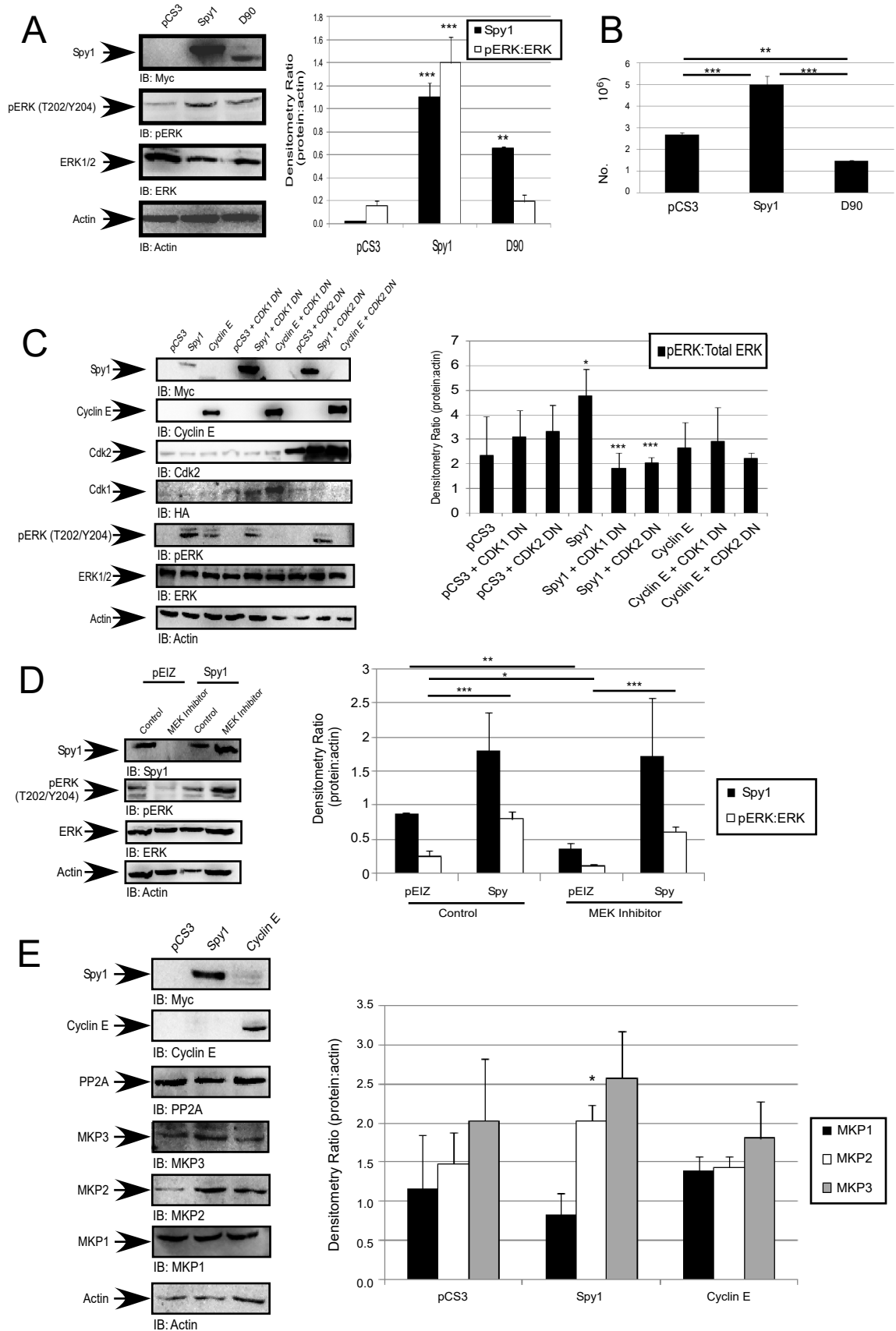
Figure 2. Spy1-mediated ERK phosphorylation is CDK dependent, MAPK phosphatase independent. (A-B) Cells were transfected with pCS3, Spy1, and Myc-tagged-Spy1-D90A (binding mutant) vectors. (A) SDS-PAGE and IB was performed. (B) Trypan blue exclusion assay was performed to determine the number of living cells after transfection. (C) Cells were transfected alone or in combination with pCS3, Spy1, Cyclin E overexpression vector, CDK1 dominant negative, and CDK2 dominant negative. (D) Cells were stably infected with pEIZ or Spy1 vectors and treated with 10 ug of control inhibitor or MEK inhibitor (U0126). This was followed by SDS-PAGE and IB. (E) Cells were transfected with pCS3, Spy1, and Cyclin E overexpression vectors, followed by SDS-PAGE and IB. (A;C-E) A representative blot is shown (left panel) and the densitometry ratio of protein to loading control actin is shown (right panel). (A-E) Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05, **p<0.002, ***p<0.001.

Activation of ERK1/2 by Spy1 is dependent on Ras and Raf.

To determine whether alternate upstream activators of ERK1/2 are important in Spy1-mediated effects we tested the consequences of Spy1 overexpression in the presence of Ras and Raf inhibitors, Farnesyl Thiosalicyclic Acid and GW5074, respectively. When c-Raf is inhibited we see a slight decrease in pERK protein levels as compared to the control; however, there is a significant decrease of pERK protein levels when the Ras inhibitor is used, indicating that the regulation of pERK by Spy1 requires both Raf and Ras activation (Figure 3A). Moeller et al. (2003) showed Ras activation requires the inhibition of p27. p27 controls the formation of the Grb2/SOS complex which activates Ras (Moeller et al., 2003). Spy1-CDK2 can bind to and inhibit p27 (McAndrew et al., 2007; Porter et al., 2003). Spy1 inhibits p27 levels in the absence and presence of Raf/Ras inhibitors (Figure 3A), demonstrating that this aspect of Spy1 activity is still intact and that ERK-mediated effects reside downstream of p27 degradation. To investigate the importance of the direct Spy1-p27 interaction, a Spy1-p27 binding mutant (R170) was utilized (AlSorkhy et al., 2015, unpublished data). When R170 lentivirus was infected into cells, there was a significant increase in p27 levels as compared to the empty vector (pEIZ) control and the pEIZ-Spy1 overexpression vector (Figure 3B). Interestingly, R170 expression in these cells causes a significant decrease in the activation of ERK as compared to Spy1 overexpression (Figure 3C). Finally, when R170 is used in conjunction with the Ras inhibitor, we see no significant change in the phosphorylation status of ERK, further supporting that activation of ERK resides downstream of Spy1-mediated effects on p27 protein levels (Figure 3C). These data show the Spy1-CDK complex requires the activation of Ras through the downregulation of p27

to significantly increase pERK protein levels. However, the question of how Spy1 could activate pERK through the Ras pathway independent of MEK1/2 remained.

Spy1 overexpression may function through RIPK2.

Receptor-interacting serine-threonine kinase 2 (RIPK2) is activated through Ras-activated Raf kinase. Activation of RIPK2 can directly phosphorylate ERK1/2 *in vitro* and *in vivo* (Navas et al., 1999). To determine whether the effects of Spy1 overexpression on ERK1/2 could be mediated through RIPK2, cells were transfected with Myc-tagged-Spy1 followed by infection with lentivirus packaging either scrambled control shRNA (pLKO) or shRNA targeting two different regions of the RIPK2 mRNA (shRIPK2.1, shRIPK2.2) (Figure 3D). RIPK2 knockdown significantly abrogated the ability of Spy1 to enhance phosphorylation of ERK1/2 (Figure 3D). Collectively, these results suggest Spy1 is activating ERK1/2 indirectly through the Ras-Raf-RIPK2 pathway. Spy1 plays a role in normal growth and development of the breast, and elevated levels promote rapid tumorigenesis in mouse models (Golipour et al., 2008). Spy1 is found at elevated levels in aggressive forms of breast cancer (Al Sorkhy et al., 2012). Given the role of ERK signalling in driving tamoxifen resistance (Chen et al., 2013; Riggins et al., 2007); we sought to determine whether Spy1-mediated activation of ERK1/2 in breast cells could be implicated in sensitivity to tamoxifen.

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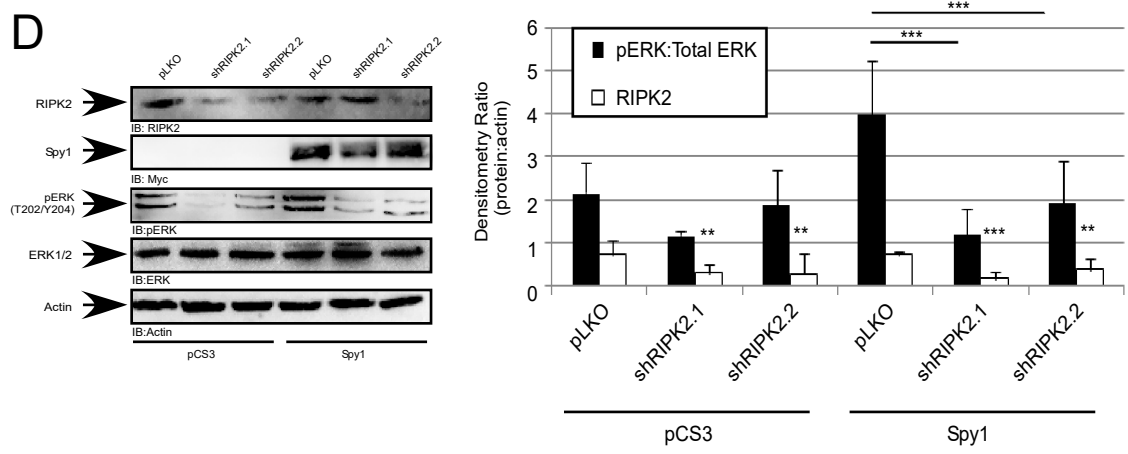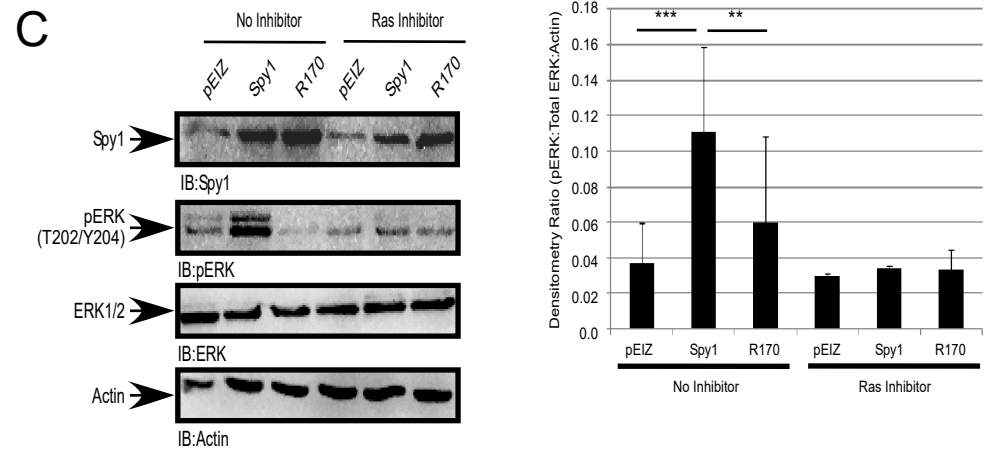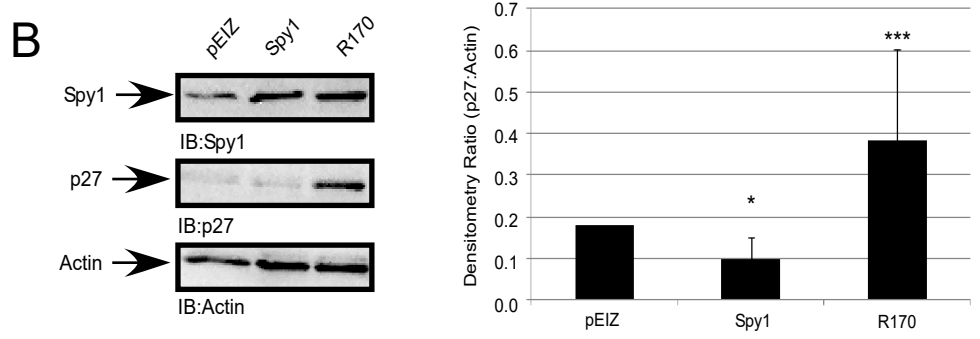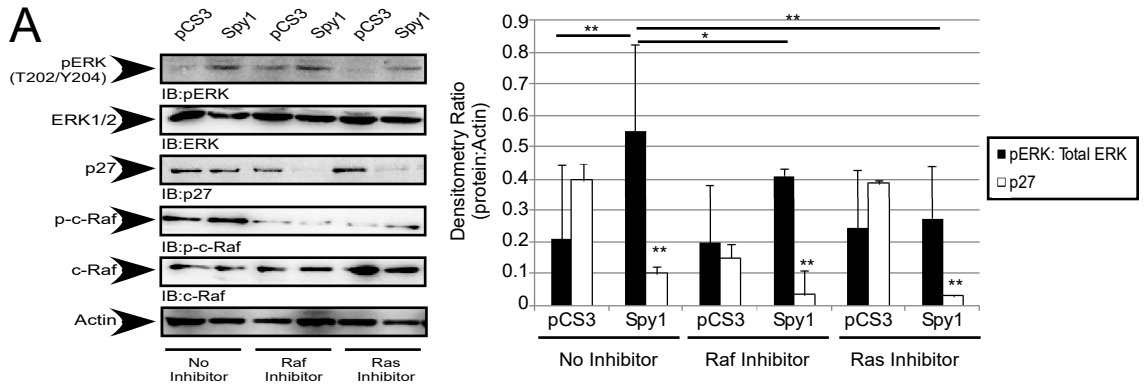
Figure 3. Activation of ERK1/2 by Spy1 is dependent on Ras and Raf. (A) Cells were transfected with pCS3 and Spy1, followed by 20 ug of Raf inhibitor or Ras inhibitor. This was followed by SDS-PAGE and IB. (B-C) Cells were infected with pEIZ control, pEIZ-Spy1, or pEIZ-Spy1-R170 (p27 binding mutant) vectors. (B) This was followed by SDS-PAGE and IB. (C) 20 ug of Ras inhibitor was added after infection. This was followed by SDS-PAGE and IB. (D) Cells were transfected with pCS3 and Spy1. Following transfection, cells were infected with pLKO-shScrambled (denoted pLKO) and shRIPK2 constructs (shRIPK2.1 and shRIPK2.2). After puromycin selection cells were subjected to SDS-PAGE and IB. (A-D) A representative blot is shown (left panel) and the densitometry ratio of protein to loading control actin is shown (right panel). Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05,**p<0.01, ***p<0.001.

Spy1 is upregulated upon activation of the estrogen receptor.

To determine whether Spy1 expression is endogenously regulated downstream of ERα activation, ERα positive MCF7 cells were treated with 50 nM of E2 for 0 to 6 hours. The phosphorylated protein level of ERα was significantly elevated as was the level of Spy1 (Figure 4A). To confirm this finding, ER negative HEK-293 cells were transfected with an ERα vector and treated with E2 over time (Figure 4B). Spy1 protein levels were significantly elevated in response to E2 treatment (Figure 4C). These data indicate that Spy1 is downstream of the estrogen signalling pathway.

Increased levels of Spy1 regulate ERK1/2 feedback to ERα in breast cells.

To determine whether Spy1 can affect the phosphorylation of ERα on the ERK phosphorylation site (S118), we infected MCF7 cells with constructs to overexpress Spy1 or Cyclin E. Increased levels of Spy1 significantly increased the level of phosphorylation of ERα on S118 (Figure 4D). These cells were then treated with tamoxifen in the presence or absence of the MEK inhibitor, U0126. When Spy1 levels were elevated, even in the presence of 100 nM tamoxifen, the levels of pERα-S118 were significantly increased as compared to control and Cyclin E overexpression and this occurred in a MEK-independent fashion (Figure 4E). We then wanted to determine if inhibiting ERK1/2 directly would alter the effect of Spy1 overexpression on pERα-S118, in the presence or absence of tamoxifen treatment. MCF7 cells overexpessing Spy1 were treated with 10 µM ERK1/2 inhibitor (SCH772984), either alone or in combination with 100 nM tamoxifen. Initially, cells were counted for viability after treatment using the trypan blue exclusion assay. We show that the use of the ERK1/2 inhibitor alone significantly reduces the number of viable cells in both control and Spy1 overexpressing

cells (Figure 4F). Furthermore, Spy1-mediated proliferation was not inhibited by tamoxifen alone but ERK inhibition prevented Spy1-mediated effects on growth. The same experiment was also carried out looking at pERα-S118 status and comparing to overexpression of Cyclin E (Figure 4G). Spy1 significantly increases pERα-S118 in control situations, whereas Cyclin E had no notable effect. Spy1-mediated ER phosphorylation occurs in the presence of tamoxifen but not in the presence of the ERK inhibitor. These data indicate that Spy1 effects on ERα are mediated through a feedback to ERK1/2 .
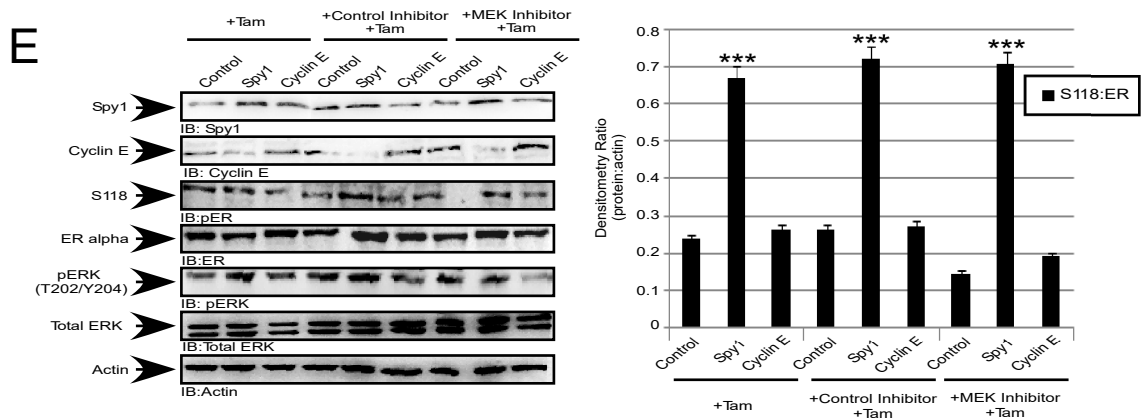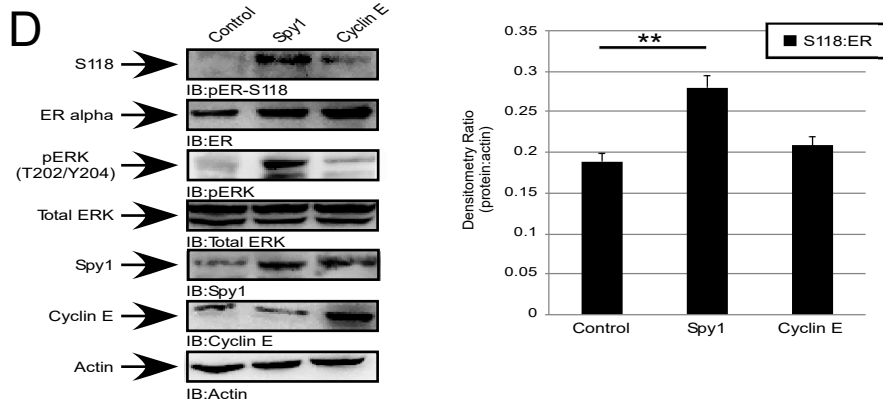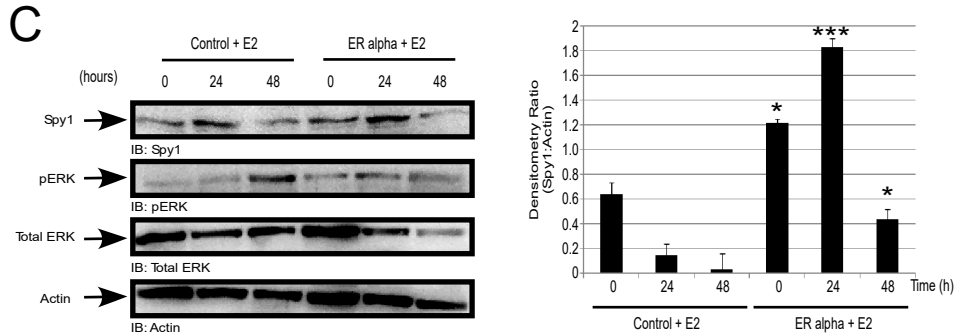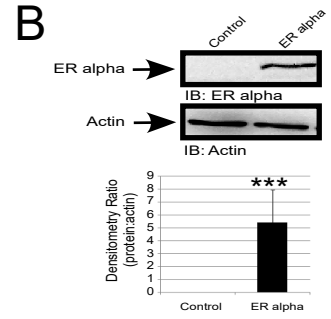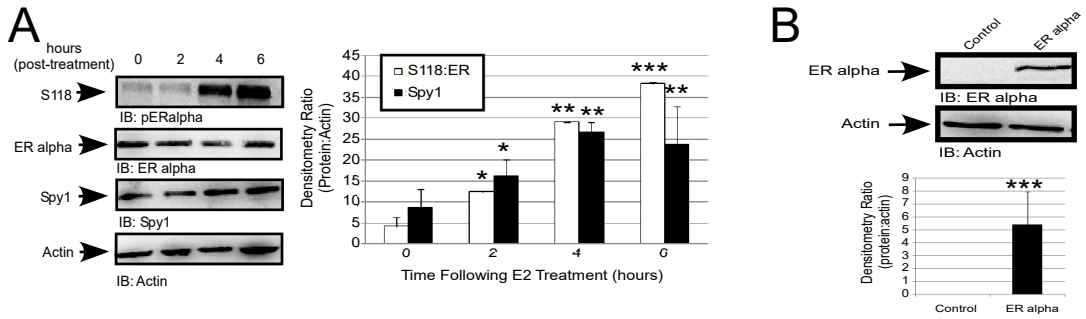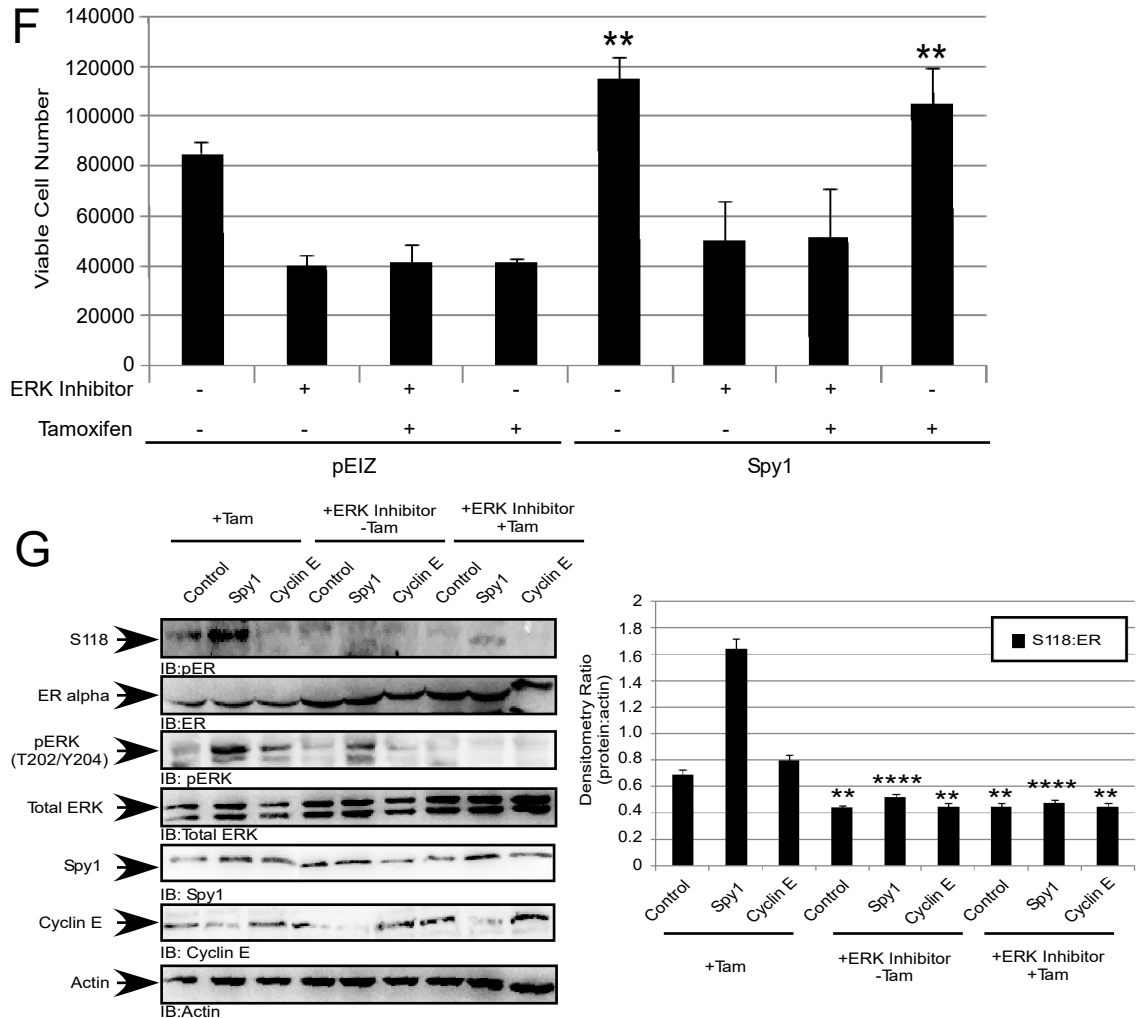
Figure 4. Spy1 is upregulated upon activation of the estrogen receptor. (A) MCF7 cells were treated with 50 nM of estradiol (E2) or vehicle control (DMSO) over the indicated time course; followed by SDS-PAGE and IB. (B-C) Hek-293 cells were transfected with the estrogen receptor (ER). (B) Transfection was confirmed by SDS-PAGE and IB. (C) Hek-293 cells transfected with ER or control were treated with 50 nM of E2 over the indicated time course; followed by SDS-PAGE and IB. (D-G) MCF7 cells were infected with control, Spy1, or Cyclin E, (D) followed by SDS-PAGE and IB. (E) Cells were then treated with 10 uM MEK1/2 inhibitor, U0126, control inhibitor, or 100 nM of tamoxifen. SDS-PAGE and IB followed. (F) Spy1 overexpressed cells were treated with 10 uM ERK1/2 inhibitor with or without 100 nM tamoxifen and subjected to trypan blue exclusion assay. (G) Spy1 and Cyclin E overexpressed cells were treated with 100 nM tamoxifen with or without 10 uM ERK1/2 inhibitor, followed by SDS-PAGE and IB. (A-E; G) A representative blot is shown (left panel) and the densitometry ratio is shown (right panel). Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Spy1 levels regulate the response of cells to tamoxifen treatment.

To test the effect of Spy1 levels on tamoxifen response, ERα positive MCF7 cells were infected with pEIZ-Spy1 and empty vector control (pEIZ) (Figure 5A). Cells were treated with 100 nM tamoxifen for 24 hours and subjected to the trypan blue exclusion assay. As expected, Spy1 overexpression significantly increased cell number as compared to pEIZ control in untreated cells. When tamoxifen was added, Spy1 continued to drive cell proliferation, but pEIZ control populations failed to proliferate (Figure 5B). Previous studies have demonstrated that combination treatment of CDK inhibitors (CKIs) with tamoxifen on MCF7 cells significantly arrested cells in G1 and G2 phases of the cell cycle (Wesierska-Gadek et al., 2011). This group showed that the level of phosphorylation on ERα was significantly decreased as was subsequent ERα activation. We tested the effect of elevated levels of Spy1 on these drug combinations (Figure 5C). Two CKIs were used; roscovitine is a pan-inhibitor, inhibiting CDKs 1, 2, 5, 7, and 9, and it is currently in phase II clinical trials (Bach et al., 2005; Nair et al., 2011) and NU-2058 is a specific CDK2 inhibitor that has shown potential as a therapeutic in prostate cancer (Harrison et al., 2009; Rigas et al., 2007). In control populations, the combination of CKIs and tamoxifen decreases total cell numbers significantly; however, Spy1 overexpression significantly abrogates this effect (Figure 5C). LCC9 cells are a MCF7-derived cell line that have been treated over time with tamoxifen and other anti-estrogens making them tamoxifen resistant even in the presence of ERα (Brunner et al., 1997). Endogenous expression of Spy1 is higher in LCC9 cells as compared to their parental MCF7 cell line (Figure 5D). To determine if Spy1 levels dictate any of the resistant characteristics of these cells, Spy1 was knocked down using shRNA against Spy1

(pLKO-shSpy1), a scrambled construct was used as a control (pLKO) (Figure 5E; left panel). Cells were then treated with tamoxifen and counted using trypan blue exclusion assay. While tamoxifen had no effect on the resistant LCC9 cells over time, Spy1 knockdown had a surprising impact on cell number in response to tamoxifen, with cells depleted almost 10 fold by 48 hours (Figure 5E; right panel).
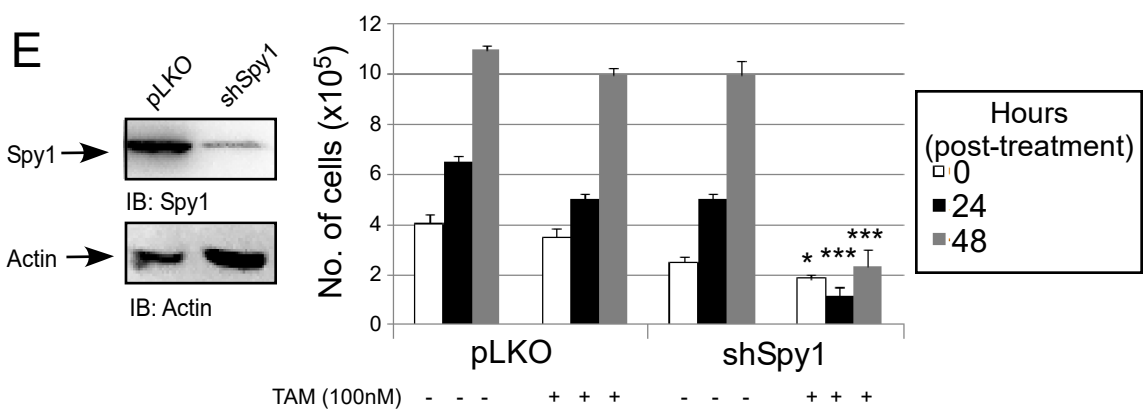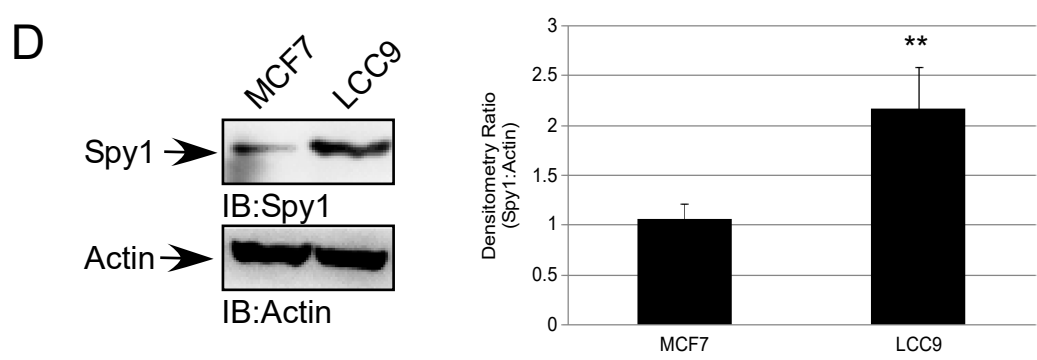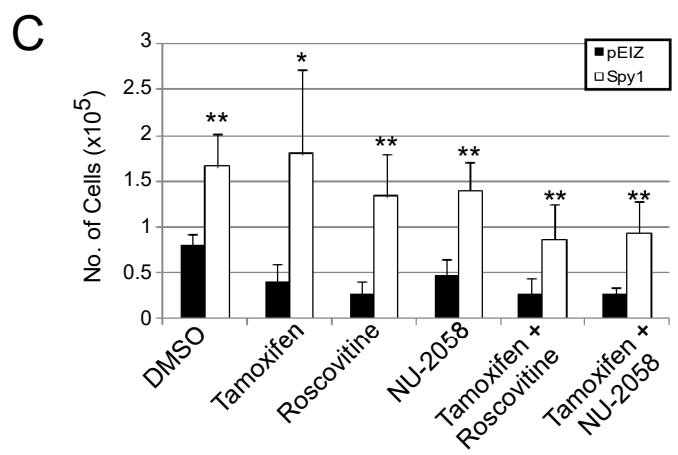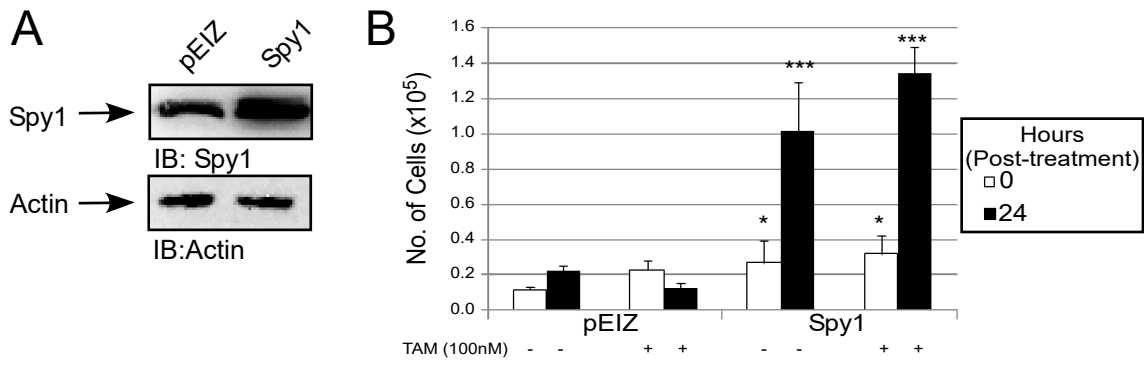
Figure 5. Spy1 levels regulate the response of cells to tamoxifen treatment. (A-B) MCF7 cells were infected with pEIZ or Spy1; followed by treatment with 100 nM of tamoxifen or vehicle control (DMSO). (A) Confirmation of overexpression was determined through SDS-PAGE and IB. (B) Proliferation after treatment was assayed using the trypan blue exlcusion assay. (C) Infected cells were treated with 100 nM tamoxifen, 20 uM roscovitine, 25 uM NU-2058, or a combination of these treatments; followed by trypan blue exclusion assay. (D) MCF7 and LCC9 cells were measured for endogenous levels of Spy1 through SDS-PAGE and IB. A representative blot (left panel) and densitometry ratio of Spy1 to loading control actin (right panel) is shown. (E) LCC9 cells were infected with shScrambled (denoted pLKO) and shSpy1, followed by treatment with 100 nM of tamoxifen or vehicle control. Confirmation of knockdown was seen through SDS-PAGE and IB (left panel) and proliferation after treatment was assayed using trypan blue exclusion assay. (A-E) Cell counts are over triplicate experiments. Error bars reflect SE between triplicate experiments. Student's t-test was performed; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Spy1 levels affect tamoxifen response in vivo.

We implemented a zebrafish xenograft model to elucidate whether Spy1 levels can increase or decrease sensitivity to tamoxifen *in vivo*. To validate the model MCF7 or LCC9 cells were injected into embryos at 48 hours post-fertilization (hpf) and treated with tamoxifen for 24 hours (Figure 6). Fish were imaged and quantified using automated software to align many fish from similar treatments into one plane. Tumour foci at 24 hours post-treatment (hpt) demonstrate that while the MCF7 cells respond to treatment the LCC9 cells are resistant to tamoxifen treatment (Figure 6A). Sensitive MCF7 cells were then infected with pEIZ control or pEIZ-Spy1 vectors (Figures 6B-D). Embryos were imaged at 0 hpt (48 hours post-implantation (hpi)) and tumour foci at 0 hpt were normalized between control and Spy1 injected cells. Tamoxifen was then administered to the fish over 24 hours and change in tumour burden recorded (Figure 6D). Spy1 overexpression significantly decreases sensitivity to tamoxifen treatment *in vivo* (Figure 6D).

A

Tamoxifen Treatment

B

0 hpt   24 hpt

DMSO

Tamoxifen

C

0 hpt   24 hpt

DMSO

Tamoxifen

D
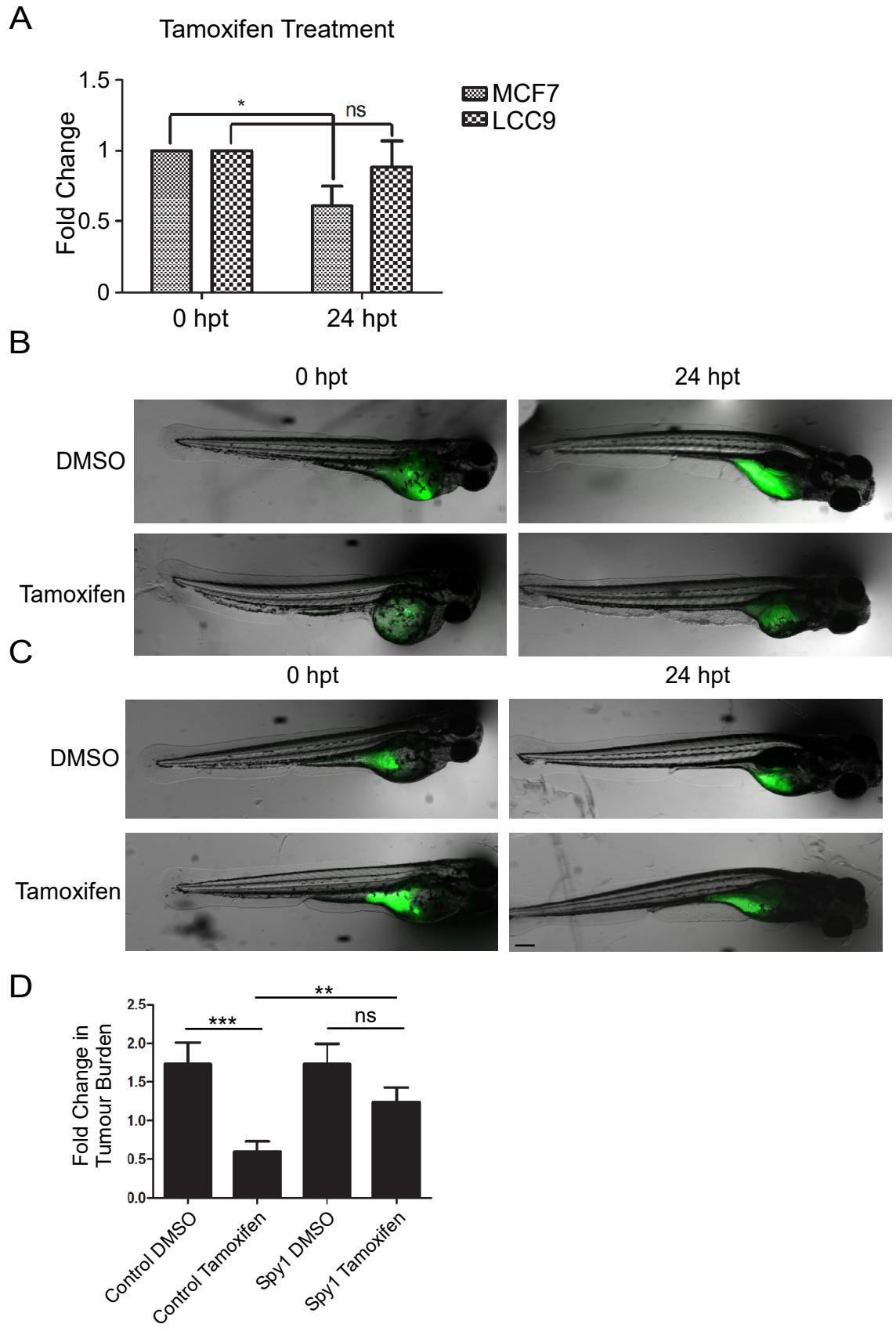
Figure 6. Spy1 levels affect tamoxifen response *in vivo*. (A) At 48 hpf, zebrafish were injected with either MCF7 or LCC9 cells. 24 hpi, 10 uM tamoxifen was added to the fish water and tumour foci per fish were counted for 24 hpt and the fold change, normalized to 0 dpt, was calculated. *p<0.05. (B-C) Representative images of cells expressing either (B) empty control vector or (C) Spy1 overexpression vector before (0 hpt) and after (24 hpt) treatment with either DMSO or 10 uM tamoxifen. (D) Graph representing the mean fold change in tumour burden from 0 hpt to 24 hpt. ns= not significant, **p<0.01, ***p<0.001. Scale bar=200 um.

DISCUSSION

The response of breast cancer cells to E2 increases the rate of proliferation through upregulation of genes required for the cell cycle, such as c-Myc and Cyclin D (Castro-Rivera et al., 2001; Maminta et al., 1991). This is achieved through the activity of ERα which can signal both genomically, to transcriptionally regulate targets directly, or non-genomically, by activating downstream pathways, such as MAPK (Bunone et al., 1996; Chen et al., 2000; Chen et al., 2002). The MAPK pathway influences the transcription of a plethora of genes, one of which is the atypical cyclin, Spy1. Spy1 is important for mammary gland development and is a driver of mammary carcinogenesis (Golipour et al., 2008). Our work demonstrates that Spy1 is upregulated downstream of activated ERα. Whether Spy1 is an essential mediator of ERα signalling in mammary development and whether Spy1 upregulation by ERα is mediated entirely through MAPK signalling are interesting questions for further exploration.

ERα proliferative signalling in breast cells is dependent upon ligand binding and/or post-translational modification to enable signalling in the absence of a ligand. Ligand-independent modifications to ERα, such as the phosphorylation on S118, can render the receptor resistant to anti-estrogen therapies like tamoxifen (Chen et al., 2000). This work demonstrates that Spy1 levels correlate with an increase in phosphorylation of ERα on S118. Cellular cycling influences the phosphorylation status of ERα, all dependent upon mitogen stimulation (Barone et al., 2006; Planas-Silva and Weinberg, 1997). Inhibiting this cycling by inhibition of CDKs using synthetic CKIs has become an important target in breast cancer therapy (Nair et al., 2011; Wesierska-Gadek et al., 2011). The use of a pan-CDK inhibitor, roscovitine, in an ERα positive cell line was

shown to reduce the level of basal phosphorylation of S118 in the presence or absence of E2 and/or tamoxifen; which at times led to the downregulation of ERα (Wesierska-Gadek et al., 2011). Our work showed elevated levels of Spy1 can protect ERα positive cells from tamoxifen treatment in combination with CKIs, roscovitine and NU-2058. Hence, the level of Spy1 may be prognostic to response to both tamoxifen and CKI therapy.

Phosphorylation on S118 is known to be regulated by ERK1/2 (Chen et al., 2000; Chen et al., 2013; Cheng et al., 2007), this work demonstrates for the first time that Spy1 is capable of activating this pathway in human cells. Lenormand et al. (1999) showed that Spy1 could activate MAPK in *Xenopus* oocytes. Unlike the Lenormand data, however, Spy1 activation of ERK1/2 appears to be MEK independent in human breast cells (Figure 7). We have further resolved some of this pathway demonstrating that Spy1-mediated ERK activation requires the direct binding of Spy1 to CDK and p27, and is mediated through the Ras pathway (Figure 7). We have further demonstrated that elevated Spy1 levels and activated ERK1/2 leads to a significant elevation in specific phosphatase levels, specifically MKP2, potentially indicating an attempt to restore homeostatic balance. Mutations within Ras and Raf, or hyperactivation of MEK1/2 has influenced the production of upstream inhibitors of the MAPK pathway; however, resistance and relapse occurs within 6 to 7 months of treatment (Morris et al., 2013). Indeed, we have shown elevated levels of Spy1 alone can override MEK1/2 inhibitors and significantly increase the phosphorylation status of ERα on S118. A significant decrease in cell viability and pERα-S118 levels in the presence of elevated Spy1 was seen only when ERK1/2 was directly inhibited. These data show that Spy1 overexpression decreases sensitivity of cells to treatment commonly used in the clinic. The discovery of a new inhibitor specifically

inhibiting the ATP-binding site of ERK1/2 has shown promising results with respect to solid tumours (Hatzivassiliou et al., 2012; Morris et al., 2013), and, in the presence of elevated levels of Spy1 within ERα positive breast cells, we see a complete abrogation in phosphorylated S118 levels and a significant response to tamoxifen treatment. Our data shows Spy1 alters the post-translational status of the ERα and inhibits response to hormone therapy through ERK1/2 activation (Figure 7). Novel therapies focusing on the direct inhibition of ERK1/2 in patient populations harbouring elevated levels of Spy1 may represent a novel therapeutic direction for both treating drug resistant patients and preventing/decreasing the incidence of resistance in ERα-positive patients.
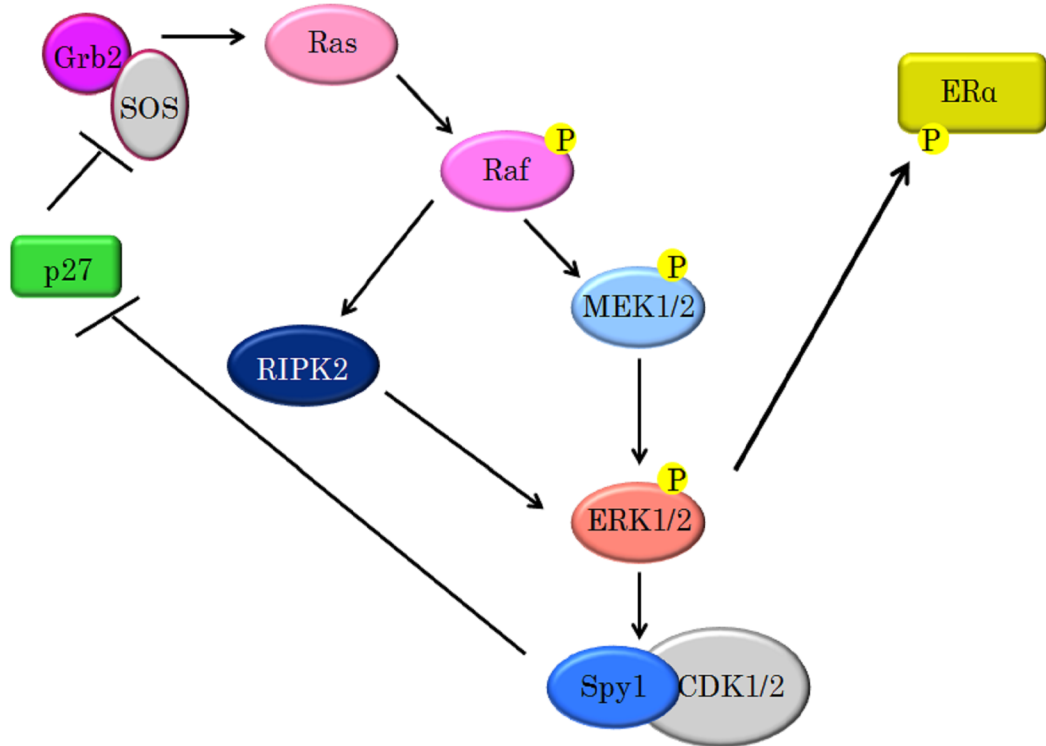
Figure 7. Schematic diagram of proposed pathway. Spy1 has been shown to be downstream of the MAPK pathway (Golipour et al., 2008). Our data further demonstrates elevated levels of Spy1 increase the activation of ERK1/2 through a MEK-independent pathway, which includes inhibition of p27, the subsequent activation of Ras and Raf, and the activation of the kinase RIPK2. The increase in ERK1/2 activation also promotes the phosphorylation of the ER on S118, which is implicated in resistance to tamoxifen treatment.

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CHAPTER 3

THE STABILIZATAION OF C-MYC BY THE NOVEL CELL CYCLE REGULATOR, SPY1, DECREASES EFFICACY OF BREAST CANCER TREATMENTS

INTRODUCTION

Breast cancer is a diverse set of diseases, with both intra- and inter-tumoral heterogeneity affecting response to treatment. Intertumoral heterogeneity can be classified into genomic subgroups; however, in the clinic breast cancers remain classified largely by the presence or absence of hormone/growth factor receptors. Triple negative breast cancer (TNBC) is one such group, identified by the lack of expression for the estrogen receptor alpha (ERα), progesterone receptor (PR), and the HER2/neu protein. TNBCs can fall under different genomic subgroups; Her2-enriched, claudin-low or basal breast cancers, one indicator of the varying mechanisms that can drive the phenotype for this form of breast cancer (Malhotra et al., 2010; Perou, 2010; Perou et al., 2000; Prat et al., 2010). Patients who lack ERα, including TNBC patients, are not candidates for hormone therapy and generally have a less favourable prognosis (Lumachi et al., 2013). Loss of ERα occurs in about 15-20% of resistant breast cancers (Riggins et al., 2007). While the mechanism for this downregulation remains to be fully elucidated, ERα levels can be manipulated epigenetically with histone modification and DNA methylation (Yang et al., 2001). Understanding the molecular pathways regulating the expression of ERα may provide novel mechanisms of sensitising ERα-negative patients, including those that are triple negative, to available therapies.

As early as 1984, gene amplification and overexpression of the transcription factor c-Myc was shown in an array of aggressive breast cancers of varying receptor/growth factor status (Escot et al., 1986; Kniazev et al., 1986; Kozbor and Croce, 1984; Perou et al., 2000). Several studies show that c-Myc overexpression occurs frequently in ERα-negative breast cancers (Persons et al., 1997; Xu et al., 2010) and c-

Myc is highly elevated and implicated as an important driver of TNBC (Kang et al., 2014; Peddi et al., 2012; Perou, 2010). Only around 20% of c-Myc amplification in breast cancer is detectable at the mRNA level, implicating regulation at the RNA stability, translation or protein level (Bieche et al., 1999). c-Myc regulates approximately 10% to 15% of the human genome, having potent effects on cell proliferation, differentiation, apoptosis, and senescence (Amati and Land, 1994; Amati et al., 1993; Evan et al., 1994; Evan et al., 1992). In the breast, c-Myc has multiple functions in normal development and is implicated in the initiation and progression of breast cancer (Blakely et al., 2005; Liao and Dickson, 2000; Littlewood and Evan, 1990; Schmidt, 1999). Although the exact mechanism of how ERα levels become downregulated is unknown, long-term overexpression of c-Myc has become a suggested mechanism towards the development of estrogen-independence (Xu et al., 2010). Increased transcriptional activity of c-Myc mimics ERα response to estradiol, specifically through the activation of ERα-targeted genes (Alles et al., 2009; Dadiani et al., 2009). This chapter studies the implications of persistent c-Myc signalling on ERα status.

While c-Myc is an attractive target for several forms of cancer, mechanistically achieving this goal has been a challenge. The dominant negative mutant, Omomyc, has the potential to regress *in vivo* tumours, but tumours quickly re-establish when Omomyc is removed (Soucek et al., 2002). Targeting c-Myc effectors has demonstrated some effectiveness. Of particular interest, inhibiting the G2/M cyclin dependent kinase, CDK1, in TNBC has shown impressive synthetic lethality in cell systems (Horiuchi et al., 2012; Kang et al., 2014). CDK1 is critical for cell proliferation and this limits the ability to

achieve a safe therapeutic window; hence, exploiting ways to target specific aspects of CDK1 driven activity is required to make this a viable strategy.

One aspect of CDK inhibitors that has been overlooked in the clinic is the presence of 'cyclin-like' proteins capable of binding and activating CDKs in the presence of traditional inhibitors (Nebreda, 2006). Spy1 (gene *SPDYA*) is one member of this family. Spy1 can bind and activate both G1/S and G2/M CDKs independent of the activating phosphorylation on the T-loop and in the presence of inhibitory phosphates to enhance proliferation and promote the degradation of p27^{Kip1} (Cheng et al., 2005a; McAndrew et al., 2007). Spy1-bound CDKs appear to take on a different conformation with unique substrate preferences (Cheng et al., 2005b). Spy1 levels are low in most adult tissues, being selectively expressed in regenerative populations and elevated downstream of c-Myc in several human cancers, including invasive ductal carcinoma of the breast (Al Sorkhy et al., 2012; Golipour et al., 2008; Hang et al., 2012; Huang et al., 2009; Ke et al., 2009; Lubanska and Porter, 2014b; Porter et al., 2002; Zucchi et al., 2004). Hence, Spy1-CDKs may represent a unique mechanism to target for selective treatment of specific cancers. Indeed Spy1 levels specifically override apoptosis following DNA damage and this mechanism may be particularly potent in drug resistant tumours (Barnes et al., 2003; Gastwirt et al., 2006).

Herein, we demonstrate that persistent elevation of c-Myc correlates with an increased expression of Spy1 and a reduction in ERα levels. Knockdown of Spy1 reduces c-Myc levels and enhances sensitivity to both hormone and chemotherapy treatment. Hence, specifically targeting Spy1-directed CDKs may be an effective strategy in ERα-negative breast cancers with elevated c-Myc.

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MATERIALS AND METHODS

Cell Culture. Primary MMTV-Myc cells were isolated from the fourth mammary gland of MMTV-Myc mice and were subcultured in DMEM-F12 media supplemented with 10% FBS, 30,000 units penicillin/streptomycin solution, 5ng/ml EGF, 0.5 μg/ml Hydrocortisone, and 5 μg Insulin. HEK-293, MCF7, MDA-MB-231, and MDA-MB-468 cells were purchased from ATCC and were subcultured in DMEM media containing 10% FBS and 30,000 units penicillin streptomycin solution. LCC9 cells (Lombardi Comprehensive Cancer Center, Georgetown University) were routinely subcultured in DMEM phenol red free media supplemented with 1 mM L-glutamine, 30,000 units penicillin/streptomycin, and 10% charcoal treated FBS. All cells were maintained under normoxic conditions (5% CO_2) at 37°C.

Plasmids. Creation of the Myc-Spy1-pCS3 was described previously (Porter et al., 2002). Rc-CMV-Cyclin E (#8963) and lentiviral constructs pLB (#11619) and pLKO-scrambled control (#8453) were purchased from Addgene. shRNA oligos for Spy1, Cyclin E and a scrambled control were ligated into the pLB vector, as previously described, and were previously determined to be specific using multiple constructs of each and rescue constructs (Lubanska and Porter, 2014b).

Immunoblotting (IB). Cells were harvested in NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) containing protease inhibitors (100 μg/ml PMSF, 5 μg/ml aprotinin, and 2 μg/ml leupeptin) for 1 hour on ice. Bradford reagent was used for protein concentration as per manufacturer's instructions (Sigma). 80-100 μg of protein were subjected to electrophoresis on denaturing 10% SDS polyacrylamide gels and transferred to PVDF-Plus 0.45 micron transfer membrane (Osmonics Inc.) for 2

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hours at 30 volts using a wet transfer method. Blots were blocked for 1 hour in 1% BSA solution at room temperature. Primary antibodies were reconstituted in blocker and incubated overnight at 4°C, secondary antibodies were used at 1:10000 dilution in blocker for 1 hour at room temperature. Blots were washed 3 times in TBST for 3 minutes following incubation with primary and secondary antibodies. Chemiluminescent Peroxidase Substrate was used for visualization following manufacturer's instruction (Pierce). Chemiluminescence was quantified on an AlphaInnotech HD2 (Fisher) using AlphaEase FC software. The proper antibodies were used at the following concentrations: Actin MAB150 1R (Chemicon-Millipore; 1:1000), Spy1 (ThermoScientific; 1:1000), c-Myc (Sigma; 1:1000), anti-phospho-c-Myc-S62 (Abcam; 1:1000), anti-phospho-c-Myc-T58 (Santa Cruz Biotechnology; 1:1000), Cyclin E1 (Abcam; 1:1000), anti-p-ERK 1/2 [Thr 202/Tyr 204] (Cell Signaling; 1:1000), and anti-ERK1/2 (Santa Cruz Biotechnology; 1:1000).

Lentiviral Production. VSV-G pseudotyped lentivirus was produced by transient transfection of HEK293 LentiX cells with transfer vector and the multi-deleted packaging plasmids (pMDG, pMDL2, pRSV) using polyethylenimine (PEI) (Sigma) reagent with 1:3 DNA to PEI ratio and incubation for 5 hours at 37°C, 5% CO_2. The virus was collected the next day and concentrated for 3 hours at 4°C using an ultracentrifuge. The titer for pEIZ and pLB were determined by transducing 293T cells and analysis of eGFP protein expression by flow cytometry at 72 hours post transduction. The titer for pLKO lentivirus was assessed by puromycin selection followed by crystal violet staining and quantification of resistant colonies. The titered virus was filter sterilized and stored at -80°C.

Transfection/Infection. *Transfection:* Cells were transfected using PEI branched reagent. In brief, 10 μg of DNA was mixed with 3 μL of 10 mg/ml PEI for 10 minutes then added to a 10 cm tissue culture plate. Transfection media was changed after 24 hours. *Lentiviral Infection:* 8000 cells were seeded in fully supplemented growth media in 96-well plates for 2 hours. Cells were starved by removing serum and penicillin/streptomycin from the media, followed by the use of 1 mg/ml polybrene (Santa Cruz Biotechnology) and MOI 3 of the specific vector used. Infected media was changed to fully supplemented media 24 hours after infection.

Drug Treatments. Treatments included vehicle control dimethyl sulfoxide (DMSO), 100 nM 4-Hydroxytamoxifen (Sigma), 25 nM doxorubicin (Sigma), 100nM paclitaxel, 6mM cyclophosphamide (Sigma) for specified time points.

Proliferation Assay. Cells were seeded at 5×10^4 cell density in 24-well plate. Following treatments cells were collected at specified time points, pelleted and resuspending in 1mL of media. 10 μl sample was collected, trypan blue was added and counted using a haemocytometer.

BrdU Assay. BrdU stock (10 mM) was dissolved in 10 mL of culture medium to produce a 10 μM labelling solution. Infected and non-infected cells were seeded at 8×10^3 cell density in 96-well plate, after 24 hours the culture medium was replaced with BrdU labelling solution for 30 minutes in CO_2 incubator at 37°C. The labelling solution was removed and washed 2 times with 1x PBS for 2 minutes each. PBS was removed and 3.7% formaldehyde in PBS was added to each well and incubated for 30 minutes at room temperature. Cells were washed with 1xPBS 3 times for 2 minutes each. Cells were immersed in 0.07N NaOH for 2 minutes, then in 1xPBS (pH 8.5) to neutralize the base.

20 µl anti-BrdU (Becton Dickinson) was mixed with 50 µl of 0.1% Tween 20/PBS. For indirect immunofluorescence staining, the diluted unconjugated anti-BrdU was added to the cells and incubated for 30 minutes in a humidified chamber. Cells were washed with 1xPBS. 50 µl 0.1% Tween 20/PBS was added to cells. Alexa mouse (1:1000) was added for 30 minutes at room temperature. Cells were washed with 1xPBS and incubated with Hoechst. Cells were washed with water and air dried.

Cyclohexamide (CHX) Treatment. After transfection cells were treated with 50 µg/mL cycloheximide (Sigma) to block *de novo* protein synthesis. After 0.25 to 2 hours, cells were pelleted, harvested and subjected to SDS-PAGE analysis and IB.

Tissue Microarray (TMA) Construction. Embedded tissue samples were received from Windsor Regional Cancer Centre and constructed into TMAs using an Arraymold Inc., TMA mold with 72 1.5 mm cores (20015D). Briefly, paraplast X-tra paraffin (Sigma) was added to the mold, an embedding ring was placed on top and filled with paraffin. After an hour incubation on ice the mold was separated from the embedding ring. 3-4 cores were taken from each embedded sample and placed into the TMA mold. Following 10 minutes of incubation in 65°C oven, the mold was placed on ice. The cores were left overnight prior to sectioning. TMAs were sectioned using a Leica microtome at 10 µm and placed on Fisherbrand Superfrost Plus microscope slides (12-550-15) and heated for 10 minutes.

Immunohistochemistry (IHC). TMA sectioned slides were deparaffinized and rehydrated using 3 changes of xylene for 3 minutes each, followed by 3 changes of 100% EtOH for 2 minutes each, and 95%, 80%, and 70% EtOH for 2 minutes. Slides were washed in 1x PBS for 5 minutes. Sodium citrate antigen retrieval was performed. Briefly,

slides were placed in a rack in 600 ml of 10 mM Sodium Citrate (pH 6.0) in a 2L tupperware container, and microwaved for 20 minutes total, replacing evaporated water every 10 minutes. Slides were cooled for 20 minutes, washed 3 times in distilled water for 5 minutes each and in 1x PBS for 5 minutes. Slides were blocked for endogenous peroxidases using 90 ml methanol/10 ml 30% H_2O_2 for 10-15 minutes at room temperature, followed by 3 washes in 1x PBS for 5 minutes each. After circling sections with a PAP pen, blocking buffer (3%BSA/0.1% Tween in 1xPBS) was added for 1 hour at room temperature in a humidified chamber. Primary antibody was diluted 1:200 in blocking buffer and added to slides overnight at 4°C. After incubation, primary antibody was washed in 1x PBS 3 times for 10 minutes each. Biotinylated secondary antibody was diluted 1:500 in blocking buffer and added to the slides for 1 hour at room temperature in a humidified chamber. Secondary antibody was washed 3 times for 10 minutes in 1x PBS. ABC reagent (Vector Labs) was added to each slides and incubated for 30 minutes at room temperature. Slides were washed in 1x PBS 3 times for 5 minutes each. DAB (Vector Labs) was added to each slide and was removed once colour change was seen (approximately 1-10 minutes). Slides were washed in distilled water for 5 minutes. Haematoxylin was used as a counterstained for 1-2 minutes followed by a 1 minute wash in 1x PBS and then one wash in distilled water for 1 minute. Slides were dehydrated in 95% EtOH 3 times for 5 minutes, followed by 3 changes of 100% EtOH for 5 minutes. Lastly, slides were put through 3 changes of xylene for 15 minutes each, coverslip was added after final xylene change.

Microscopy. Slides were imaged using Leica Stereoscope M205FA. Using Leica LAS V4.3 program, images were taken at 79.7x magnification using 1x stereoscope objective and 159x magnification using 2x stereoscope objective.

TMA Quantification. Quantification of Spy1 and c-Myc immunostaining intensity was performed using Adobe Photoshop CC 2014 (Adobe Systems Inc. San Jose, CA) using nine random samplings of 10 x 10 pixels each, based on a previously reported densitometry method (Goenka et al., 2013; Matkowskyj et al., 2000).

RESULTS

Primary MMTV-Myc cells acquire resistance to tamoxifen over time in culture.

Primary MMTV-Myc cells were passaged over time and their response to tamoxifen over 24 hours measured using BrdU incorporation analysis. In early passages (P10-P35) tamoxifen treatment reduced the number of cells going through DNA synthesis by 80-90%, but in late passages (P45-P85) tamoxifen had no significant effect (Figure 1A). Response was also recorded as a product of percent change in overall cell number compared to vehicle control. Early passage cells (P34) decrease in cell number by ~35% in response to treatment, while late passage cells (P80) do not respond to tamoxifen treatment (Figure 1B). We then used this model to determine any effects on ERα levels with persistent c-Myc signalling (Figure 1C). We find that the protein levels of ERα begin to decrease dramatically between P30-P80. As shown previously, levels of Spy1 are high in the presence of c-Myc signalling (Golipour et al., 2008), and we show that they remain at an elevated level at all passages.
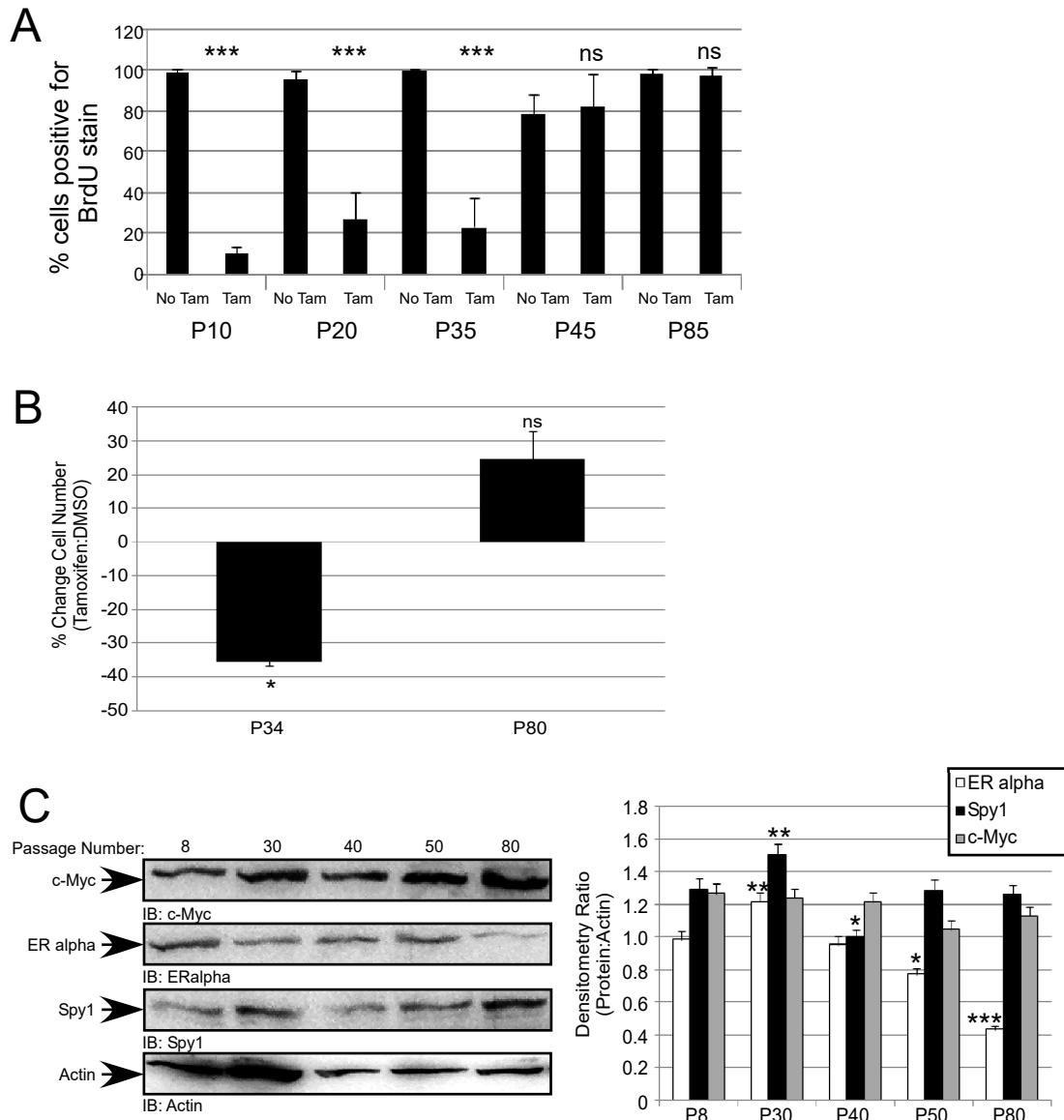
Figure 1. Primary MMTV-Myc cells acquire resistance to tamoxifen over time in culture. (A) Primary MMTV-Myc cells at passage (P) 34 and 80 were treated with 100 nM of tamoxifen or vehicle control (DMSO); followed by BrdU analysis. The percent BrdU positive cells are depicted on y-axis (B) Primary MMTV-Myc cells at various passages were treated with 100 nM of tamoxifen or vehicle control (DMSO) followed by trypan blue exclusion assay. Percent difference between tamoxifen treated and DMSO control depicted on y-axis. (C) Primary MMTV-Myc cells were passaged and collected, followed by SDS-PAGE and IB. A representative blot is shown (left panel) and densitometry of protein to loading control actin is shown (right panel). (A-C) Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05, **p<0.01, ***p<0.001.

Spy1 levels affect cellular response to tamoxifen treatment.

To determine if Spy1 levels could affect the treatment response of late passage cells (P80), which showed an acquired resistance to tamoxifen, we infected P80 cells with either shScrambled (pLB) or Spy1 knockdown (shSpy1). Knockdown of Spy1 significantly decreased c-Myc protein levels (Figure 2A). Cells with Spy1 knockdown had a significant reduction in cell proliferation over time compared to the aggressive control population (Figure 2B). Spy1 knockdown cells and control cells were also treated with 100 nM tamoxifen and viability measured. After 24 hours, Spy1 knockdown significantly decreased the number of viable cells by ~33% in response to tamoxifen (Figure 2C). This was also reflected by a significant difference in BrdU incorporation in P80-Spy1 knockdown vs. control knockdown (pLB) cells treated with tamoxifen (Figure 2D).
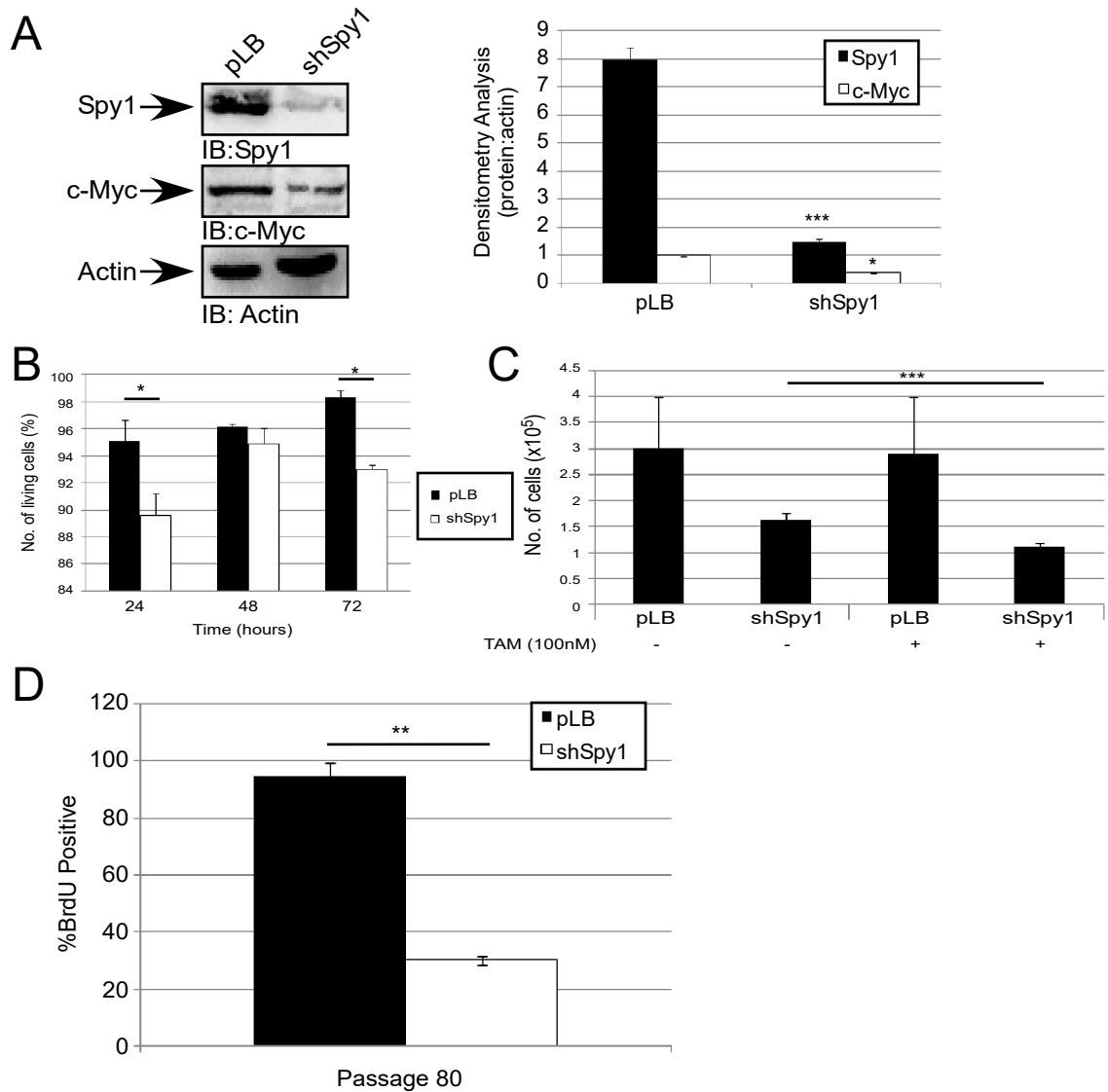
Figure 2. Spy1 levels affect cellular response to tamoxifen. Cells were infected with pLB empty vector or pLB-shSpy1 vector. (A) SDS-PAGE and IB was performed. A representative blot is shown (left panel) and densitometry ratio of protein to loading control actin is shown (right panel). (B) Infected cells were subjected to trypan blue exclusion assay for the indicated time course. (C) Infected cells were treated with 100 nM of tamoxifen or vehicle control (DMSO). Proliferation after treatment was assayed using trypan blue exclusion assay. (D) BrdU analysis of P80 cells infected with pLB or shSpy1. The percent of BrdU positive cells is depicted on the y-axis. (A-D) Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05,**p<0.01,***p<0.001.

Spy1 has a role in stabilizing c-Myc.

TNBC is associated with elevated levels of c-Myc (Horiuchi et al., 2012). We tested whether Spy1 levels correlated with that of c-Myc in TNBC cells (MDA-MB-231 and MDA-MB-468) as compared to ERα-positive MCF7 or their hormone resistant counterpart LCC9 (Figure 3A) (Hydbring et al., 2010). TNBC cells have higher levels of both Spy1 and c-Myc as compared to ERα positive breast cancer cell lines. Interestingly, LCC9 resistant cells also have higher levels of both c-Myc and Spy1 than hormone sensitive MCF7 cells. To elucidate whether Spy1 is essential for elevated c-Myc levels, we infected MDA-MB-231 cells with shScrambled or shSpy1 and collected 24 to 72 hours after infection. Spy1 knockdown significantly reduced c-Myc protein levels (Figure 3B). To determine if Spy1 overexpression can affect the stabilization of c-Myc protein, HEK-293 cells were manipulated to overexpress Spy1 or an empty vector control (pCS3) followed by treatment with cycloheximide to block *de novo* protein synthesis and c-Myc protein half-life ($t_{1/2}$) was monitored by western blot over time. Spy1 increases the half-life of c-Myc almost 2 fold (Figure 3C). Half-life of the phosphorylated form was shorter than that of overall levels of c-Myc but also demonstrates a significant increase in the presence of Spy1 (Figure 3C, lower graph).
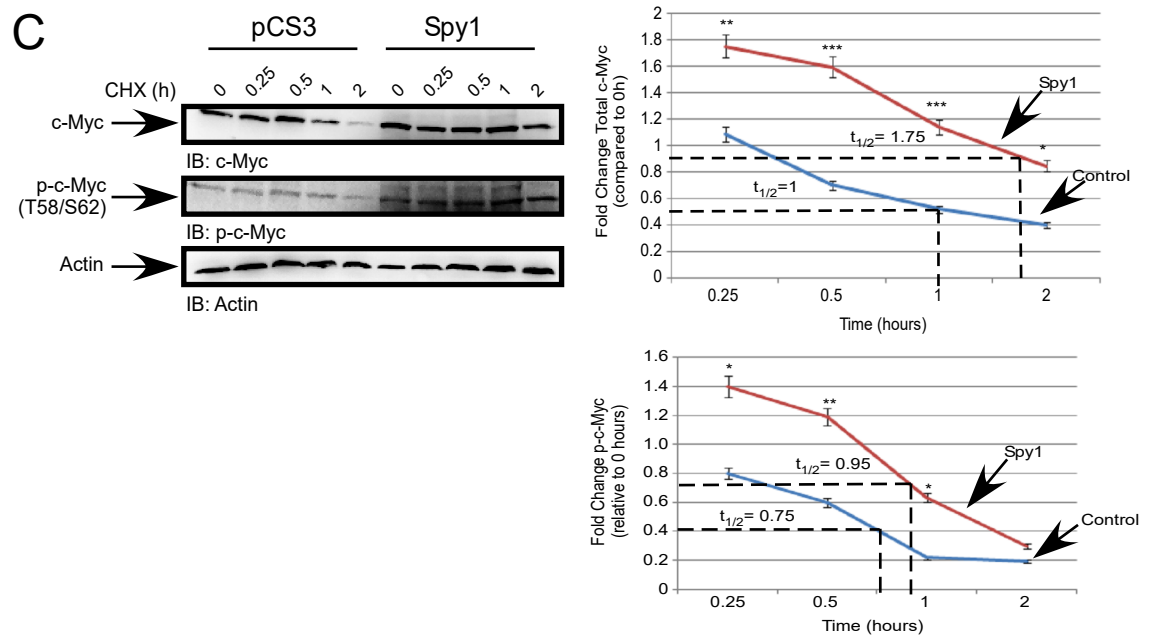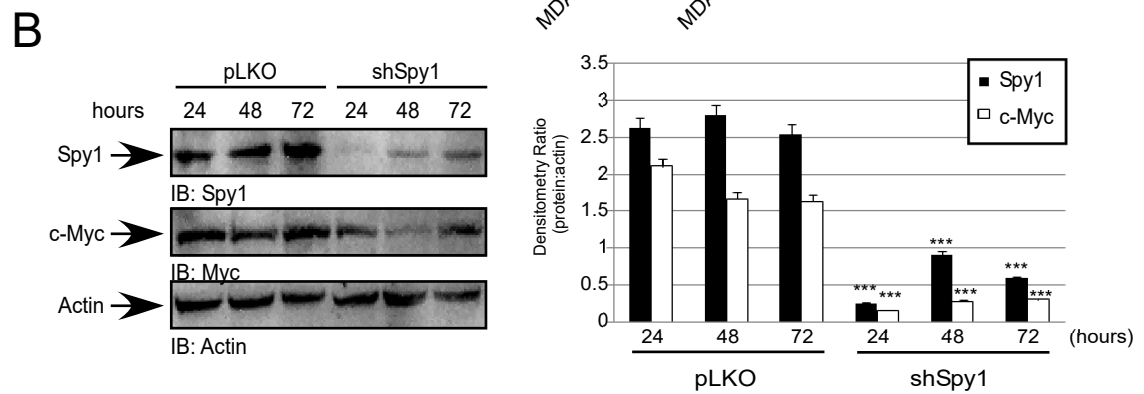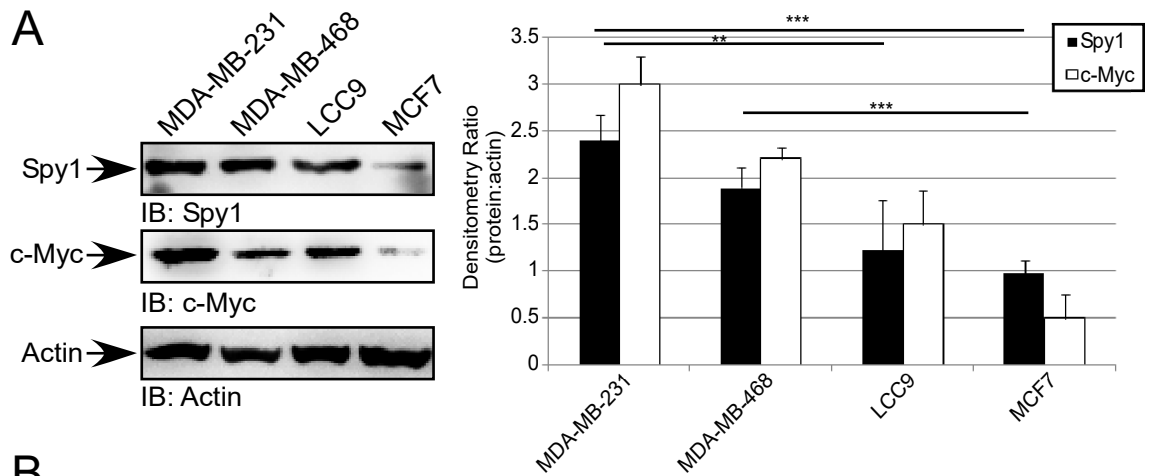
Figure 3. Spy1 has a role in stabilizing c-Myc. (A) A panel of breast cancer cell lines were subjected to protein extraction and SDS-PAGE analysis followed by IB to determine overall Spy1 and c-Myc levels. (B) MDA-MB-231 cells were infected with shScrambled (denoted pLKO) or shSpy1 (denoted shSpy1); followed by SDS-PAGE and IB. (C) Hek-293 cells were transfected with pCS3, myc-tagged Spy1 followed by treatment with 50 ug/ml cyclohexamide. After 0.25, 0.5, 1, and 2 hours, cells were collected and subjected to SDS-PAGE analysis and IB. Fold change of control and overexpression to time 0 hours was calculated and graphed to determine the overall effect on c-Myc stabilization by overexpression of Spy1. (A-C) A representative blot is shown (left panel) and densitometry ratio of Spy1 to actin loading control is shown (right panel). Error bars reflect SE between triplicate experiments. Student's t-test was performed; $*p<0.05, **p<0.01, ***p<0.001$.

Stabilization of c-Myc requires Spy1 and Cyclin E.

c-Myc protein levels are, in part, regulated post-translationally via phosphorylation on serine (S)-62 leading to subsequent protein stabilization. S62 can be phosphorylated by CDK2, CDK1, and ERK1/2 (Adhikary and Eilers, 2005; Amati, 2004; Amati et al., 1998; Sears et al., 2000). To determine the effect of Spy1 on the stabilization of c-Myc, Spy1 was overexpressed in HEK-293 cells and Cyclin E1 was used as a positive control. Our data confirms the literature that Cyclin E overexpression results in an increased phosphorylation of c-Myc at S62 (Figure 4A). Comparably, we show that overexpression of Spy1 also leads to the phosphorylation of c-Myc (Figure 4A). We further investigated whether Spy1 was a necessary mediator of c-Myc stabilization by knocking down either Spy1 or Cyclin E1 in HEK-293 cells. Knockdown of either gene in HEK-293 significantly decreased the level of p-c-Myc as compared to control (Figure 4B).
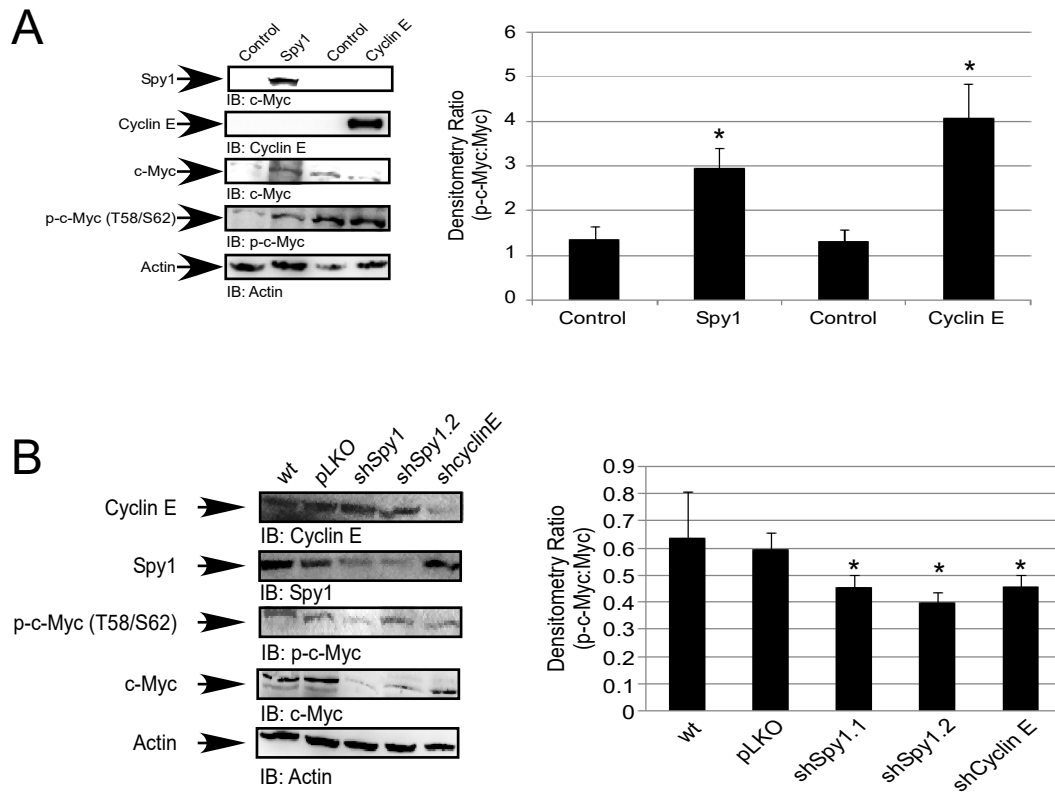
Figure 4. Stabilization of c-Myc requires Spy1 and Cyclin E. (A) Cells were transfected with pCS3, myc-tagged pCS3-Spy1, pCMV, or pCMV-Cyclin E vector; followed by SDS-PAGE and IB. (B) Cells were infected with shScrambled control (denoted pLKO), two shSpy1 constructs (shSpy1.1 and shSpy1.2), and shCyclin E; followed by SDS-PAGE and IB. (A-B) A representative blot is shown (left panel). Densitometry ratio of protein to actin loading control is shown (right panel). Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05.

Spy1 levels are elevated in human TNBC tumour tissue.

Spy1 levels are elevated in invasive ductal carcinoma of the breast (Al Sorkhy et al., 2012). To determine the levels of Spy1 in TNBC, frozen adjacent pair-matched normal and tumour TNBC tumour samples were obtained from the Ontario Tumour Bank and subjected to protein extraction and IB analysis. Spy1 levels are significantly elevated in TNBC patient samples as compared to adjacent pair-matched normal tissue (Figure 5A). To increase our sample size TNBC embedded patient samples were collected, subject to haematoxylin and eosin (H&E) staining to designate tumour versus stroma area and 1.5 mm cores used in a TMA construction. At least 3 normal patient samples were included on each array and IHC/DAB staining was performed for Spy1 (Figure 5B-C) and c-Myc protein levels (Figure 5D-E). Spy1 protein levels are significantly higher than in control, with increases in intensity of over 2 fold over all samples (Figure 5C). c-Myc protein levels were also significantly upregulated as compared normal tissue, being greater than 2 fold elevated (Figure 5E). The data collected from this study has also been individualized for each patient sample and will be used to determine whether there are any correlations between Spy1 and/or c-Myc protein levels and response to therapy.

A

Spy1 →

IB: Spy1

Actin →

IB: Actin

B

i

negative

Patient #
HS13-13850

ii

iii

Patient #
HS13-3215

iv

v

Normal

vi

vii

C
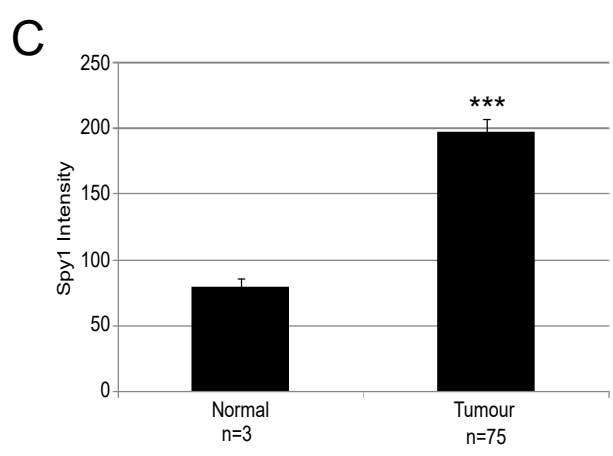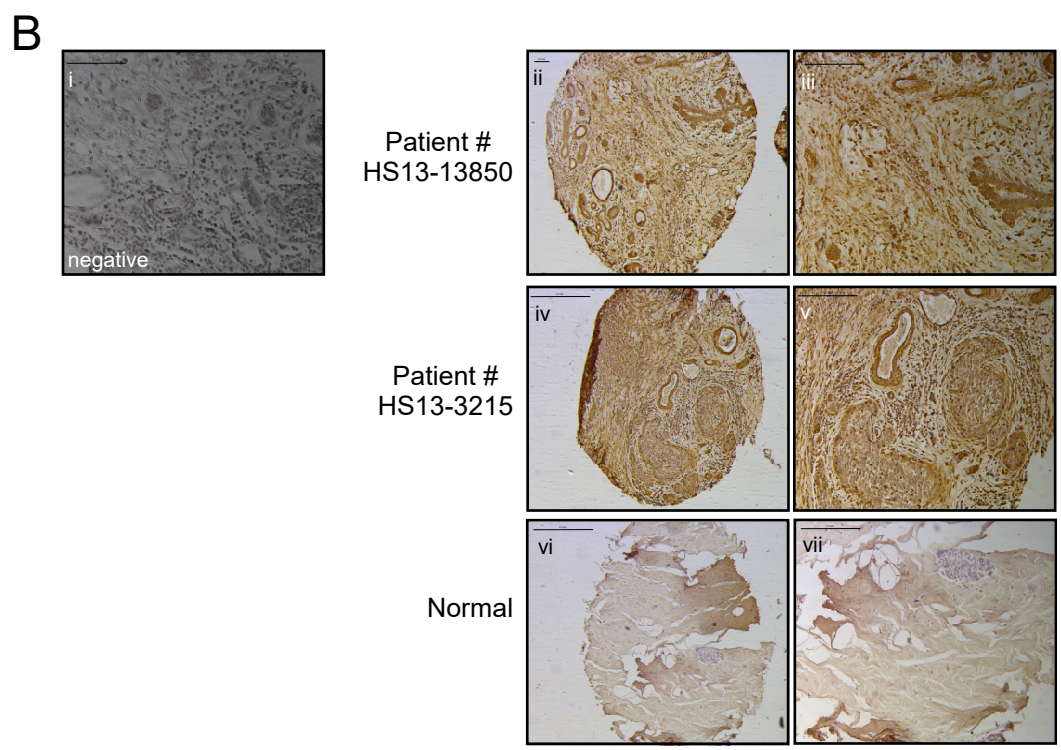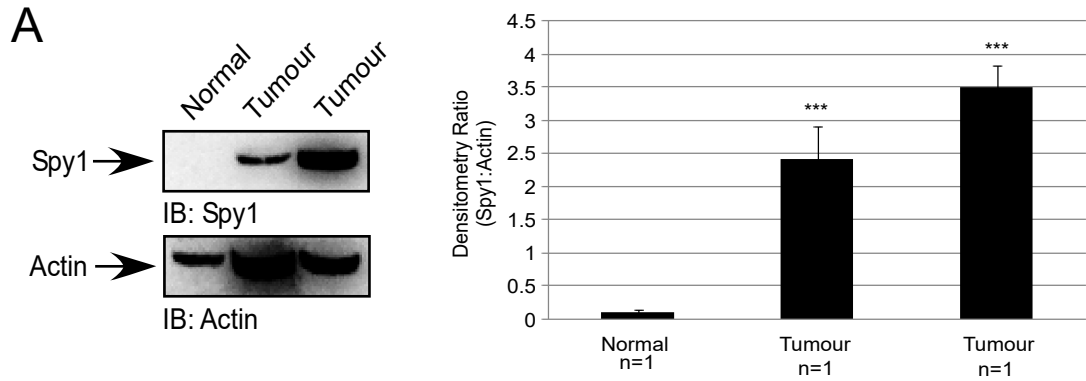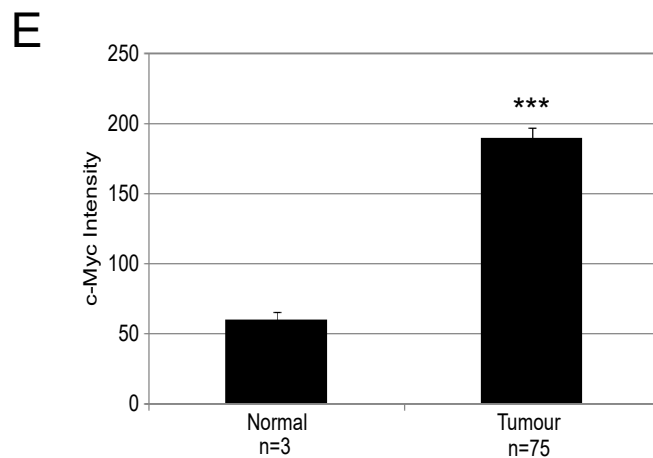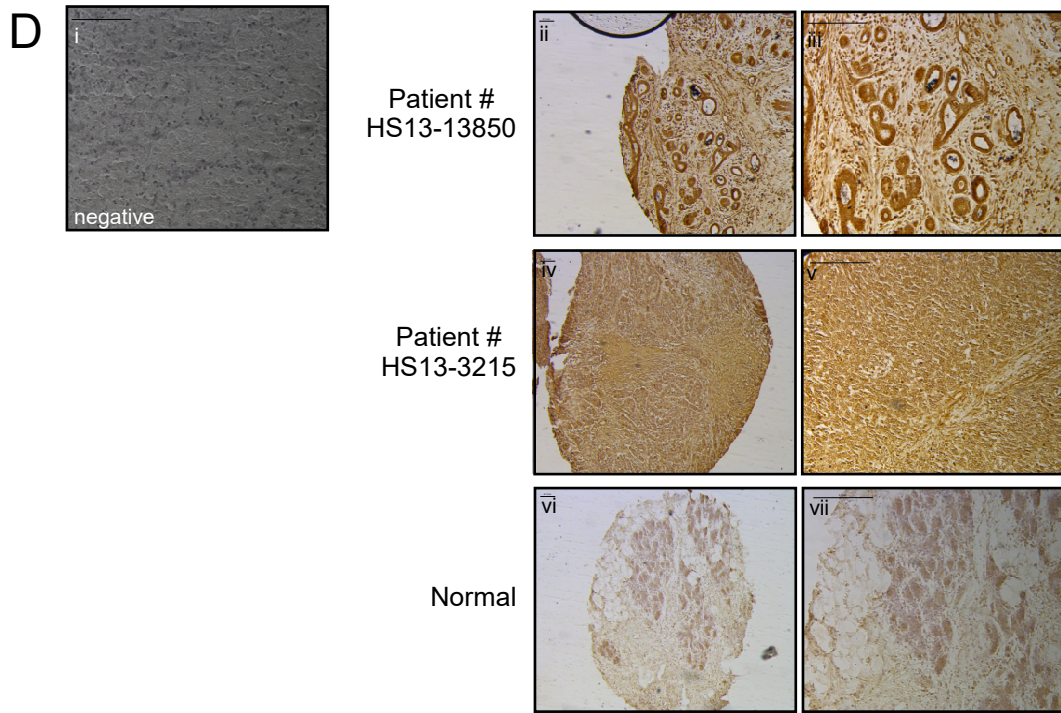
Figure 5. Spy1 levels elevated in human TNBC tumour tissue. (A) Protein from frozen tumour tissue was extracted and analysed by SDS-PAGE and IB. Densitometry analysis for Spy1 levels is seen in the right panel. TMAs were constructed and stained for Spy1 protein (B-C) or c-Myc protein (D-E). (B) Representative images of patient samples stained for Spy1 protein. (i) negative control. (ii, iv & vi) Image taken at 79.7x using a 1x stereoscope. (iii, v & vii) Image taken at 159x using a 2x stereoscope. (C) Average Spy1 intensity over 75 patient samples obtained by random sampling of 10x10 pixels each and quantified in Adobe Photoshop. (D) Representative images of samples stained for c-Myc protein. (i) negative control. (ii, iv & vi) Image taken at 79.7x using a 1x stereoscope. (iii, v & vii) Image taken at 159x using a 2x stereoscope. (E) Average c-Myc intensity over 75 patient samples obtained by random sampling of 10x10 pixels each and quantified in Adobe Photoshop. (A,C, & E) Error bars reflect SE between triplicate cores. Student's t-test was performed; ***p<0.001.

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Spy1 knockdown increases TNBC cell line response to chemotherapy treatment.

ERα-negative breast cancers, including TNBC, undergo a chemotherapy regimen, one common standard of care regimen includes an anthracyclin, cyclophosphamide, and taxol combination, also known as AC/T (Citron et al., 2003). To determine whether chemotherapy regimens can work more effectively *in vitro* when Spy1 levels have been depleted or are low, MDA-MB-231 cells were infected to knockdown either Spy1 or Cyclin E1 (Figure 6A). Knockdown of either gene significantly reduced levels of c-Myc. However, Spy1 knockdown also demonstrated a significant decrease in percent cell viability with the use of each drug treatment alone or in combination (AC/T). This effect was not consistently seen with Cyclin E1 knockdown, especially with the individual use of paclitaxel (Figure 6B), indicating this is a trait unique to Spy1.
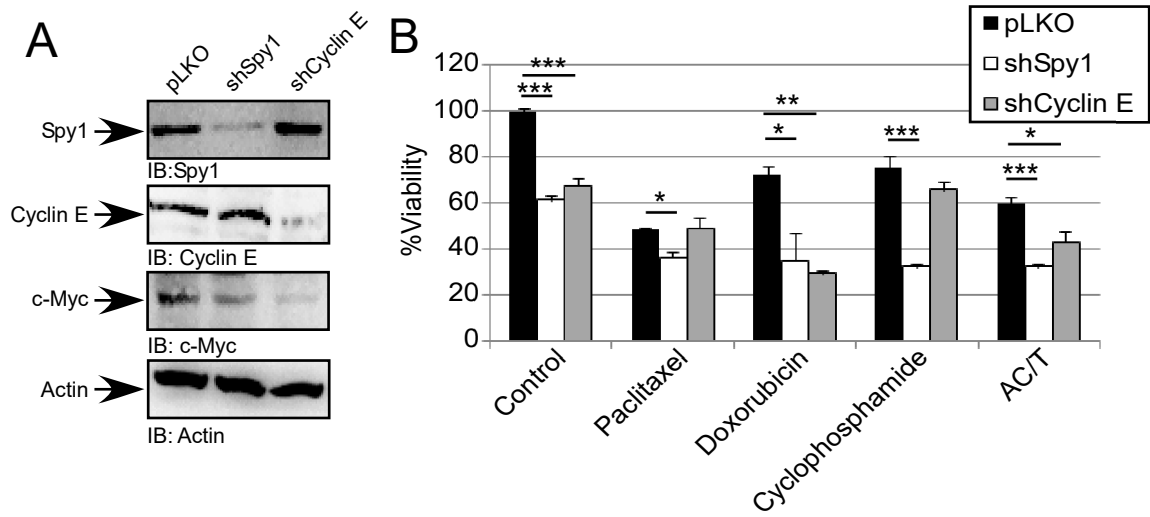
Figure 6. Spy1 knockdown increases TNBC cell line response to chemotherapy treatment. (A-C) Cells were infected using pLKO, shSpy1, and shCyclin E. (A) Confirmation of knockdown was seen through SDS-PAGE and IB analysis. (B) Cells were treated with 100 nM paclitaxel, 25 nM doxorubicin, 6 mM cyclophosphamide, or a combination of the three (AC/T). Following incubation times, cells were subjected to trypan blue exclusion assay. Error bars reflect SE between triplicate experiments. Two-way ANOVA was performed; *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Breast cancer is a heterogenous disease, divided into 5 main subtypes. Each subtype is defined by its molecular signature; with 3 important proteins to aid in its classification; ERα, PR, and Her2/neu (Dolle et al., 2009). The presence of at least one of these three proteins enables a patient to receive targeted therapies; which have significantly increased the overall 5-year survival rate of breast cancer patients to 88% (CBCF, 2014). However, a subset of patients cannot respond to these forms of treatments or become resistant to therapies through the loss of ERα, PR, or Her2/neu (Dolle et al., 2009). Loss/absence of all three genes is classified as the highly aggressive TNBC group, with a majority of patients being younger than 50 years of age (Dolle et al., 2009). The aggressiveness of this subtype is also in part due to the upregulation of various genes, such as the oncogene c-Myc or mutation of the tumour suppressor gene *p53* (Alles et al., 2009; Perou, 2010).

Transcriptional upregulation of c-Myc over time can confer control over ERα-targeted genes (Alles et al., 2009; Dadiani et al., 2009). This characteristic of c-Myc has been correlated with the basal breast cancer subtype and acquired resistance to hormone therapies, such as tamoxifen (Dimitrakakis et al., 2006; Musgrove et al., 2008a; Musgrove et al., 2008b). Our data shows that persistent c-Myc signalling using the MMTV-Myc model results in a decreased response to tamoxifen over time, leading to a resistant phenotype; which correlates with a loss of ERα protein expression. The mechanism behind this differential response and loss of expression is currently unknown. There is data to suggest, however, that a loss/downregulation of ERα may be through the activation of downstream c-Myc targets that feedback to the ERα (Dimitrakakis et al.,

2006). Much research has focused on targeting c-Myc; however, this has proven challenging due to the vast number of c-Myc targets, directing multiple biological functions, including proliferation, differentiation, apoptosis, and senescence (Amati and Land, 1994; Amati et al., 1993). For instance, c-Myc null cells have a 2.5 fold decrease in protein synthesis which leads to growth defects due to a delay in both G1 and G2 phases of the cell cycle (Schmidt, 1999). Whether targeting specific downstream targets, such as the atypical cyclin Spy1, can avoid lethality of healthy cells and provide adequate targeting to cancer cells is a hypothesis that this work supports.

Spy1 has been found to follow a similar expression profile as c-Myc within the developing mammary gland (Golipour et al., 2008), and we showed Spy1 protein levels remain upregulated in c-Myc driven breast cancer cells in culture as they acquire resistance to hormone therapy. Spy1 and c-Myc are upregulated in similar cancers, such as neuroblastoma and invasive breast carcinomas (Al Sorkhy et al., 2012; Golipour et al., 2008; Kniazev et al., 1986; Lubanska and Porter, 2014b; Perou, 2010; Perou et al., 2000; Xu et al., 2010; Zucchi et al., 2004). We show, for the first time, that Spy1 and c-Myc are co-regulated in TNBC patient samples and cell lines and that Spy1 levels influence the protein stabilization of c-Myc. Importantly, in c-Myc overexpressing MMTV-Myc mammary carcinoma cells, knockdown of Spy1 not only significantly decreases the rate of proliferation, but it also sensitized late passage resistant cells to tamoxifen. ERα-negative tumours, specifically TNBC, are known to have no response to hormone therapies and are dependent on a chemotherapeutic regimen (Dent et al., 2007; Peddi et al., 2012). Standard of care for many cancers is through the use of doxorubicin, cyclophosphamide, and paclitaxel, commonly referred to as AC/T (Citron et al., 2003).

Knockdown of Spy1 in TNBC cells significantly sensitized cells to both single agent as well as combination AC/T therapy. Importantly, the sensitivity seen with Spy1 knockdown was unique; similar effects were not seen in all treatments when Cyclin E1 was knocked down. We have shown that although Spy1 functions similar to Cyclin E1 in stabilizing and influencing the levels of c-Myc, manipulation of Spy1 levels uniquely enhances sensitivity to treatment. Spy1 has a unique set of substrates and overrides CDKs using a mechanism different than that of classical cyclin-CDKs (Cheng et al., 2005b; Gastwirt et al., 2007; Karaiskou et al., 2001); hence, targeting Spy1-driven CDKs in cancers with high expression levels of both c-Myc and Spy1 could promote novel, and potentially specific therapeutics.

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CHAPTER 4

**SPY1 PROTEIN LEVELS SENSITIZE TRIPLE NEGATIVE BREAST CANCER
CELLS TO COMBINATION CISPLATIN AND CDK INHIBITOR TREATMENT**

INTRODUCTION

Triple negative breast cancer (TNBC) is clinically characterized by the lack of expression, or low levels of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2/Neu) (Musgrove et al., 1993; Perou et al., 2000). Absence of these receptors renders TNBC typically unresponsive to existing targeted therapies; hence, chemotherapy remains the standard of care for these patients (Perou, 2010; Perou et al., 2000). TNBC represents approximately 10-20% of the breast cancer population, patients are statistically younger and have a worse prognosis over all subtypes of breast cancer (Boyle, 2012). Hence, there is an urgent need to improve therapy options for TNBC patients.

One of the current chemotherapy regimens for TNBC includes treatment with doxorubicin, cyclophosphamide, and paclitaxel at varying doses and time points dependent upon a patient's mass and severity/grade of cancer. These drugs directly bind to either the DNA (doxorubicin, cyclophosphamide) or microtubules (paclitaxel) to prevent cell cycle progression and ultimately trigger apoptosis (Hall and Tilby, 1992; Jordan and Wilson, 2004; Stordal et al., 2007). One clinical trial has shown that the administration of four cycles of AC every two weeks followed by paclitaxel administration every 2 weeks significantly improved the overall disease free survival of patients (Citron et al., 2003). Furthermore, there is evidence to support the administration of an anthracycline based chemotherapy regimen in TNBC patients that shows higher chances of disease free and overall survival (Miyoshi et al., 2010). The response rate to chemotherapy is impressive; however, the high recurrence rate shows there is a need for further investigations into less cytotoxic, more effective therapies.

Data support that combining platinum drugs, such as cisplatin or carboplatin, may represent a valuable new combination therapy for TNBC that has the potential to increase efficacy without increasing toxicity (Liu et al., 2015; Pruefer et al., 2008; Rosenberg et al., 1969; Siddik, 2003; Wang and Lippard, 2005). Platinum drugs function through the formation of DNA adducts, usually in the S-phase of the cell cycle. Torrissi *et al.* (2008) showed the addition of cisplatin to the chemotherapy regimen of TNBC patients increased the pathologic complete response (pCR); which correlates with a better prognosis (Torrisi et al., 2008). Although the results from this study showed promising results when combining a platinum to standard of care chemotherapy, the long-term benefits and molecular stratification of patients were not elucidated.

At a molecular level, overcoming, or preventing, resistance to standard chemotherapy reagents may be achieved by sensitizing cells to trigger apoptosis in response to DNA damage. During the DNA damage response (DDR) the drivers of the cell cycle, the cyclin dependent kinases (CDKs) and cyclin partners, are inhibited via upregulation of CDK inhibitors (CKIs), such as p21^{Cip1} (Arellano and Moreno, 1997). Prolonged arrest of the cell cycle due to inappropriate repair of DNA leads to triggering of the apoptotic machinery and long-term expression of p21^{Cip1}, and its family of inhibitors (Di Leonardo et al., 1994). Paradoxical to this goal, however, most cancers, including breast cancer, have upregulated drivers of the cell cycle and decreased levels of CKIs to permit the evolving accumulation of DNA mutations (Bandoh et al., 2005; Caldon et al., 2012; Zhang et al., 2011). Strategies to reinforce this important cell cycle checkpoint with synthetic CKIs have been established over the past decade. Pan-CKIs, such as roscovitine, have shown some success to date in pre-clinical trials, but

demonstrate low efficacy in the clinic, with a high toxicity rate (Bach et al., 2005; Byrd et al., 2007; Nair et al., 2011). Better results have been achieved using more selective inhibitors (Finn et al., 2009) and, indeed, there are panels of second and third generation inhibitors that remain to be tested. Selectivity of CKIs is challenging due to similarities in the active site of the CDKs and clinical results suggest that it is important to identify the patients who will benefit from select CKI treatment (Finn et al., 2009; Malinkova et al., 2015). Yet another complication to this field is the presence of non-cyclin proteins that bind to and activate CDKs in a unique manner (Nebreda, 2006). One such protein, Spy1 (gene *SPDYA*, also called RINGO, Spy1A1, Speedy), does not require the removal of inhibitory phosphorylation on threonine (T)-14 and tyrosine (Y)-15 from CDKs and it can lead to the activation of CDKs even in the absence of the activating phosphorylation in the T-loop of the active site of the CDK or presence of natural CKIs (Cheng et al., 2005a; Karaiskou et al., 2001; Porter et al., 2003). Based on these characteristics, the presence of Spy1 may alter sensitivity to synthetic CKIs. Elevated levels of Spy1 are found in several human cancers, including invasive breast cancers (Al Sorkhy et al., 2012; Hang et al., 2012; Ke et al., 2009; Lubanska and Porter, 2014b) and overexpression in cell systems is a potent mechanism of overriding apoptosis and cell cycle arrest triggered by the DNA damage response (Barnes et al., 2003; Gastwirt et al., 2006). Herein, we investigated whether Spy1 levels are implicated in the sensitivity of TNBC to emerging chemotherapy treatments. We show that Spy1 levels are elevated in TNBC cell lines and we demonstrate that manipulating Spy1 levels can sensitize TNBC cells to treatment with cisplatin and available CKI treatment.

MATERIALS AND METHODS

Cell Culture. MDA-MB-231 and MCF7 cells were purchased from ATCC and were cultured in DMEM media supplemented with 10% FBS and 30,000 units penicillin/30,000 µg streptomycin solution. Cells were maintained under normoxic conditions (5% CO_2) at 37°C.

Plasmids. pLKO-scrambled control (#8453) was purchased from Addgene. pLKO-shSpy1 and pLKO-shCyclin E1 were cloned to express a short hairpin previously described to knockdown Spy1 specifically or Cyclin E1 specifically in the place of the scrambled sequence; previously determined to be specific using multiple constructs of each and rescue constructs (Lubanska and Porter, 2014b). pEIZ vector was generously donated from Dr. B. Welm. The creation of pEIZ-Spy1 was completed by inserting Spy1 oligo into the *Eco*RI and *Xba*I sites of pEIZ.

Immunoblotting (IB). Cells were lysed in NP-40 buffer (1% NP-40, 50 mMTris-HCl pH 7.5, 1 mM EDTA, 150 mMNaCl) containing protease inhibitors (100 µg/ml PMSF, 5 µg/ml aprotinin, and 2 µg/ml leupeptin) for 1 hour on ice. Bradford reagent was used to determine the concentration of protein following the manufacturer's instructions (Sigma). 80-100 µg of protein were subjected to electrophoresis on denaturing 10% SDS polyacrylamide gels and transferred to PVDF-Plus 0.45 micron transfer membrane (Osmonics Inc.) for 2 hours at 30 volts using a wet transfer method. Blots were blocked for 1 hour in 1% BSA solution at room temperature. Primary antibodies were reconstituted in blocker and incubated overnight at 4°C, secondary antibodies were used at 1:10000 dilution in blocker for 1 hour at room temperature. Blots were washed 3 times in TBST for 3 minutes following incubation with primary and secondary antibodies.

Chemiluminescent Peroxidase Substrate was used for visualization following manufacturer's instruction (Pierce). Chemiluminescence was quantified on an AlphaInnotech HD2 (Fisher) using AlphaEase FC software. The proper antibodies were used at the following concentrations: Actin MAB150 1R (Chemicon-Millipore; 1:1000), human Spy1 (ThermoScientific; 1:1000), and Cyclin E1 (Abcam; 1:1000).

Lentiviral Production and Infection: VSV-G pseudotyped lentivirus was produced by transient transfection of HEK293 LentiX cells with transfer vector and the multi-deleted packaging plasmids (pMDG, pMDL2, pRSV) using polyethylenimine (PEI) (Sigma) reagent with 1:3 DNA to PEI ratio and incubation for 5 hours at 37°C, 5% CO_2. The virus was collected the next day and concentrated for 3 hours at 4°C using an ultracentrifuge. The titer for pEIZ was determined by transducing 293T cells and analysis of eGFP protein expression by flowcytometry at 72 hours post transduction. The titer for pLKO lentivirus was assessed by puromycin selection followed by crystal violet staining and quantification of resistant colonies. The titered virus was filter sterilized and stored at -80°C. 80,000 cells were seeded in fully supplemented growth media in 24-well plates for 2 hours. Cells were starved by removing serum and penicillin/streptomycin from the media, followed by the use of 1 mg/ml polybrene (Santa Cruz Biotechnology) and MOI 3 of the specific virus used. Infected cells were changed to fully supplemented media 24 hours after infection. For knockdown, cells were incubated with 1mg/ml puromycin (Sigma) 48 hours after infection for 72 hours to allow for puromycin selection. Media is thereafter changed every 48 hours with puromycin included.

Drug Dosage. Cells were treated with 25 nM doxorubicin (Sigma), 100 nM paclitaxel (Sigma), 6 mM cyclophosphamide (Sigma), or 43 μM cisplatin (Sigma) for 24 hours

when used alone. AC/T treatment (doxorubicin, cyclophosphamide treatment first for 24 hours, recover for 24 hours, followed by paclitaxel treatment for 24 hours). Addition of cisplatin treatment to AC/T regimen occurs concurrently with paclitaxel treatment. CDK inhibitors included 20 μM roscovitine (Santa Cruz Biotechnology) or 25 μM NU-2058 (Tocris) for 48 hours, unless otherwise indicated.

Proliferation Assay. Cells were seeded at 5×10^5 cell density in 24-well plate. Following incubation times with drug treatments, indicated above, cells were collected, pelleted and resuspended in 1 ml of media. 10 μl samples were collected, trypan blue was added and live cells were counted using a haemocytometer.

MTT Assay. Cell numbers were optimized to ensure log phase of growth was used for each MTT thereafter. Cells were seeded at 8×10^3 cell density in a 96-well plate. Following incubation times with drug treatments, indicated above, 5 mg/ml MTT was added. The plate was incubated for 3 hours in 5% CO_2 at 37°C. After incubation, 100 μl extraction buffer (20% sodium dodecyl sulfate [SDS] in 50% N,N-dimethylformamide [DMF], containing 0.5% [v:v] 80% acetic acid and 0.4% [v:v] 1N HCl) was added. The plate was incubated for 30 minutes-1 hour in 5% CO_2 at 37°C and then read on a Wallac Victor 1420 plate reader (PerkinElmer, software Workout 2.0).

RESULTS

Spy1 enhances the efficacy of CKIs.

The TNBC cell line, MDA-MB-231, was treated with 20 μM roscovitine or 25 μM NU-2058 for 24-72 hours, followed by analysis of metabolic activity by use of MTT and trypan blue exclusion assay (Figure 1A). The percent viability was calculated at each time-point in comparison to vehicle control (DMSO). There was a significant decrease in viability at the earliest time-point with the highly specific CDK2 inhibitor, NU-2058, while roscovitine began to show a significant effect at 48 hours. Both inhibitors showed the greatest effect at 72 hours. TNBC cell lines have higher levels of Spy1 than ERα-positive luminal breast cancer cell lines (Al Sorkhy et al., 2012). Hence, triple negative MDA-MB-231 cells were infected with shScrambled, shSpy1, or shCyclin E. Following confirmation of knockdown (Figure 1B), cells were treated with the synthetic CKIs to determine if Spy1 levels could affect their efficacy. The percent viability was calculated through MTT analysis and trypan blue exclusion assay (Figure 1C) in comparison to vehicle control (DMSO). Spy1 knockdown significantly increases the efficacy of both CKIs, NU-2058 and roscovitine; however, knocking down Cyclin E shows no notable effects on the viability of the cells over that of inhibitor alone. These data suggest that Spy1 directed activity is not completely inhibited by CKIs and is still driving aspects of cell growth.
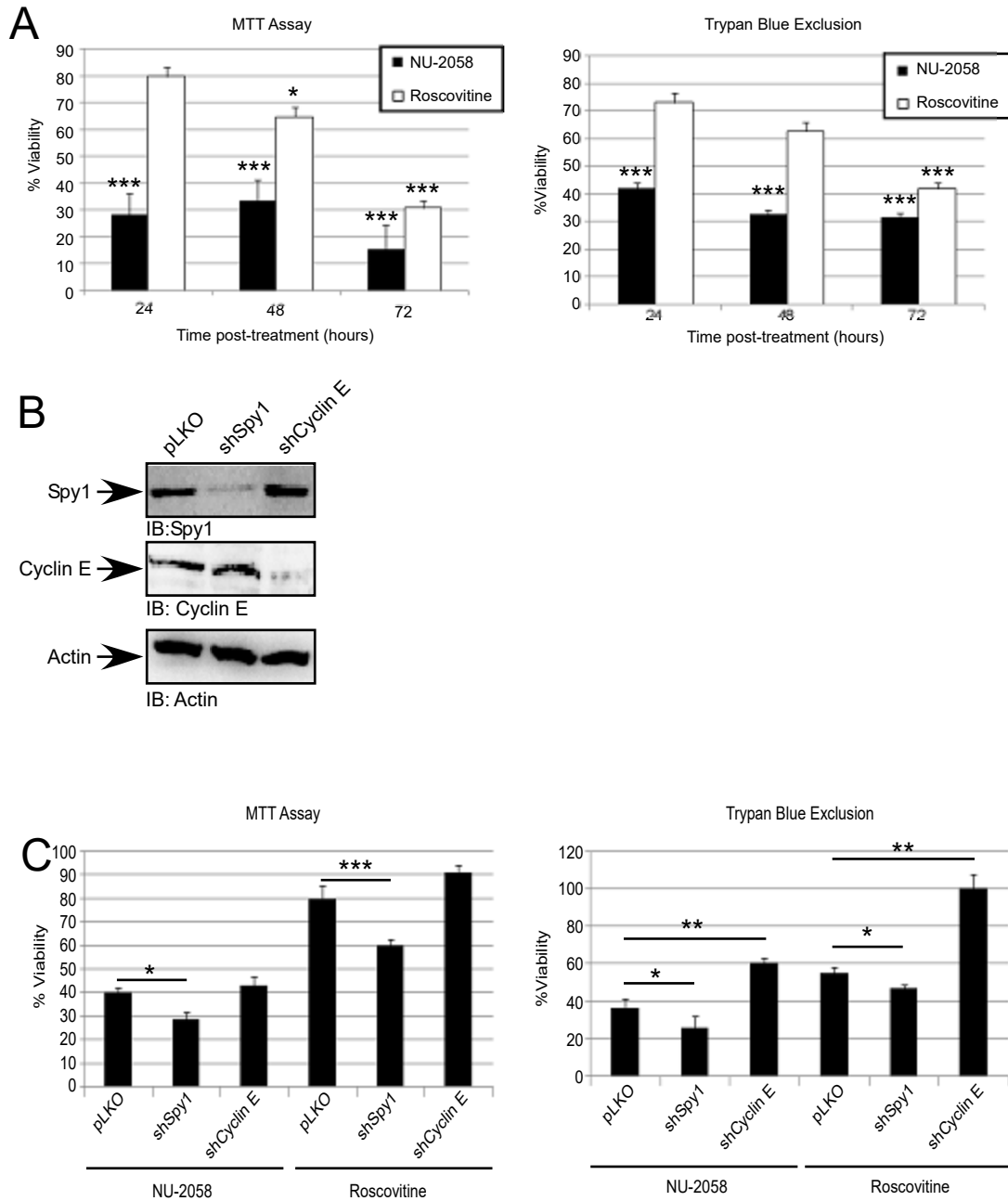
Figure 1. Spy1 enhances the efficacy of CKIs (A) Cells were treated with vehicle control (DMSO), 25 uM NU-2058 or 20 uM roscovitine for the indicated time. Following each time point the viability was assessed using MTT (left panel) and trypan blue exclusion assay (right panel). (B-C) Cells were infected with shScrambled (denoted pLKO), shSpy1, or shCyclin E. Following confirmation of knockdown (B), cells were treated with vehicle control (DMSO), 25 uM NU-2058, or 20 uM roscovitine. (C) Following 48 hour incubation, cells were subjected to MTT assay (left panel) or trypan blue exclusion assay (right panel). (A,C) Percent viability is determined as percent of vehicle control. Error bars reflect SE between triplicate experiments. Student's t-test was performed; *p<0.05, ***p<0.001.

Synthetic CKIs have a synergistic effect when combined with cisplatin.

To determine if the efficacy of cisplatin treatment on TNBC cells could be enhanced when in combination with CKIs, MDA-MB-231 cells were treated with the inhibitors (roscovitine or NU-2058) or cisplatin, or a combination of cisplatin with each inhibitor. As seen in Figure 2A and 2C, when each CKI is used alone there is a significant decrease in cell viability. Furthermore, when cisplatin treatment is combined with CKI treatment, percent viability is further decreased. We have shown that the addition of a CKI can sensitize these cells to cisplatin treatment. Moreover, using CompuSyn software, the data shows that the use of the combination of drugs can induce a synergistic effect as shown by a combination index (CI) value less than 1 (Figure 2B and 2D). CI values for the combination of cisplatin and roscovitine as well as cisplatin and NU-2058 are summarized in Table I and Table III, respectively. CompuSyn was also able to provide the CI values for the effective dose (ED) from ED_{50} to ED_{95}, which are summarized for each combination in Table II and Table IV. In short, these data conclude that 50% to 100% inhibition of the population of cells, provided by the combination of cisplatin and a synthetic CKI, shows a synergistic effect.
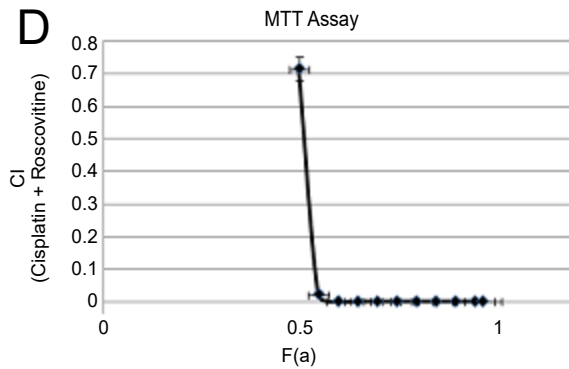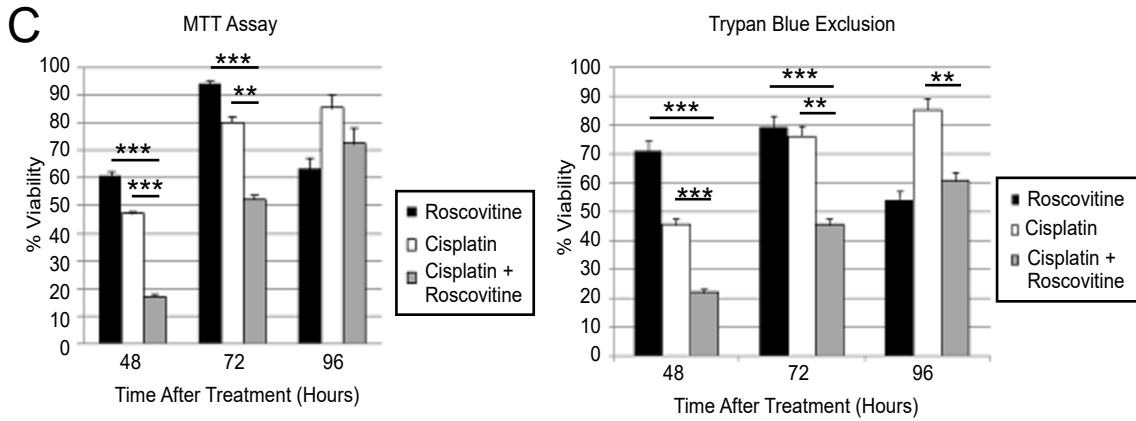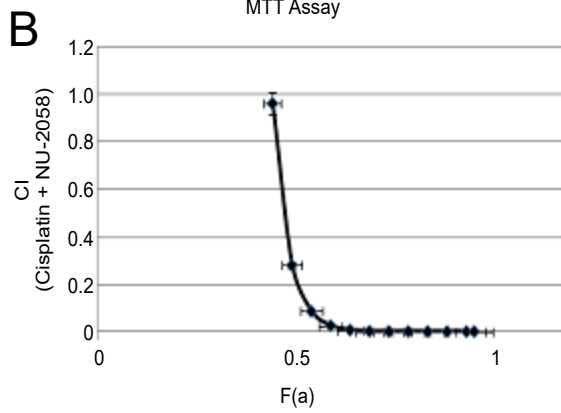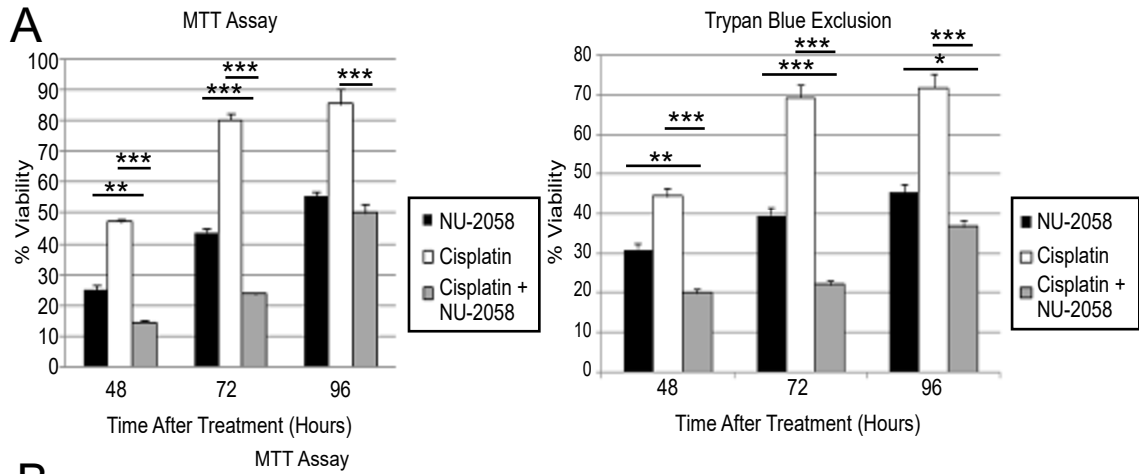
Figure 2. Synthetic CKIs have a synergistic effect when treated with cisplatin. (A) Cells were treated with vehicle control (DMSO), 25 uM NU-2058, 45 uM cisplatin, or a combination for the indicated time. (B) Output graph of MTT results from CompuSyn program showing synergistic effect (CI<1) at 48 hours when 50% to 100% of the population is affected (F(a)). (C) Cells were treated with vehicle control (DMSO), 20 uM roscovitine, 45 uM cisplatin, or a combination for the indicated time. (A,C) Following each incubation, cells were assessed through MTT analysis (left panel) or trypan blue exclusion assay (right panel). (D) Output graph of MTT results from CompuSyn program showing synergistic effect (CI<1) at 48 hours when 50% to 100% of the population is affected. (A-D) Percent viability is determined as percent of vehicle control. Error bars reflect SE between triplicate experiments. Student's t-test was performed; $*p<0.05$, $**p<0.01$, $***p<0.001$.

Fraction Affected (Fa)	Combination Index	Interaction
0.45	23.012	Antagonistic
0.5	0.71357	Synergistic
0.55	2.23×10^{-2}	Synergistic
0.6	6.54×10^{-4}	Synergistic
0.65	1.6×10^{-5}	Synergistic
0.7	3.3×10^{-7}	Synergistic
0.75	4.46×10^{-9}	Synergistic
0.8	3.2×10^{-11}	Synergistic
0.85	8.5×10^{-14}	Synergistic
0.9	3.2×10^{-17}	Synergistic
0.95	9.4×10^{-23}	Synergistic
0.97	1.1×10^{-26}	Synergistic

Table I. Combined effects of cisplatin and roscovitine.

Ratio (cisplatin:roscovitine)	Effective Dose (ED)	Combination Index (CI)	Interaction
1:1	ED50	0.71357	Synergistic
	ED75	4.46×10^{-9}	Synergistic
	ED90	3.2×10^{-17}	Synergistic
	ED95	9.4×10^{-23}	Synergistic

Table II. Effective dose effects of cisplatin and roscovitine at a molar concentration of 1:1.

Fraction Affected (Fa)	Combination Index	Interaction
0.45	0.96	Synergistic
0.5	0.28645	Synergistic
0.55	0.08588	Synergistic
0.6	0.02513	Synergistic
0.65	0.00698	Synergistic
0.7	0.00177	Synergistic
0.75	3.93×10^{-4}	Synergistic
0.8	6.99×10^{-5}	Synergistic
0.85	8.65×10^{-6}	Synergistic
0.9	5.39×10^{-7}	Synergistic
0.95	6.09×10^{-9}	Synergistic
0.97	2.5×10^{-10}	Synergistic

Table III. Combined effects of cisplatin and NU-2058.

Ratio (Cisplatin:NU-2058)	Effective Dose (ED)	Combination Index (CI)	Interaction
1:1	ED50	0.28645	Synergistic
	ED75	3.93×10^{-4}	Synergistic
	ED90	5.39×10^{-7}	Synergistic
	ED95	6.09×10^{-9}	Synergistic

Table IV. Effective dose effects of cisplatin and NU-2058 at a molar concentration of 1:1.

Spy1 knockdown increases the effect between cisplatin and CKIs.

Elevated levels of Spy1 can confer resistance to genotoxic agents (Barnes et al., 2003); therefore, to determine if the levels of Spy1 can affect the efficacy of these drugs, Spy1 knockdown was performed followed by the use of each drug individually or in combination. We first show that Spy1 knockdown sensitizes cells to cisplatin treatment, but knockdown of Cyclin E has no significant effect and, in fact, dramatically prevents a decrease in viability (Figure 3A). When NU-2058 (Figure 3A) or roscovitine (Figure 3B) are combined with cisplatin, viability enhances over cisplatin alone, supporting that combination therapy with these reagents needs to be avoided or conducted in sequential cycles. Interestingly, while knockdown of Cyclin E significantly enhances viability in the face of each combination, knockdown of Spy1 significantly sensitized cells to combinations of cisplatin and CKIs. To determine whether Spy1 knockdown had effects on cisplatin in combination with standard of care chemotherapy, cells were treated with the combination of doxorubicin, cyclophosphamide, and paclitaxel (AC/T) and percent viability was determined through MTT analysis. While both Spy1 and Cyclin E knockdown significantly decrease the viability of the cells to AC/T treatment, the addition of cisplatin significantly decreases cell viability when Spy1 is knocked down, an effect not seen with Cyclin E knockdown (Figure 3C). To resolve whether an ERα cell line with overexpression of Spy1 or Cyclin E would respond to standard of care treatment with or without cisplatin, MCF7 cells were treated with each regimen. In both instances, overexpression of Spy1 and Cyclin E show an increase in cell viability with AC/T treatment; however, addition of cisplatin to Cyclin E overexpressing cells significantly decreases percent viability back to control levels (Figure 3D). These data suggest that

levels of Spy1 can change the efficacy of cisplatin as well as the standard of care chemotherapy regimen.
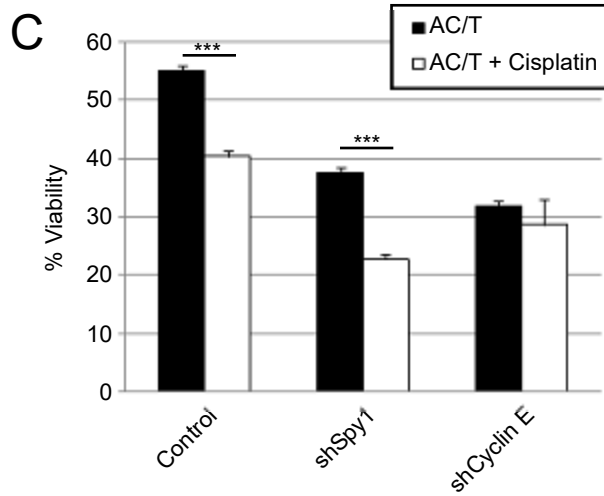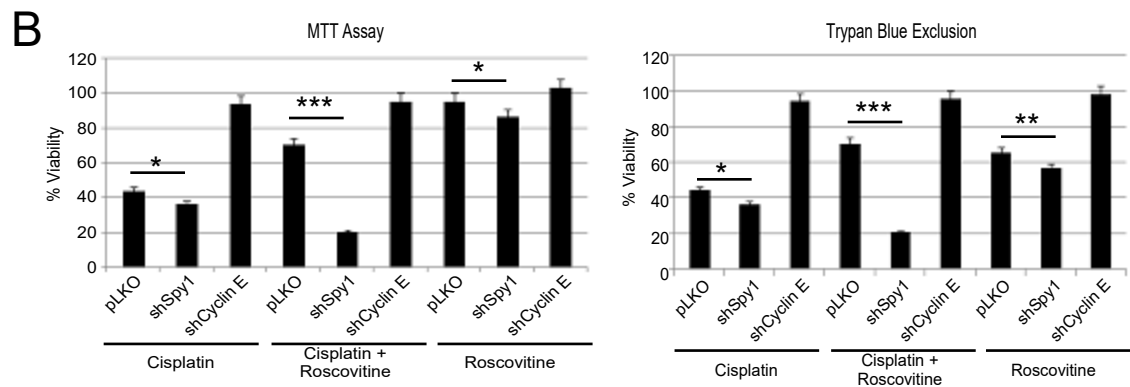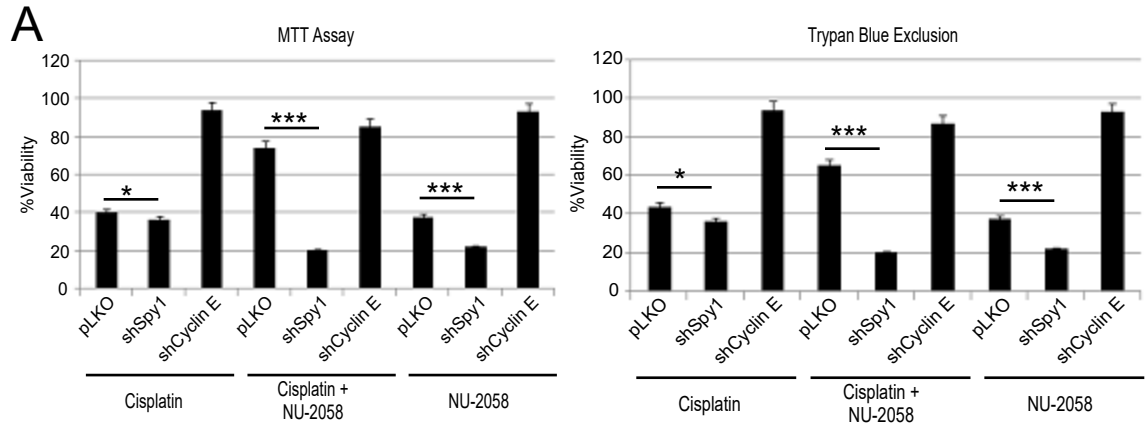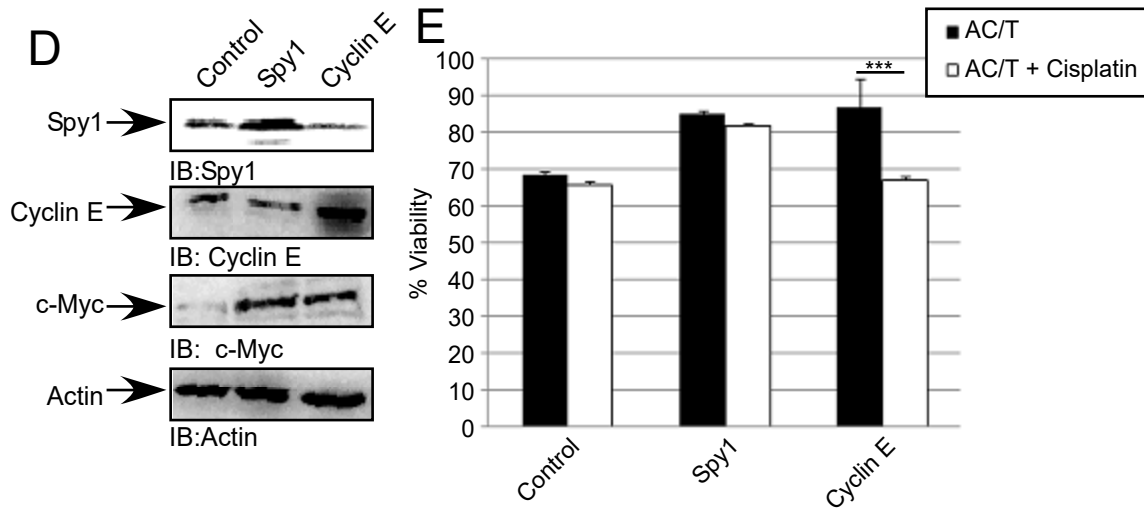
Figure 3. Spy1 knockdown increases the effect between cisplatin and CKIs. (A-C) MDA-MB-231 cells were infected with shScrambled (denoted pLKO), shSpy1, or shCyclin E. (A) Infected cells were treated with vehicle control (DMSO), 25 uM NU-2058, 45 uM cisplatin, or a combination. Cells were assessed using MTT analysis (left panel) or trypan blue exclusion assay (right panel). (B) Infected cells were treated with vehicle control (DMSO), 20 uM roscovitine, 45 uM cisplatin, or a combination. Cells were assessed using MTT analysis (left panel) or trypan blue exclusion assay (right panel). (C) Cells were treated with AC/T or AC/T + cisplatin and viability was assessed using MTT analysis. (D-E) MCF7 cells were infected with pEIZ, Spy1, or Cyclin E. Following confirmation of overexpression (D), cells were treated with AC/T or AC/T + cisplatin and assessed using MTT analysis. (A-C & E) Percent viability is determined as percent of vehicle control. Error bars reflect SE between triplicate experiments. Two-way ANOVA was performed; *p<0.05,**p<0.01, ***p<0.001.

DISCUSSION

TNBC is a highly aggressive disease with no targeted treatment options available. Chemotherapy drugs that inhibit the cell cycle have had a huge impact on the overall survival rate; however, TNBC patients still risk a high rate of relapse within 3 years after therapy (Pogoda et al., 2013). Resolving the key regulators of resistance can uncover novel treatment options and offer new promise to this group of patients.

Chemotherapy is highly dependent on actively dividing cells and works by inhibiting the cell cycle at various stages and then triggering apoptosis (Bharadwaj and Yu, 2004; Emadi et al., 2009; Hall and Tilby, 1992; Jordan and Wilson, 2004; Minotti et al., 2004). Within the last decade, many regimens have added the use of platinum drugs, such as cisplatin, in combination with standard of care chemotherapy. Cisplatin is standard of care for stomach and ovarian cancer and has been receiving attention in the treatment of TNBC (Helm and States, 2009; Liu et al., 2015; Roth et al., 2007). Cisplatin forms DNA adducts, usually during S phase of the cell cycle, that alter DNA conformation triggering the initiation of the DNA damage response, followed by apoptosis (Rosenberg et al., 1969; Wang and Lippard, 2005). The use of platinum drugs as a single agent therapy or in combination with chemotherapy regimens came about by the presence of frequent mutations in the *BRCA1* gene in TNBC patients. *BRCA1* mutations leads to a decreased ability for DNA repair and, therefore, the tumour is unable to fully recover from DNA-damaging agents, promoting tumour cell apoptosis (Hill et al., 2014). Indeed, our work demonstrates that the inclusion of cisplatin in the treatment of TNBC cell lines significantly decreases cell viability over an AC/T regimen alone. Resistance has been shown to develop over time in ovarian cancer patients (Siddik,

2003); however, since cisplatin is not routinely used for breast cancer therapies, there are no clinical trials yet to determine whether TNBC patients develop cisplatin resistance. In this study, we explore whether targeting the cell cycle with CKIs may be a valuable mechanism of optimizing cisplatin combinations to avoid this problem.

During initiation of carcinogenesis, the increase in DNA damage and mutations is significant. This elicits the DNA damage response in hopes of repairing the damage. Chemotherapy promotes damage to induce cell cycle arrest and apoptosis. If the repair pathway can be superseded, then chemotherapy stops working (Johnson and Shapiro, 2010). The unique 'cyclin-like' protein, Spy1, has been shown to override the DNA damage pathway and when Spy1 is overexpressed it can override the apoptotic effects of p53 and the DNA repair pathway (Barnes et al., 2003; Gastwirt et al., 2006). Furthermore, elevated levels of Spy1 have shown a resistant phenotype to chemotherapy and genotoxic agents (Barnes et al., 2003). Spy1 is elevated in several human cancers, including invasive breast cancers (Al Sorkhy et al., 2012; Hang et al., 2012; Ke et al., 2009; Lubanska and Porter, 2014b), and, hence, may represent a potent mechanism for inducing chemotherapy resistance. In this study, we demonstrate that Spy1 knockdown sensitizes TNBC cells to existing CKI drugs, NU-2058 and roscovitine, as well as to cisplatin. Interestingly, our results support that this is not through classically defined activation of the G1/S CDK, CDK2, as Cyclin E knockdown does not mimic these effects.

Synthetic CKIs have been added into the regimen for chemotherapy in many clinical trials to further inhibit cell cycle progression (Deep and Agarwal, 2008). These inhibitors were originally modelled after the Cyclin A-CDK2 crystal structure (Brown et

al., 1995), and have shown some effectiveness in pre-clinical trials (Deep and Agarwal, 2008; Nair et al., 2011). Spy1 activates CDKs even in the presence of the CKIs, p27^{Kip1} and p21^{Cip1} (Cheng et al., 2005a; Porter et al., 2003). These results support that it is important to begin to understand how cyclin-like proteins differentially regulate CDK activity and to develop synthetic reagents to target this mechanism. Recent CKI trials in ERα-positive breast cancer patients have stratified patient populations with Cyclin D1 amplification, loss of p16, or both and utilized select CDK4/6 inhibitors (Finn et al., 2015; Finn et al., 2009) with significantly improved results. Late breaking ASCO abstracts reveal survival rates for these patient groups have doubled (Finn et al., 2015; Turner et al., 2015). It is an important next step for the TNBC field to conduct trials stratifying patient populations for levels of cyclins, cyclin-like proteins, CDKs and natural CKIs and testing the efficacy of targeted second generation CKI therapies. This approach may offer radical improvements for this subset of patients.

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CHAPTER 5

SPY1 IS A SELECTIVE TARGET FOR TREATMENT WITH SINGLE AGENT PACLITAXEL IN TRIPLE NEGATIVE BREAST CANCER CELLS

INTRODUCTION

Cyclin dependent kinases (CDKs) and their cyclin binding partners regulate progression though the cell cycle to ultimately control cell proliferation. A healthy cell detects potentially harmful changes in the cellular environment, including damage to the DNA, by halting the cell cycle through the upregulation of CDK inhibitors (CKIs) to allow for repair of the situation, or initiating apoptosis in the case of irreparable damage (Johnson and Shapiro, 2010). Bypass of these protective checkpoints is an essential step in the progression of tumourigenesis (Arellano and Moreno, 1997; Nakayama, 1998). Indeed, CKIs, such as p27^{Kip1} and p21^{Cip1}, are commonly downregulated in various cancers (Abukhdeir and Park, 2008; Jeffrey et al., 1995). Chemotherapy drugs target critical aspects of cell proliferation, including damaging the DNA, and, ironically, rely heavily on cell cycle checkpoints to detect these errors and trigger apoptosis of the cancer cell (Bharadwaj and Yu, 2004; Johnson and Shapiro, 2010). Reinstalling CKI function represents an attractive mechanism for sensitizing cells to chemotherapy treatment.

Paclitaxel is a common chemotherapy drug used for many different malignancies, including prostate, breast, and lung cancers (Bharadwaj and Yu, 2004; Jordan and Wilson, 2004). Paclitaxel disrupts microtubule depolymerization by reversibly binding to tubulin, resulting in stable non-functioning microtubules. This interferes with mitosis and leads to apoptosis (Jordan and Wilson, 1998). Although paclitaxel has had success in the clinic for many cancer treatments, patients with triple negative breast cancer (TNBC) commonly present with *de novo* resistance or develop acquired paclitaxel resistance (Blanchard et al., 2015). Since TNBC patients do not express hormone or Her2/neu receptors, they are not eligible for hormone or targeted Her2/neu therapy treatment and

rely heavily on chemotherapy (Perou, 2010). The development of resistance to current chemotherapy regimens accounts for an average survival rate, calculated over all four stages, of approximately 57% for TNBC patients, compared to 73% for hormone receptor positive breast cancers (Polyak and Metzger Filho, 2012).

Synthetic CKIs have been designed to inhibit the cyclin-CDK complex by binding to the ATP binding site on the CDK (Asghar et al., 2015; Jeffrey et al., 1995). Synthetic CKIs, such as the pan-inhibitor roscovitine, have entered clinical trials but results to date have been largely disappointing (Johnson and Shapiro, 2010). Few trials have attempted to stratify patient populations according to cyclin, CDK or CKI levels and all trials have been conducted in conjunction with chemotherapies, most of which were not thoroughly tested in either pre-clinical or clinical settings. Combinations of paclitaxel and synthetic CKIs have shown particularly disappointing effects, likely because proper function of paclitaxel is dependent upon activation of cyclin-CDK complexes throughout the cell cycle to ensure the entry of cells into M-phase (Marsh et al., 2007). Nakayama *et al.* (2009) showed that activity of CDKs, specifically CDK1 and CDK2, can predict paclitaxel sensitivity and that a change in the activity of CDKs changes the efficacy of the treatment (Nakayama et al., 2009). Furthermore, Pushkarev *et al.* (2012) showed only at lower than physiologically relevant concentrations of paclitaxel does the combination of paclitaxel and CDK inhibitors have a promising outcome for colon and anaplastic thryroid cancer in the clinic. They documented that as paclitaxel concentrations increase closer to physiologically relevant levels, the addition of CKIs produced antagonistic outcomes in colon and anaplastic thyroid cancer cells (Pushkarev et al., 2012).

In addition, a confounding issue not yet considered in a clinical setting is the existence of cyclin-like proteins that activate CDKs in an atypical manner insensitive to natural CKIs, such as p21[Cip1] (Nebreda, 2006). One cyclin-like protein Spy1A1 (gene *SPDYA*, herein referred to as Spy1), can bind to both CDK1 and CDK2 in a novel manner, requiring no activating phosphorylation on the threonine (T)-160/161 residue in the T-loop and without the necessary dephosphorylation of threonine (T)-14 and tyrosine (Y)-15 residues (Cheng et al., 2005a). This unique binding may alter the normal conformation of the CDK and may not allow for the binding of the synthetic CKI. Our data supports the work of Nakayama *et al.* (2009) that CKIs, roscovitine and NU-2058, do not significantly enhance the effectiveness of paclitaxel. We show that knockdown of Spy1 in TNBC cells increases the efficacy of TNBC cells to paclitaxel alone, however, neither knockdown of Spy1 or Cyclin E improved combination therapy with CKI treatment and paclitaxel.

MATERIALS AND METHODS

Cell Culture. MDA-MB-231 were purchased from ATCC and were subcultured in DMEM media supplemented with 10% FBS and 30,000 units penicillin/30,000 μg streptomycin solution. Cells were maintained under normoxic conditions (5% CO_2) at 37°C.

Plasmids. pLKO-scrambled control (#8453) was purchased from Addgene. pLKO-shSpy1 and pLKO-shCyclin E1 were cloned to express a short hairpin previously described to knockdown Spy1 or Cyclin E1 specifically in the place of the scrambled sequence; previously determined to be specific using multiple constructs of each and rescue constructs (Lubanska and Porter, 2014b).

Immunoblotting (IB). Total protein was isolated from cell cultures using NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) containing protease inhibitors (100 μg/ml PMSF, 5 μg/ml aprotinin, and 2 μg/ml leupeptin) for 1 hour on ice. Bradford reagent was used to determine the concentration of protein following the manufacturer's instructions (Sigma). 80-100 μg of protein were subjected to electrophoresis on denaturing 10% SDS polyacrylamide gels and transferred to PVDF-Plus 0.45 micron transfer membrane (Osmonics Inc.) for 2 hours at 30 volts using a wet transfer method. Blots were blocked for 1 hour in 1% BSA solution at room temperature. Primary antibodies were reconstituted in blocker and incubated overnight at 4°C, secondary antibodies were used at 1:10000 dilution in blocker for 1 hour at room temperature. Blots were washed three times in TBST for three minutes following incubation with primary and secondary antibodies. Chemiluminescent Peroxidase Substrate was used for visualization following manufacturer's instruction (Pierce).

Chemiluminescence was quantified on an AlphaInnotech HD2 (Fisher) using AlphaEase FC software. The proper antibodies were used at the following concentrations: Actin MAB150 1R (Chemicon-Millipore; 1:1000), human Spy1 (ThermoScientific; 1:1000), and Cyclin E1 (Abcam; 1:1000).

Lentiviral Production and Infection. VSV-G pseudotyped lentivirus was produced by transient transfection of HEK293 LentiX cells with transfer vector and the multi-deleted packaging plasmids (pMDG, pMDL2, pRSV) using polyethylenimine (PEI) (408719, Sigma) reagent with 1:3 DNA to PEI ratio and incubation for 5 hours at 37°C, 5% CO_2. The virus was collected the next day and concentrated for 3 hours at 4°C using an ultracentrifuge. The titer for pLKO lentivirus was assessed by puromycin selection followed by crystal violet staining and quantification of resistant colonies. The titered virus was filter sterilized and stored at -80°C. 80,000 cells were seeded in fully supplemented growth media in 24-well plates for 2 hours. Cells were starved by removing serum and penicillin/streptomycin from the media, followed by addition of 1 mg/ml polybrene (Santa Cruz Biotechnology) and MOI 3 of the specific vector used. Infected media was changed to fully supplemented media 24 hours after infection. Cells were incubated with 1mg/ml puromycin (Sigma) 48 hours after infection for 72 hours to allow for puromycin selection. Media is thereafter changed every 48 hours with puromycin included.

Drug Dosage. Cells were treated with 100 nM paclitaxel (Sigma), with or without CDK inhibitors; 20 μM roscovitine (Santa Cruz Biotechnology) or 25 μM NU-2058 (Tocris). Paclitaxel treatment occurred for 24 hours and CDK inhibitors treatment occurred for 48 hours.

Proliferation Assay. Cells were seeded at 5×10^5 cell density in 24-well plate. Following incubation times with drug treatments, indicated as above, cells were collected, pelleted and resuspended in 1 ml of media. 10 µl samples were collected and trypan blue added and counted using a haemocytometer.

MTT Assay. Cells were seeded at 8×10^3 cell density in a 96-well plate. Following incubation times with drug treatments, indicated as above, 5 mg/ml MTT was added. The plate was incubated for 3 hours in 5% CO_2 at 37°C. After incubation, 100 µl extraction buffer (20% sodium dodecyl sulfate [SDS] in 50% N,N-dimethylformamide [DMF], containing 0.5% [v:v] 80% acetic acid and 0.4% [v:v] 1N HCl) was added. The plate was incubated for 30 minutes-1 hour in 5% CO_2 at 37°C.

RESULTS

CKIs decrease the efficacy of paclitaxel.

To determine whether paclitaxel can continue to be an optimal treatment for TNBC when CDK activity is inhibited by synthetic CKIs, we treated MDA-MB-231 cells with each CDK inhibitor alone (roscovitine or NU-2058), paclitaxel alone, or with the combination of roscovitine and paclitaxel or NU-2058 and paclitaxel. Here we show that TNBC cells treated with paclitaxel and CDK inhibitors alone respond through a decrease seen in the percent viability of the cells (Figure 1A). However, in combination with the pan-CDK inhibitor roscovitine, we show that at an effect on 50% to 95% of the population there is an antagonistic effect on the viability of this TNBC cell lines, through the use of the CompuSyn software (Figure 1B and Table I). Furthermore, the effective dose (ED50 to 95) were also shown to be antagonistic (Table II). In contrast, there is a small decrease in cell viability when the specific CDK2 inhibitor, NU-2058, is used in combination with paclitaxel (Figure 1A). Using CompuSyn software, CI values were found to be larger than 1, indicating the relationship is antagonistic and have non-synergistic interaction (Table III).
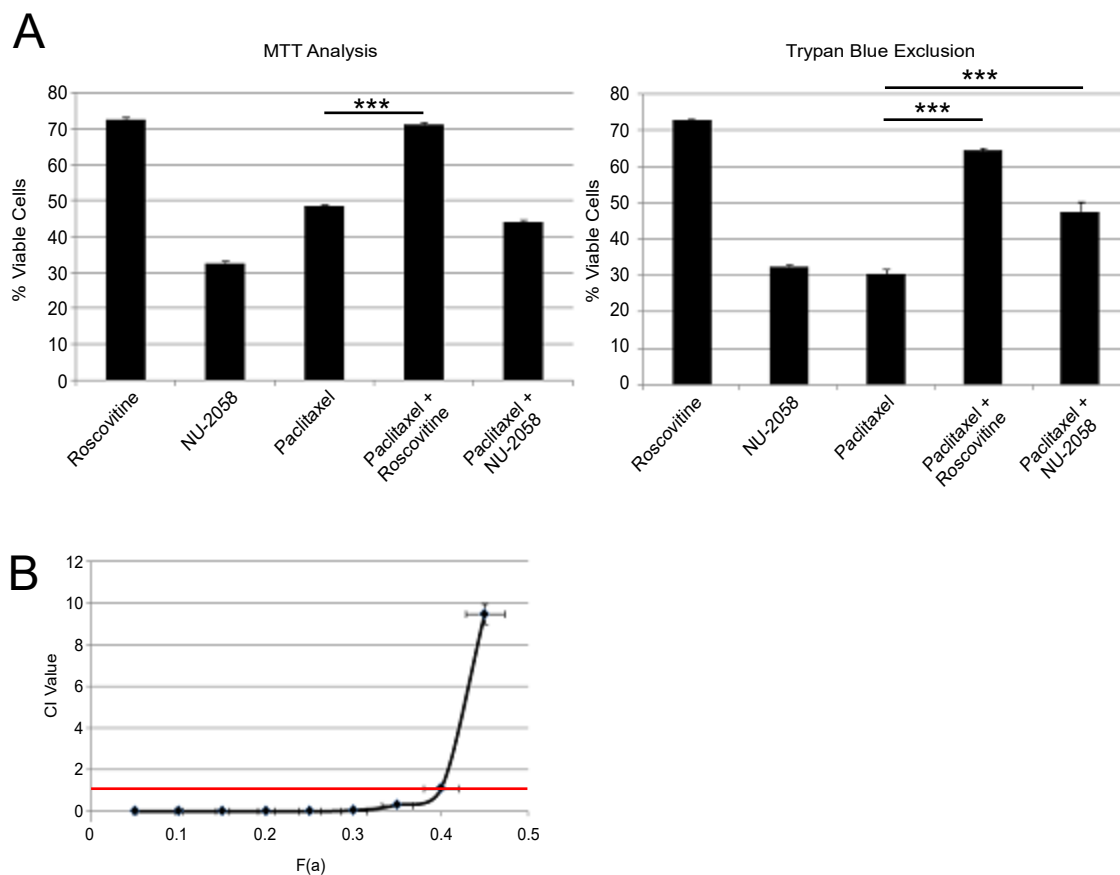
Figure 1. CKIs decrease the efficacy of paclitaxel. (A-B) Cells were treated with vehicle control (DMSO), 20 uM roscovitine, 25 uM NU-2058, 100 nM paclitaxel, or a combination of paclitaxel with CDK inhibitors. (A) Following appropriate incubation time, cells were assessed using MTT analysis (left panel) and trypan blue exclusion assay (right panel). Cell viability was determined in comparison to vehicle control. Error bars reflect SE between triplicate experiments. Student's t-test was performed; ***p<0.001. (B) CompuSyn software was used to determine the effect between paclitaxel and roscovitine. CI>1 above 40% fraction affected, showing an antagonistic effect between the two drugs.

Fraction Affected (Fa)	Combination Index	Interaction
0.05	9.5×10^{-10}	Synergistic
0.1	5.04×10^{-7}	Synergistic
0.15	2.45×10^{-5}	Synergistic
0.2	4.55×10^{-4}	Synergistic
0.25	0.00510	Synergistic
0.3	0.04202	Synergistic
0.35	0.28547	Synergistic
0.4	1.1	Additive
0.45	9.56628	Antagonistic
0.6	51.5586	Antagonistic
0.65	277.882	Antagonistic
0.7	1550.44	Antagonistic
0.75	63271.9	Antagonistic
0.9	5.467×10^{9}	Antagonistic
0.95	5.93×10^{12}	Antagonistic

Table I. Combined effects of paclitaxel and roscovitine.

Ratio (Paclitaxel:Roscovitine)	Effective Dose (ED)	Combination Index (CI)	Interaction
1:1	ED50	51.5586	Antagonistic
	ED75	521749	Antagonistic
	ED90	5.467×10^{9}	Antagonistic
	ED95	5.93×10^{12}	Antagonistic

Table II. Effective dose effects of paclitaxel and roscovitine at a molar concentration of 1:1.

Fraction Affected (Fa)	Combination Index	Interaction
0.45	8961.62	Antagonistic
0.5	16155.7	Antagonistic
0.55	31862.4	Antagonistic
0.6	63847.9	Antagonistic
0.65	131818	Antagonistic
0.7	286054	Antagonistic
0.95	3.53×10^8	Antagonistic

Table III. Combined effects of paclitaxel and NU-2058.

Ratio (Paclitaxel:NU-2058)	Effective Dose (ED)	Combination Index (CI)	Interaction
1:1	ED50	16155.7	Antagonistic
	ED75	671288	Antagonistic
	ED90	2.79×10^7	Antagonistic
	ED95	3.53×10^8	Antagonistic

Table IV. Effective dose effects of paclitaxel and NU-2058 at a molar concentration of 1:1.

Spy1 levels affect the efficacy of single agent paclitaxel and CKI treatment.

Previously published data has shown Spy1 protein levels to be high in TNBC cell lines (Al Sorkhy et al., 2012). To determine the effect of Spy1 on combination therapy for TNBC cells, MDA-MB-231 cells were infected to knockdown either Spy1 or Cyclin E. When Spy1 levels were knocked down, TNBC cells responded greater to CDK inhibitor treatment alone as well as paclitaxel treatment alone (Figure 2A and 2B). Although there is a decrease in cell viability when the combination of paclitaxel and CKIs were used during Spy1 knockdown, in comparison to paclitaxel alone, the percentage of living cells increases (Figure 2A and 2B). During control or knockdown of either Spy1 or Cyclin E, a combination of the two drugs leads to antagonism. Cyclin E knockdown, however, has no significant decrease in cell viability when CDK inhibitors are used. This data suggests targeting Spy1 alone or in combination with paclitaxel and/or CDK inhibitors could be beneficial in treating TNBC patients.
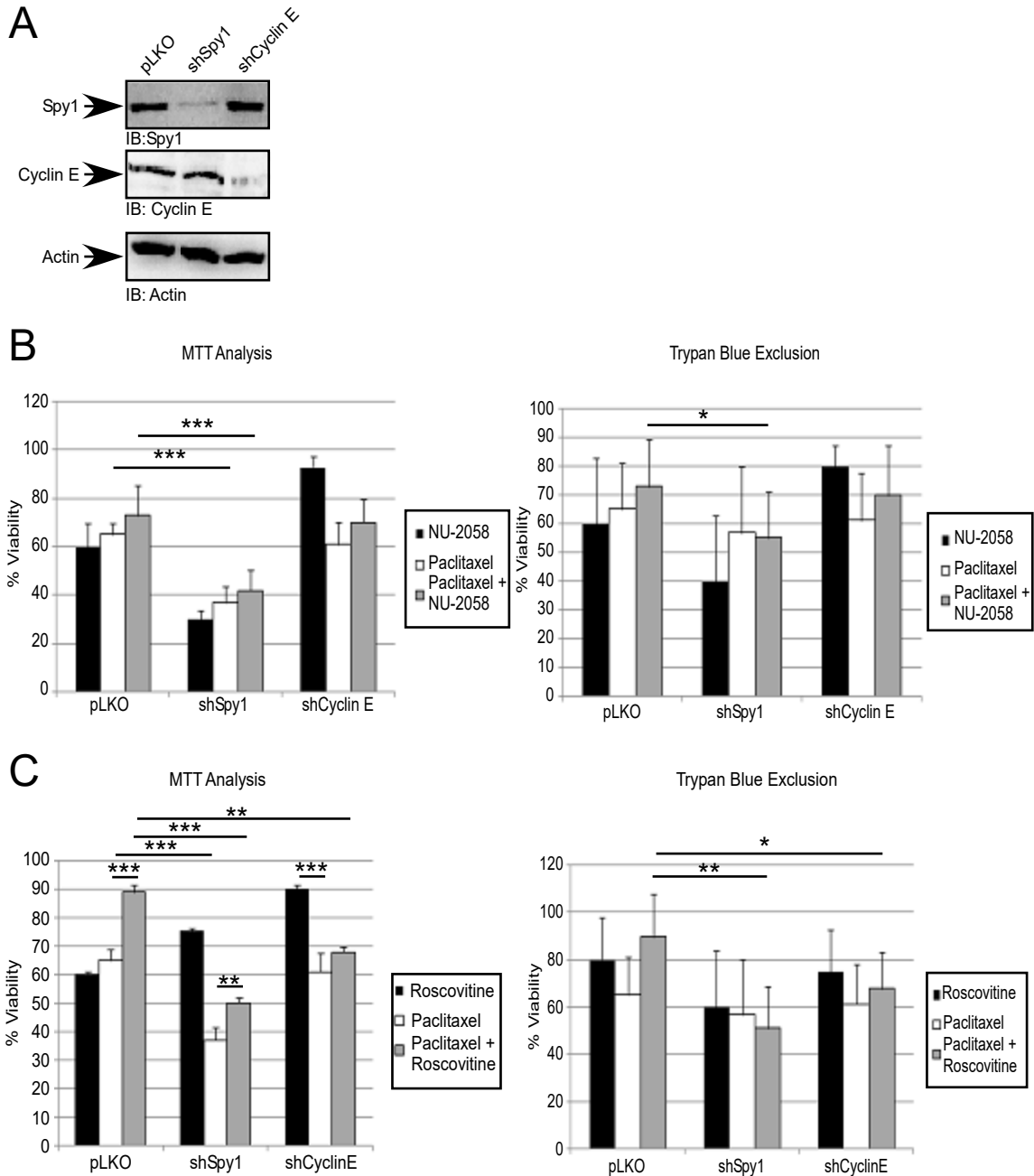
Figure 2. Spy1 levels affect the efficacy of single agent paclitaxel and CKI treatment. (A-C) Cells were infected with shScrambled (denoted pLKO), shSpy1, or shCyclin E. (A) Confirmation of knockdown assessed by SDS-PAGE and IB. (B) Infected cells were treated with vehicle control (DMSO), 25 uM NU-2058, 100 nM paclitaxel, or a combination. (B) Infected cells were treated with vehicle control (DMSO), 20 uM Roscovitine, 100 nM paclitaxel, or a combination. (A-B) Following the appropriate incubation, cells were assessed using MTT analysis (left panel) and trypan blue exclusion assay (right panel) and percent viability was measured in comparison to DMSO. Error bars reflect SE between triplicate experiments. Two-way ANOVA was performed; *p<0.05,**p<0.01, ***p<0.001.

DISCUSSION

The G1/S cyclin is frequently upregulated in many solid cancers, including TNBC (Caldon et al., 2012; Dhillon and Mudryj, 2002) and CKIs, such as $p27^{Kip1}$ and $p21^{Cip1}$, are significantly downregulated (Abukhdeir and Park, 2008; Bandoh et al., 2005). Synthetic CKIs have been modelled against the binding of $p21^{Cip1}$ to the Cyclin A-CDK2 complex to compensate for the loss of the natural CKIs (Jeffrey et al., 1995). Many of the synthetic CKIs tested have shown promising efficiency in pre-clinical trials; however, in the clinic they showed little to no efficacy and have had severe adverse effects in patients due to dosing problems, administrative schedules and target specificity (Asghar et al., 2015; Byrd et al., 2007; Deep and Agarwal, 2008). Current clinical trials have added CKIs in combination with standard of care chemotherapy regimens without thorough *in vitro* testing of each drug individually (Byrd et al., 2007; Harrison et al., 2009; Johnson and Shapiro, 2010). A more thorough assessment of how to combine these drugs into existing regimens is required.

Clinical trials with the pan-CDK inhibitor flavopiridol have shown the change in efficacy of chemotherapeutic agents is dependent on time of dosage (Motwani et al., 1999). Administration of flavopiridol prior to paclitaxel decreases the efficacy of paclitaxel due to the prevention of cells into M-phase. However, when gastric and breast cells are treated with flavopiridol after paclitaxel treatment there was an increase in apoptosis (Motwani et al., 1999). Our data shows addition of the pan-inhibitor, roscovitine, after paclitaxel decreases the efficacy of paclitaxel in TNBC cells. When a selective CDK2 inhibitor, NU-2058, was added after paclitaxel, there was also a significant decrease in cell viability. In refractory malignancies such as lung, prostate,

and esophageal cancers, phase I clinical trials with flavopiridol and paclitaxel have shown promising results (Schwartz et al., 2002); however, breast cancer shows only partial responses to this treatment (Motwani et al., 1999; Nair et al., 2011). Roscovitine is presently in phase II clinical trials for breast cancer and has also only shown partial response to treatment due to its toxic effect on normal cells (Nair et al., 2011). While most clinical trials focus on the use of pan-inhibitors, it may be valuable to focus on selective inhibitors that may selectively reinforce essential checkpoints while allowing cells to proceed through the phase of the cell cycle required for chemotherapy to function. Our current data support the literature that combination therapy with CKIs following paclitaxel treatment is not an effective regimen.

Synthetic CKIs continue to evolve by changing one or two chemical groups on the previous model. CDK4 inhibitors have begun to show great success in estrogen receptor alpha (ERα) positive breast cancers (Finn et al., 2009) and have also shown to protect mammary gland cells from Ras or Her2 induced tumorigenesis, but not c-Myc-induced tumourigenesis (De Falco and De Luca, 2010). Hence, the driving molecular signature plays a role in the sensitivity of the cell cycle to select inhibitors, likely due to the composition of cyclin-CDKs, and cyclin-like proteins expressed in individual cancers. TNBC cell lines have high levels of the atypical cyclin-like protein, Spy1 (Al Sorkhy et al., 2012). Spy1 can bind to and activate CDKs in the presence of the CKIs, p21^{Cip1} and p27^{Kip1} (Cheng et al., 2005a) and, hence, elevated levels of Spy1 could contribute to the efficacy of synthetic CKIs. We show here that the knockdown of Spy1 in TNBC cells decreases cell viability alone and enhances the efficacy of paclitaxel and CKIs individually, particularly enhancing the effects of the select CDK2 inhibitor, NU-2058.

These results were not noted with the G1/S classical cyclin, Cyclin E and, hence, suggests that this is due to the unique ability of Spy1 to override these select checkpoints.

Chemotherapeutic regimens have had an immense impact on overall survival for aggressive cancers, such as TNBC. However, recurrence and relapse can occur quickly after chemotherapy treatment. One study showed 33.9% of TNBC patients showed distant recurrence in 2.6 years after chemotherapy treatment in comparison to "other" breast cancers, which showed a 20.4% distant recurrence rate in 5 years (Dent et al., 2007). Furthermore, the adverse effects of chemotherapy have led clinicians to minimize these inconsistencies by combining the chemotherapy with other cell cycle regulator inhibitors (Deep and Agarwal, 2008). Currently, targeting the cell cycle in combination with chemotherapy is plagued with inconsistent data in patients due in large part to the lack of solid information regarding the timing and dosing of each reagent and how to stratify patients appropriately. Our data supports that high levels of the cyclin-like protein Spy1 may be prognostic for response to paclitaxel and CKI treatment and selective targeting of this mechanism may sensitize patients to these reagents alone. Further work is required, however, to determine whether CKIs can be used safely in combination with paclitaxel.

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CHAPTER 6

DISCUSSION & FUTURE DIRECTIONS

This work demonstrates the role of the 'cyclin-like' protein, Spy1 (Speedy, RINGO, Spy1A1; gene *SPDYA*) in regulating signalling of the estrogen receptor alpha (ERα), including both c-Myc and mitogen activated protein kinase (MAPK) pathways (Bunone et al., 1996; Liao and Dickson, 2000; Musgrove et al., 2008a). Both c-Myc and MAPK signalling are highly elevated in breast carcinogenesis and are linked to treatment resistance (Adeyinka et al., 2002; Liao and Dickson, 2000). Previous work demonstrated that Spy1 is a downstream target of both c-Myc and MAPK during normal mammary growth and development (Golipour et al., 2008; Lenormand et al., 1999). Hence, we sought to determine whether Spy1 is directly affected by estrogen signalling or has an independent role, and to dissect the involvement of the c-Myc and MAPK pathways. We have found that estradiol (E2) binding to ERα upregulates Spy1 protein levels and that persistent Spy1 signalling correlates with a downregulation and/or altered post-translational modification of ERα. Mechanistically, altered post-translational modification of ERα occurs in a MEK1/2-independent fashion and is mediated via an activation of ERK1/2, a result unique to Spy1 and not demonstrated by elevated levels of a classical cyclin, Cyclin E. This increase in ERK1/2 activity was very interesting and exciting data that indicated a possible feedback loop to the MAPK pathway. Previous work showed MAPK activation downstream of Spy1 microinjections in *Xenopous* oocytes (Lenormand et al., 1999); however, this is the first demonstration that Spy1-CDKs can influence MAPK signalling in a human cell system.

Spy1-CDK complexes activate substrates with non-canonical sequence motifs; specifically non-basic residues at the +3 position in the ((S/T)PX(K/R)) (Cheng and Solomon, 2008). The site of phosphorylation known in literature as the main activation

site of ERK1/2 is threonine (T)-202/tyrosine (Y)-204 (TXY) (Raman et al., 2007). Although the TXY motif is not a canonical consensus sequence for CDKs, this combination of sites has not been tested as a potential substrate for Spy1 bound to CDK. Furthermore, a computer generated program showing non-canonical phosphorylation sites predicted a site, SPSQ, close to the TXY sequence which has potential to be phosphorylated by CDK complexes (data not shown). Future work needs to be performed through mutations of the canonical and non-canonical sites in the presence of overexpressed Spy1 to determine if there is an increase in phosphorylated ERK1/2 and at which site this may take place. Crystallographic studies demonstrating how Spy1 binds to CDKs are also of the utmost importance to elucidate whether conformational changes of the CDK are responsible for this unique activation of ERK1/2.

Ras and Raf signalling promote ERK1/2 activation and activating mutations in either one of these genes significantly increases ERK1/2 phosphorylation, driving proliferative cell programs. Cancer treatments aim to inhibit this pathway by specifically inhibiting one of these two genes (Roberts and Der, 2007). In the presence of Spy1 overexpression, Ras and Raf inhibitors decrease the activation of pERK1/2, supporting that Spy1 requires these pathways. In 2003, Moeller *et al.* showed that Ras activation was dependent upon the inhibition of p27 (Moeller et al., 2003). p27 is a CKI that also functions in cell adhesion, apoptosis, and senescence, and some data support that these properties could be independent of CDK (Lim and Kaldis, 2013). In the Moeller study, p27 was isolated as a Grb2 (growth factor receptor bound protein 2) interacting protein and shown to compete for SOS (son of sevenless) (Moeller et al., 2003). Grb-SOS complex formation activates Ras, and, hence, this competition inhibits Ras activation.

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Spy1 promotes the binding and degradation of p27 (McAndrew et al., 2007; Porter et al., 2003) and, hence, we questioned whether this could be an important link to the feedback to ERK1/2. We have shown that Spy1 mutants unable to interact with p27 have a significant reduction in ERK1/2 activation. These data support the conclusion that Spy1-mediated degradation of p27 enables the activation of Ras/Raf and downstream activation of ERK1/2. How this can occur in a MEK1/2 independent fashion was still a mystery, however. Receptor-interacting serine/threonine protein kinase (RIPK)2 is a kinase activated by Raf and has been shown to directly phosphorylate ERK1/2 on the TXY sequence, independent of MEK1/2 (Navas et al., 1999). We have further shown that overexpression of Spy1 works independently through RIPK2 to phosphorylate ERK1/2. Hence, our data supports that in at least subsets of breast cancer cells, Spy1 is activated downstream of ERα and persistent signalling activates ERK1/2 via a unique MEK-independent mechanism dependent on Spy1 binding to p27 and activation of the kinase RIPK2.

Increased pERK1/2 protein levels have been correlated in various human cancers, including hepatocellular carcinoma and breast cancer (Adeyinka et al., 2002; Huynh et al., 2003). Similarly, Spy1 has been found to be upregulated in both of these cancers and has been shown to be one of the fifty most upregulated genes in invasive carcinomas of the breast (Al Sorkhy et al., 2012; Ke et al., 2009; Zucchi et al., 2004). ERK1/2 activation is associated with the development of an tamoxifen-resistant phenotype, primarily mediated through the phosphorylation of ERα on serine 118 (S118) (Kato et al., 1995). Hence, we questioned whether elevated levels of Spy1 would play a role in driving this functional outcome. We find that Spy1 overexpression leads to a significant increase in

pERα-S118 and increased proliferation and this leads to tamoxifen resistance both *in vivo* and *in vitro*. Our data further support that manipulation of Spy1 levels may re-sensitize select resistant breast cancer cells to treatment. The future implications of this, both for prognosis and treatment, is an exciting and important direction.

Downregulation of ERα and patients presenting without hormone receptors (triple negative breast cancer, TNBC), cannot be treated with tamoxifen or targeted therapies and, therefore, chemotherapy is the only option (Prat and Perou, 2011). Understanding how a patient may acquire this status is important in preventing this progression in patient populations. Understanding the pathways driving growth in an ERα-negative cell system also provides novel opportunity for therapeutic intervention. The protein levels of the proto-oncogene, c-Myc, have been correlated with breast cancers that initially begin with a positive hormone receptor status, but, following extended c-Myc transcriptional activity and/or amplification, resemble the basal-like breast cancer subtype (Dimitrakakis et al., 2006; Liao and Dickson, 2000; Musgrove et al., 2008b). Mechanistic insight into this phenomenon has been lacking. It has been speculated that c-Myc controls the activation of genes that can increase the phosphorylation of ERα, which leads to its degradation (de Leeuw et al., 2013; Dimitrakakis et al., 2006). Our study designed a cell model system where prolonged c-Myc signalling demonstrated a marked downregulation of the ERα and subsequent resistance to hormone therapy. We demonstrated using this system and in human cell systems that elevated levels of Spy1 downstream of c-Myc play an important role in stabilizing c-Myc protein via phosphorylation on serine 62 (S62). CDK2 and ERK1/2 have a stabilizing effect on c-Myc and it is through the S62 phosphorylation site that c-Myc can mediate strong effects on driving proliferative programs (Amati, 2004;

Campaner et al., 2010). Our data explored the biological relevance of the ability of Spy1 to regulate c-Myc stability. We have demonstrated that Spy1 knockdown re-establishes response to tamoxifen in resistant cells expressing high levels of c-Myc, showing a possible correlation between Spy1, c-Myc, and ERα protein levels and response to hormone therapies.

While chemotherapy has increased survival of TNBC patients, they still show a significantly high rate of relapse within the first 3 years after treatment and have a high metastasis rate (Perou, 2010). A newly emerging form of cancer therapeutic is the reintroduction of lost/downregulated CKIs through the use of synthetic CKIs (Asghar et al., 2015). Synthetic CKIs are purine-based drugs designed to mimic the mechanism of p21$^{\text{Cip1}}$ in the ATP-binding site of the CDK, blocking full CDK activation (Bach et al., 2005; Deep and Agarwal, 2008; Harrison et al., 2009). Synthetic CKIs, if effective, can be an invaluable tool in promoting a homeostatic state to inhibit the cell cycle of a cancerous cell and trigger apoptosis in response to damaged DNA; however, problems and inconsistencies with treatment have resulted in suboptimal data in patient populations. CKIs have had a high success rate in pre-clinical trials, but to date have had high cytotoxic effects in clinical trials (Byrd et al., 2007; Harrison et al., 2009; Nair et al., 2011; Rigas et al., 2007). This downfall is partly due to the lack of understanding regarding the exact mechanism of how CKIs work and which CDKs they inhibit. The specificity of CKI activity is lacking. Initial clinical trials have all focused on pan-CKIs capable of inhibiting a very wide variety of CDKs. Second and third generation CKIs are now attempting to increase specificity (Asghar et al., 2015; Deep and Agarwal, 2008; Malinkova et al., 2015). Given the essentiality of CDKs in cell growth it is important to

determine how to properly direct specificity to reduce cytotoxic effects and enhance long-term benefits (Asghar et al., 2015). Importantly, the majority of clinical trials assessing the efficacy of synthetic CKIs have failed to stratify patient populations in any significant manner (Asghar et al., 2015; Deep and Agarwal, 2008). The first clinical trial to stratify ERα positive breast cancers for amplified Cyclin D, loss of p16, or both has just released a late breaking ASCO abstract showing that the addition of a select CDK4/6 inhibitor nearly doubles 5-year disease free survival rates (Turner et al., 2015); solidifying that stratification and directing specified CKIs is a critical step to optimize the use of this class of drugs.

Most clinical trials testing CKIs in breast cancer to date have focused on ERα positive populations with or without Her2/neu expression (Turner et al., 2015, Finn et al., 2015); the implications of these drugs in resistant populations and in TNBC populations remain to be thoroughly tested. *In vitro* studies on TNBC indicate that those populations with an amplification of c-Myc may respond specifically to CKIs blocking CDK1 (Kang et al., 2014). This presents a complication as CDK1 is an essential gene and this strategy may risk high toxicities. We explored the possibility of targeting Spy1-directed CDKs, which utilize an alternate mechanism and may enable a more specific approach with reduced toxicities. Importantly, Spy1-directed CDKs can override p53- and p21-dependent apoptosis and can bypass cell cycle checkpoints, hence, this approach may sensitize drug resistant cells to existing therapies. We show here, for the first time, that Spy1 levels are high in TNBC patient samples and that Spy1 knockdown significantly increased the sensitivity of cells to CKIs alone and in combination with known chemotherapeutic agents, including cisplatin and paclitaxel. Spy1 knockdown showed a

synergistic effect when used in combination with chemotherapies and CKIs; this was a unique observation as Cyclin E knockdown did not show this effect. In fact, under specific conditions Cyclin E knockdown increased the viability of TNBC cells with treatment, stressing the potential importance of fine-tuning the targeting of the CKIs.

It is an important next step to test these interactions within *in vivo* models prior to movement into patient populations. These experiments classically weigh heavily on assessment in mouse model systems. We have established the efficacy of using zebrafish models to assess human cancer cell response to drug treatments (Chapter 2, Figure 6). One true benefit of this system is that the model has an intact adaptive immune system and microenvironment similar to that of a patient (Novoa and Figueras, 2012) and, hence, can provide us with a better understanding of the response, sensitivity, and metabolism of the treatment within a patient. Our work to date has focused on available cell systems, or an established primary mouse cancer cell system; future work will also focus on using primary cells derived from patient samples of ERα positive and TNBC patients. It is important to test the effects of Spy1 manipulation on human patient samples in the presence and absence of synthetic CKIs and chemotherapy. It is also extremely important to determine if Spy1 levels can predict a patient's response to therapy, this could then represent an important prognostic marker for guiding therapeutic decisions.

Further elucidating the biology underlying Spy1-mediated effects on treatment sensitivity in conjunction with *in vivo* studies remains a crucial step in determining the role of Spy1 in mammary tumourigenesis and in effectively targeting Spy1-directed effects in the correct patient population. Further dissecting the biochemistry of the Spy1 structure when bound to specific CKIs, and how and why Spy1 levels are elevated in

aggressive breast cancer phenotypes are important questions that require further research. These basic research questions are critical in moving studies forward into the clinic to benefit patients.

In summary, the data from this study supports the hypothesis that Spy1 levels accumulate downstream of ERα-signalling and that Spy1 is capable of feeding back to activate the ERK1/2 pathway to modify ERα and subsequent response to hormone therapy. Furthermore, we demonstrate that Spy1 levels correlate with that of c-Myc in TNBC patients, and that Spy1 plays an active role in maintaining accumulated levels of c-Myc in this aggressive form of breast cancer. We show that manipulating the levels of Spy1 can sensitize TNBC cells to current chemotherapy treatment as well as to both a pan-CKI and a second generation CDK1/2 inhibitor. Our data supports that CKIs can sensitize drug resistant and TNBC cells to hormone and chemotherapy regimens, but that the stratification and specification of the CKI is an important step in optimizing the addition of this treatment. Much work remains to be done; however, this body of data moves forward our understanding of how to better direct research efforts to continue to improve the care available for breast cancer patients.

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APPENDICES

Appendix A- Permissions

To Whom It May Concern

Dear Sir/Madam,

Please accept this letter as my authorization for Ms. **Rosa-Maria Ferraiuolo** to use the data from our co-authored paper in preparation, "**The Cyclin-like Protein, Spy1, Regulates the ER alpha and ERK1/2 Pathways Promoting Tamoxifen Resistance**", in her doctoral thesis within her second chapter. Please do not hesitate to contact me for further clarification.

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