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The Role of SRC Kinase in Estrogen Receptor-positive Breast Cancer Cell Proliferation and Tumor Growth

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Christopher Arif Abdullah

Committee in charge:

Professor Jean Y. J. Wang, Chair
Professor Sara A. Courtneidge, Co-Chair
Professor Mark P. Kamps
Professor Jens Lykke-Andersen
Professor Alexandra C. Newton

2017

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The dissertation of Christopher Arif Abdullah is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2017

Dedication

To my family and friends

I have been supported in every way possible by my family over the years and during my time in graduate school. They have helped to shape me into the person that I am today and will continue to do the same into the future. My parents have always let me carve out my own path, but they were right there behind me as I did it. My sister always has helped to keep me grounded through little messages, inside jokes, or at sporting events over the years. I am grateful to my family for the support they have given me and for their continued support in the future.

Most of my studies have been away from my traditional family, but I have had the amazing support of a great bunch of friends throughout childhood, college, and graduate school. They are an incredibly diverse group of people that constantly teach me something new and are always supportive of everything that I do. I am grateful to all of them.

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List of Common Abbreviations

ER: Estrogen receptor

E2: 17β-estradiol, estrogen

IMP1: Insulin-like growth factor 2 mRNA-binding protein 1

mRNA: Messenger ribonucleic acid

SFK: SRC family kinase

shRNA: Short hairpin ribonucleic acid

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ABSTRACT OF THE DISSERTATION

The Role of SRC Kinase in Estrogen Receptor-positive Breast Cancer Cell Proliferation and Tumor Growth

By

Christopher Arif Abdullah

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2017

Professor Jean Y. J. Wang, Chair
Professor Sara A. Courtneidge, Co-Chair

Non-genomic estrogen (E2) signaling has previously been implicated in promoting E2-dependent proliferation, but the mechanism by which this occurs is not known. Here, we found a requirement for the protein tyrosine kinase, SRC, in cell cycle progression and proliferation in quiescent estrogen receptor (ER)-positive

breast cancer cells stimulated with E2 *in vitro*. Using a SRC family kinase (SFK) inhibitor, SU11333, and shRNA mediated knockdown, we demonstrated that SRC, but not YES, is required for cell cycle progression and proliferation *in vitro*. Using the SRC/ABL selective kinase inhibitor, saracatinib, we also demonstrated that SFKs were required for tumor growth *in vivo* in an orthotopic xenograft model. Expression of *MYC* is required for E2-dependent proliferation, and *MYC* mRNA levels are induced by SRC by stabilization of *MYC* mRNA rather than activation of transcription. This stabilization required the RNA-binding protein ΔN-IMP1 which we found to be expressed in several ER-positive breast cancer cell lines. ΔN-IMP1 stabilization of *MYC* mRNA required SRC phosphorylation of Y260.

We also observed a role for SRC in overcoming a p53 block to cell cycle progression after E2 stimulation. Loss of p53 in MCF7 cells abrogated a need for SRC in promoting cell cycle progression and proliferation *in vitro.* In an *in vivo* MCF7 orthotopic xenograft model, we found that saracatinib inhibited growth of tumors regardless of p53 expression. Using shRNA-mediated knockdown of SRC, we also demonstrated that SRC was required for tumor grafting in mice. We also used an inducible shRNA system to knockdown SRC in established tumors. Here, we show SRC inhibition combined with loss of p53 led to static tumors whereas SRC inhibition in the presence of p53 leads to tumor regression. Loss of p53 led to an increase in *MYC* mRNA due to increased basal mRNA stabilization and an increase in ΔN-IMP1 protein levels. Loss of p53 also abrogated the ability of E2 to stimulate *MYC* mRNA stabilization. Lastly, a defect in proliferation was observed in MCF7 cells overexpressing mutations of known SRC target tyrosines in MDM2 (Y281F/Y302F) suggesting a possibility for MDM2 as mediator of SRC inhibition of p53 function.

Part I

Chapter I:

Introduction:

Important Effectors for Estrogen Receptor-Positive Breast Cancer Cell Proliferation

Introduction and Summary

Estrogen Signaling and its Role in Estrogen Receptor-positive Breast Cancer

Breast cancer is the most common cancer in women in the United States between 2009-2013 with an age-standardized incidence rate of 125.5 per 100,000 (Jemal et al., 2017). Breast cancer can be classified into several clinical sub-types dependent upon the expression of three cellular receptors including the estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor type 2 (HER2); or lack of expression of these receptors, classified as triple-negative breast cancer. The most common sub-type, estrogen receptor (ER)-positive breast cancer, accounts for the majority of breast cancer cases, between 60-80% of all diagnosed cases of breast cancer (Ali and Coombes, 2000; Miyoshi et al., 2010). This sub-type is characterized clinically by increased protein expression of the ER in tumor cells. Although increased expression of ER is used as a clinical marker, the mechanism by which the ER is upregulated is poorly understood. Several mechanisms including increased activation of the promoter of the gene, stabilization of the protein, and amplification of the gene have been studied (Ali and Coombes, 2000; Carroll, 2016; Miyoshi et al., 2010). Despite lack of a complete understanding of this mechanism, therapeutics designed to disrupt the activity

of the ER have been effective in the clinic at treating ER-positive breast cancer.

The ER is a nuclear hormone receptor that is activated upon binding of its natural ligand, 17-β-estradiol (estrogen, E2), and this activation has been shown to regulate the expression of target genes and promote the growth of ER-positive tumors (Marino et al., 2006). E2 acts as an agonist of the ER and upon binding, the ER is internalized by the cell, forms a homodimer, and translocates to the nucleus (DeMayo et al., 2002). In the nucleus, the ER homodimer binds to genomic DNA at estrogen responsive elements (EREs) and regulates its transcriptional gene targets. The ER can also form heterocomplexes with other transcription factors and be required for activation of transcription targets at other genomic sites. The transcriptional regulation of gene targets has been termed the E2 canonical, or genomic, signaling pathway (DeMayo et al., 2002; Fox et al., 2009; Marino et al., 2006; Sanchez et al., 2002).

In addition to the canonical pathway, the ER has been described to be involved in rapid action, or non-genomic, signaling pathways (Ali and Coombes, 2000; Fox et al., 2009; Marino et al., 2006; Sanchez et al., 2002; Song et al., 2005; Vrtacnik et al., 2014). E2 stimulation and activation of the ER are required, however, signaling occurs in the cytoplasm rather than the nucleus. Several signaling pathways have been implicated in non-genomic E2 signaling including SRC, MAPK, GPCRs, PKC, and PI3K (Castoria et al.,

2001; Lobenhofer et al., 2000). The ER has been described to activate the MAPK pathway dependent upon an interaction between the ER and SRC (Migliaccio et al., 1996), and both the SRC and MAPK pathways may be required for E2-dependent proliferation (Castoria et al., 1999; Lobenhofer et al., 2000).

In addition to downstream signaling, post-translational modifications of the ER have also been suggested to be important for E2-dependent proliferation. The ER contains a tyrosine residue (Y537) known to be phosphorylated by SRC and to be required for subcellular localization of the receptor as well as E2-dependent cell cycle proliferation (Arnold et al., 1995; Castoria et al., 2012). Additionally, a study using a six amino acid phosphorylated peptide mimic of the ER which competes in a dominant negative manner with the endogenous receptor for SRC binding was demonstrated to block cell proliferation as well as MCF7 tumor xenografts (Varricchio et al., 2007). This peptide also did not affect the transcriptional activity of the endogenous receptor providing more evidence that non-genomic signaling is important for cell cycle progression and tumor growth. These studies taken together suggest that the both the genomic and non-genomic E2 signaling pathway are important for E2-dependent signaling, and more research would aid in understanding whether one or both of these pathways are primarily important for proliferation.

SRC in ER-positive Breast Cancer

The protein tyrosine kinase SRC has been demonstrated to be involved in the initiation, progression, maintenance, and metastasis of breast cancer (Andrechek and Muller, 2000; Biscardi et al., 2000; Irby and Yeatman, 2000). In several studies, more than 70 percent of breast cancer patient samples have been demonstrated to have increased SRC expression as compared to normal breast epithelium as analyzed by either biochemical and immunohistochemical methods (Elsberger, 2014; Ottenhoff-Kalff et al., 1992; Verbeek et al., 1996). Additionally, one study found that increased expression of cytoplasmic SRC in ER-positive breast cancer samples relative to normal tissues correlated with reduced survival (Morgan et al., 2009). Another study found that active phosphorylated pY419SRC was increased in recurrent ER-positive breast cancer (Planas-Silva et al., 2006). Taken together, these data suggest clinical relevance for SRC expression in ER-positive breast cancer.

As described above, human ER-positive breast cancer has increased expression of SRC suggesting that SRC could have an important role in this breast cancer sub-type. Indeed, several mouse studies have found important roles for SRC during mammary gland development, E2 signaling, and tumorigenesis. SRC knockout mice have defects in mammary gland development, and E2 signaling was defective in mammary epithelial cells isolated from these mice (Kim et al., 2005). In a mouse model of breast cancer that was generated by expressing a mouse mammary tumor virus

(MMTV)/polyoma middle T antigen (PyVmT) fusion gene that generates rapid tumor development and metastases (Guy et al., 1992), SRC was shown to be required for tumorigenesis when the MMTV/PyVmT mice were crossed with *src*$^{-/-}$ mice and few, if any, tumors developed (Guy et al., 1994). The same requirement was not found to be true of the SRC family kinase (SFK), YES. When *yes*$^{-/-}$ mice were crossed with the MMTV/PyVmT mouse, tumors developed at nearly the same rate as in the wild-type mouse. Another mouse model in which SRC was conditionally knocked out in mammary epithelial cells to circumvent issues with impaired mammary gland development in the knockout mouse showed a similar defect in tumorigenesis in the PyVmT mouse as well as in cell cycle progression (Marcotte et al., 2012).

These *in vivo* mouse models suggest an important role for SRC in the development and progression of breast cancer. The expression studies correlating increased SRC expression in most breast cancer, but also specifically ER-positive breast cancer also complements the findings from the mouse studies. These findings are consistent with the *in vitro* studies described above suggesting a role for SRC as a mediator of E2-dependent proliferation. Next, potential downstream effectors of ER- and SRC-mediated proliferation are discussed.

Function and Regulation of p53

In many cancer types, p53 is mutated and associated with both loss- and gain-of-functions that support tumor initiation, progression, and metastasis

(Aylon and Oren, 2007; Bargonetti and Manfredi, 2002; Goldstein et al., 2011; Woods and Vousden, 2001). Differing from most tumor types, ER-positive breast cancers typically express wild-type p53 (Caleffi et al., 1994; Dumay et al., 2013). It has been suggested that cancer cells which retain wild-type p53 suppress its activity via other mechanisms.

The tumor suppressor, p53, has been described to have critical roles in regulating cell cycle arrest and apoptosis due to environmental stress and DNA damage (Aylon and Oren, 2007; Bargonetti and Manfredi, 2002; Goldstein et al., 2011; Woods and Vousden, 2001). Full activation of p53 results in irreversible cell death, and its function is, therefore, tightly regulated. Regulation of p53 has been widely studied, including the stability of the p53 protein, post-translational modifications to the protein (such as phosphorylation, acetylation, and ubiquitination), and interactions with other cofactors or regulators, including the p53 inhibitors, MDM2 and MDMX (Kruse and Gu, 2009). The classical model for p53 regulation focuses on stabilization of the protein after cellular stresses, including DNA damage, and binding to DNA to activate transcriptional regulation of target genes (Kruse and Gu, 2009). However, more recent studies have suggested that p53 is much more complex. Basal levels of p53 are regulated by the E3 ubiquitin ligase, MDM2, which can inhibit p53 by ubiquitination to cause subcellular relocalization, proteasomal degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997) and by neddylation to inhibit transcriptional activity of

p53 (Xirodimas et al., 2004). Upon stress signaling, p53 can be stabilized, phosphorylated by Chk1, Chk2, ATM, and ATR (Appella and Anderson, 2001; Shieh et al., 2000; Shieh et al., 1997), or acetylated (Tang et al., 2008), and bound to its DNA target promoters. Additional post-translational modifications, including methylation by Set7/9 (Chuikov et al., 2004), neddylation by MDM2 (Xirodimas et al., 2004), and sumoylation (Carter et al., 2007; Melchior and Hengst, 2002) of p53, then allow for formation of distinct transcriptional complexes to form and allow for fine-tuned functional responses including senescence, cell cycle arrest, DNA repair, apoptosis, or autophagy (Kruse and Gu, 2009). Clearly, p53 activation is a very complex process being regulated at multiple levels to ensure proper maintenance of the cell. Specific cellular functions are likely governed via cooperation between multiple signaling pathways, and sequential modifications of p53 are likely required for proper responses to cellular stresses, and this field of study remains an area of active research.

Although p53 regulation is very complex, the roles of MDM2 and MDMX have been more well studied and have provided hints about their roles in p53 regulation. Although MDM2 and MDMX are structurally similar, they play different, non-overlapping roles in p53 inhibition (Marine et al., 2006). Both MDM2 (Jones et al., 1995; Montes de Oca Luna et al., 1995) and MDMX (Migliorini et al., 2002; Parant et al., 2001) knockout mice are embryonic lethal, however, inactivation of p53 in either background completely rescues the

lethal phenotype suggesting that these two proteins individually are critical inhibitors of p53. Unlike MDM2 which has E2 ligase activity towards p53, MDMX does not have ligase activity, but has been described to affect p53 transcriptional activity (Marine and Jochemsen, 2005). MDM2 and MDMX individually interact with p53 but also interact with each other, and the MDM2/MDMX complex has been shown to lead to more efficient ligase activity of MDM2 (Linares et al., 2003). An additional mouse study wherein the MDM2 interaction site on MDMX was mutated in the presence of endogenous MDM2, also resulted in embryonic lethality able to be rescued by p53 inactivation suggesting that the interaction between the two is also critical for p53 regulation (Huang et al., 2011).

The interaction of MDM2 and MDMX has been shown to be dependent upon MDM2 phosphorylation by the tyrosine kinase ABL ultimately leading to p53 inactivation (Waning et al., 2011). A knock-in mouse model was generated by mutating the ABL phosphorylation site on MDM2 Y393F, and these mice spontaneously developed tumors, however, stabilization of p53 protein was unaffected (Carr et al., 2016). Although these studies suggest a role for ABL in inhibiting p53 function, conflicting reports also suggest a role for ABL in activating p53. ABL can interfere with MDM2 interaction with p53 (Sionov et al., 2001; Sionov et al., 1999), and also phosphorylate MDMX inhibiting its interaction with p53 (Zuckerman et al., 2009).

Many of the studies described above have primarily focused on regulation of p53 in the context of the DNA damage or apoptotic response. ABL regulation of MDM2 and MDMX is of particular interest for this work, because while ABL has been implicated in DNA damage and apoptosis, ABL also has been implicated in control of the cell cycle which will be discussed in more depth below.

Role of p53 Cell Cycle Regulation in Growth Factor and Estrogen Signaling

Many functions of p53 have been elucidated using DNA damage as a stimulus, which results in stabilization of p53 protein and often a full apoptotic response (Bargonetti and Manfredi, 2002; Kruse and Gu, 2009; Woods and Vousden, 2001). Apoptosis is irreversible; however, cell cycle arrest is not suggesting that cell cycle arrest is potentially an acute function of p53 distinct from apoptosis. Less work has been focused on understanding the role of p53 in suppression of cell cycle progression. Increases in p53 expression have been observed in quiescent cells, suggesting that p53 could be causing a G0/G1 cell cycle block (Itahana et al., 2002). Correlating with this, some studies have implicated inhibition of p53 and increased expression of MDM2 in growth factor-stimulated release from quiescence (Lei et al., 2011; Leri et al., 1999; Quintavalle et al., 2010; Ries et al., 2000; Shaulian et al., 1997). These

data suggest that signaling via MDM2 may play a role in regulating a p53 cell cycle block.

Our lab has previously shown that quiescent cells stimulated with peptide growth factors require SRC to progress into the cell cycle (Roche et al., 1995; Twamley-Stein et al., 1993). However, cells lacking p53 no longer require SRC activity to enter the cell cycle upon growth factor stimulation (Broome and Courtneidge, 2000; Furstoss et al., 2002; Klinghoffer et al., 1999). SRC has also been shown to activate ABL, which is also required for proliferation only in cells expressing functional p53 (Furstoss et al., 2002). ABL is also a downstream effector required for transformation by active SRC (Sirvent et al., 2007).

Experiments in several other systems are also consistent with a role for SRC inhibition of p53. The polyoma virus large T or middle T antigens do not inhibit p53 nor promote degradation of p53 unlike other tumor viruses (Dilworth, 1990) which target p53 directly. However, transformation by polyoma middle T requires the association and activation of SRC (Courtneidge, 1985; Courtneidge and Smith, 1983) and these data taken together potentially suggest that SRC may indirectly mediate p53 inhibition. Activated SRC has also been shown to reduce expression of p53 in vascular smooth muscle cells and NIH3T3 cells to promote podosome formation (Mukhopadhyay et al., 2009). In another study using vascular smooth muscle cells, our lab showed that SRC inhibited p53-regulated miRs that regulated

podosome formation (Quintavalle et al., 2010). In a model of proliferative vitreoretinopathy, activated PDGFRα has been shown to suppress expression of p53 further implicating p53 as a downstream target of growth factor signaling (Lei et al., 2011).

In addition to growth factor inhibition of p53, several studies have also shown that E2 signaling may inhibit p53 function indirectly (Bailey et al., 2012; Berger et al., 2012; Konduri et al., 2010; Rieber and Strasberg-Rieber, 2014). A direct interaction of the ER with p53 has also been described to modulate its transcriptional activity (Liu et al., 2006; Menendez et al., 2010). Given the data that individually, the ER and SRC have been implicated in the inhibition of p53, it remains to be fully tested whether SRC mediates p53 inhibition downstream of E2 signaling. However, several clinical studies have provided hints into SRC playing a role in ER-positive breast cancer. Preclinical and clinical trials using multi-targeted kinase inhibitors that inhibit SRC, including dasatinib (Mayer et al., 2011; Mitri et al., 2016) and bosutinib (Campone et al., 2012; Hebbard et al., 2011) have been largely disappointing, but there were some hints of response in hormone receptor (HR)-positive breast cancer. These trials did not take into account p53 status of the HR-positive patients, but as we described earlier, most ER-positive breast cancer patients express wild-type p53. Additionally, loss of p53 function typically occurs during cancer progression, and many of the patients in these trials are in the late stages of progression which likely correlates with p53 loss. Thus, while SRC inhibition in

the clinic has had some limited efficacy resulting in stable disease in HR-positive breast cancer, the trials may in general been targeting the appropriate population. The data suggesting that SRC inhibits p53 function taken with the clinical trial data suggests that additional research should be conducted to test whether p53 may be an important biomarker for SRC inhibition.

Function and Regulation of MYC

MYC is a transcription factor known to promote proliferation and has also been described to be important in breast cancer (Liao and Dickson, 2000). *MYC* expression is typically high in breast cancer, however, this does not appear to be due to amplification of the gene (Deming et al., 2000). Transgenic mouse models have also shown that *MYC* expression on its own is enough to induce tumors in breast tissues (Liao and Dickson, 2000; Meyer and Penn, 2008). Because of this potent tumorigenic function of MYC, the gene and its protein product are tightly regulated.

MYC is regulated by several means including at the transcriptional (Dubik and Shiu, 1988; Ho et al., 2005; Santos et al., 1988; Zou et al., 1997), post-transcriptional (Blanchard et al., 1985; Dean et al., 1986; Kindy and Sonenshein, 1986; Nepveu et al., 1987), and post-translational (Sears et al., 1999; Sears et al., 2000) levels. Phosphorylation of MYC is known to stabilize its protein levels and direct its activity (Sears et al., 1999; Sears et al., 2000). Post-transcriptionally, *MYC* mRNA is known to be regulated by *cis*-elements within its mRNA sequence including a coding region determinant (CRD)

(Ioannidis, 2005; Ioannidis et al., 2005; Lemm and Ross, 2002; Noubissi et al., 2006; Sparanese and Lee, 2007; Weidensdorfer et al., 2009), and AU elements and miRNA regulation sites within the *MYC* 3'-UTR (Guhaniyogi and Brewer, 2001; Ross, 1995; Sachdeva et al., 2009). Several RNA-binding proteins have been shown to regulate the half-life of *MYC* mRNA via these *cis*-elements including stabilization by insulin growth factor 2 binding protein 1 (IMP1, IGF2BP1, CRD-BP, ZBP1) (Barnes et al., 2015; Ioannidis et al., 2005; Weidensdorfer et al., 2009) or destabilization by tristetraprolin (TTP) (Marderosian et al., 2006; Rounbehler et al., 2012). Interestingly, IMP1 is typically expressed during development, but has been demonstrated to be re-expressed in several tumor types and cell lines (Bell et al., 2013; Ioannidis et al., 2004; Ioannidis et al., 2003; Ioannidis et al., 2005; Köbel et al., 2007) including breast cancer. A recent report also suggests that a truncated form of the protein, ∆N-IMP1 is expressed in the MCF7 cell line, an ER-positive breast cancer cell line (Fakhraldeen et al., 2015). With many levels of regulation, mechanisms that may regulate *MYC* dependent upon ER signaling are discussed next.

MYC as an Effector of ER- and SRC-Regulated Proliferation

MYC has been shown to be a downstream effector for both the ER and SRC. *MYC* has also been described to be required for E2-dependent breast cancer cell proliferation (Shiu et al., 1993). An additional study showed that inducible *MYC* expressed in MCF7 cells could overcome cell cycle block due

to ICI182870 (Prall et al., 1998). Several studies have suggested that *MYC* is regulated transcriptionally by E2 stimulation (Dubik et al., 1987; Dubik and Shiu, 1988; Shiu et al., 1993; Wang et al., 2011), however, there has not been a canonical ERE discovered in the *MYC* promoter (Dubik and Shiu, 1992). Other studies have suggested that *MYC* is regulated post-transcriptionally by E2 (Santos et al., 1988).

In line with the possibility that *MYC* may be regulated post-transcriptionally by E2 non-genomic signaling, growth factor signaling studies have also suggested that *MYC* is regulated by this mechanism (Blanchard et al., 1985; Bromann et al., 2005; Dean et al., 1986; Greenberg and Ziff, 1984; Kelly and Siebenlist, 1986; Kindy and Sonenshein, 1986; Nepveu et al., 1987). Downstream of growth factor signaling, our lab has demonstrated a role for SRC in mediating *MYC* mRNA stability in response to PDGF stimulation (Bromann et al., 2005). Our studies have also demonstrated that *MYC* expression can overcome a cell cycle block due to SFK inhibition by dominant negative SFKs (Barone and Courtneidge, 1995) and also that *MYC* mRNA was regulated by SFK activity confirmed by use of an SFK inhibitor (Blake et al., 2000).

Concluding Remarks

E2 signaling in has been extensively studied, in particular, the genomic signaling pathway, but many open research questions remain regarding the non-genomic signaling pathway and its contribution to ER-positive breast

cancer growth and progression. Much research has focused on the roles and regulation of the ER, SRC, p53, and MYC, however, the tight and complex regulation of each remains to be fully understood. The underlying biology of these signaling pathways may serve to better inform selection of patient biomarkers (perhaps p53 status and ER-status) as well as therapeutic selection (SRC inhibition).

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Chapter II:

SRC is Required for Estrogen-dependent Proliferation in Estrogen Receptor-positive Breast Cancer Cells by Stabilizing *MYC* mRNA

Abstract

Estrogen (E2) stimulation promotes proliferation in estrogen receptor (ER)-positive breast cancer, which accounts for 60-80% of all breast cancer. The tyrosine kinase SRC is also expressed highly in ER-positive breast cancer. A non-genomic E2 signaling pathway involving SRC family kinases (SFKs) has been implicated in regulating the cell cycle downstream of the ER, however, little is known about this pathway. Using an *in vitro* ER-positive breast cancer cell model, MCF7 or ZR75-1, cells were stimulated with E2 and either pretreated with an SFK inhibitor (SU11333) or shRNA-mediated knockdown of SRC or YES. SFK inhibition blocked G1/S progression and proliferation. We tested the SRC/ABL inhibitor, saracatinib, in an orthotopic xenograft mouse model of ER-positive breast cancer and observed decreased tumor growth upon treatment.

As MYC is a known target of both the ER and SRC and a known driver of cell cycle progression, we tested whether MYC could be mediating SRC's proliferative effects in this model. Using *MYC*-targeting shRNA, we showed that MYC is required for E2-dependent cell cycle progression and proliferation. Additionally, E2 stimulates *MYC* mRNA expression dependent upon SRC. We also showed that *MYC* mRNA expression is mediated by SRC-dependent mRNA stabilization, but not transcription. We next aimed to understand the mechanism by which non-genomic ER-SRC signaling drives proliferation in ER-positive breast cancer cells. A known stabilizer of *MYC* mRNA, the RNA-

binding protein IMP1, has a truncated form (ΔN-IMP1) which we show to be expressed in ER-positive breast cancer cell lines. Our data suggests that ΔN-IMP1 is required for the SRC-mediated stabilization of *MYC* mRNA and proliferation. Taken together, these data suggest that SRC mediates cell cycle progression and proliferation through a non-genomic E2 signaling pathway by stabilizing *MYC* mRNA.

Introduction

The protein tyrosine kinase, SRC, has been studied as a mediator of estrogen (E2)-dependent proliferation for some time (Andrechek and Muller, 2000; González et al., 2006; Shupnik, 2004). In normal and cancerous breast epithelial cells, SRC has been shown to be required for E2-dependent proliferation in cells expressing the estrogen receptor (ER) (Arnold et al., 1995; Guy et al., 1992; Guy et al., 1994; Kim et al., 2005; Marcotte et al., 2012). E2 binding to the ER activates SRC as well as MAPK (Migliaccio et al., 1996; Migliaccio et al., 1998). Several studies have also suggested that SRC phosphorylates the ER at tyrosine 537 and that this phosphorylation is important for their interaction as well as proliferation (Arnold et al., 1995; Castoria et al., 2012; Song et al., 2005; Yudt et al., 1999).

SRC has largely been suggested to have a role in the non-genomic estrogen signaling pathway regulating proliferation (DeMayo et al., 2002; Fox et al., 2009; Hammes and Levin, 2007; Marino et al., 2006; Shupnik, 2004; Song et al., 2005). There is evidence that the non-genomic pathway is important for E2-dependent proliferation. Two studies in which ER mutants lacking the ability to bind DNA were exogenously expressed in cells lacking endogenous ER expression were shown to enter S phase upon E2 stimulation (DeNardo et al., 2007). Interestingly, if these cells also expressed a kinase-dead version of SRC, interfering with kinase activity by acting as a dominant-negative, both the wild-type and mutant ER-expressing cells were able to

progress through the cell cycle (Castoria et al., 1999; DeNardo et al., 2007). In addition to SRC data also suggests that the PI3K and MAPK signaling pathways are also required for E2-dependent S phase entry (Castoria et al., 2001; Migliaccio et al., 1996; Migliaccio et al., 1998). This hints that cytoplasmic signaling pathways may be important for regulating proliferation in a non-genomic signaling pathway. These data suggest that SRC-mediated E2-dependent proliferation is likely independent of the estrogen receptor's transcriptional activity. Supporting this claim, no canonical estrogen responsive element (ERE) has been found in the *MYC* promoter (Dubik and Shiu, 1992).

Both the ER (Dubik et al., 1987; Dubik and Shiu, 1988; Dubik and Shiu, 1992; Santos et al., 1988; Shiu et al., 1993; Wang et al., 2011) and SRC (Barone and Courtneidge, 1995; Blake et al., 2000; Bowman et al., 2001; Furstoss et al., 2002; Prathapam et al., 2006) have been demonstrated to regulate expression of the transcription factor, MYC, as a requirement for S phase entry. In the studies using the ER DNA-binding mutants, it was shown that E2 stimulation was still able to induce *MYC* mRNA expression (DeNardo et al., 2007). Inducing expression of *MYC* in MCF7 cells pretreated with fulvestrant, an anti-estrogen (ICI182780) is able to overcome a G1 cell cycle block (Prall et al., 1998).

MYC has been described to be important in breast cancer progression (Liao and Dickson, 2000). MYC expression is typically high in breast cancer,

however, this does not appear to be due to amplification of the gene (Deming et al., 2000). *MYC* has also been shown to be required for E2-dependent breast cancer cell proliferation (Shiu et al., 1993). *MYC* is highly regulated at the transcriptional (Dubik and Shiu, 1988; Santos et al., 1988; Zou et al., 1997), post-transcriptional (Blanchard et al., 1985; Dean et al., 1986; Kindy and Sonenshein, 1986; Nepveu et al., 1987), and post-translational (Sears et al., 1999; Sears et al., 2000) levels. Phosphorylation of MYC is known to stabilize its protein levels and direct its activity (Sears et al., 1999; Sears et al., 2000). Also, *MYC* mRNA half-life is known to be regulated by *cis*-elements within its mRNA sequence including a coding region determinant (CRD) (Ioannidis, 2005; Ioannidis et al., 2005; Lemm and Ross, 2002; Noubissi et al., 2006; Sparanese and Lee, 2007; Weidensdorfer et al., 2009), and AU elements and miRNA regulation sites within the *MYC* 3'-UTR (Guhaniyogi and Brewer, 2001; Ross, 1995). Several RNA-binding proteins have been shown to regulate the half-life of *MYC* mRNA via these *cis*-elements including stabilization by insulin growth factor 2 binding protein 1 (IMP1, IGF2BP1, CRD-BP, ZBP1) (Barnes et al., 2015; Ioannidis et al., 2005; Weidensdorfer et al., 2009) or destabilization by tristetraprolin (TTP) (Marderosian et al., 2006; Rounbehler et al., 2012). Interestingly, IMP1 is typically expressed during development, but has been demonstrated to be re-expressed during cancer progression in several tumor types and cell lines (Bell et al., 2013; Ioannidis et al., 2004; Ioannidis et al., 2003; Ioannidis et al., 2005; Köbel et al., 2007)

including breast cancer. There is also a recent report suggesting that a truncated form of the protein, ∆N-IMP1 is re-expressed in the MCF7 ER-positive breast cancer cell line and is sufficient to rescue clonal outgrowth in cells lacking both forms of IMP1 (Fakhraldeen et al., 2015).

Although the rapid, non-genomic E2 signaling pathway is often cited as part of the ER pathway, relatively little is known about it outside of the likely signaling kinases involved including MAPK, PI3K, and SRC (Castoria et al., 1999; Lobenhofer et al., 2000). Here, we characterize the role of SRC in E2-dependent proliferation in the context of ER-positive breast cancer cells.

Results

SRC is Required for E2-dependent Proliferation in vitro and in vivo.

Using the ER-positive breast cancer cell line, MCF7 (Figure 2.1), we aimed to test whether SRC family kinases (SFKs) were required for E2-dependent proliferation. Using a small molecule inhibitor of SFK activity, (SU11333), we serum-starved MCF7 cells for 48 hours to quiesce the cells prior to stimulation with E2. Upon stimulation of cells with E2, we observed an increase in levels of pY418-SRC that was abolished after pre-treatment with SU11333 (Figure 2.1). We then aimed to test whether SFK activity was required for cell cycle progression and proliferation. Using BrdU incorporation as a surrogate for G1/S transition, we E2-stimulated cells that had been pretreated with either ICI182870, an anti-estrogen, or SU11333. E2 stimulated entry into S phase as seen by an increase in BrdU incorporation (Figure 2.1).

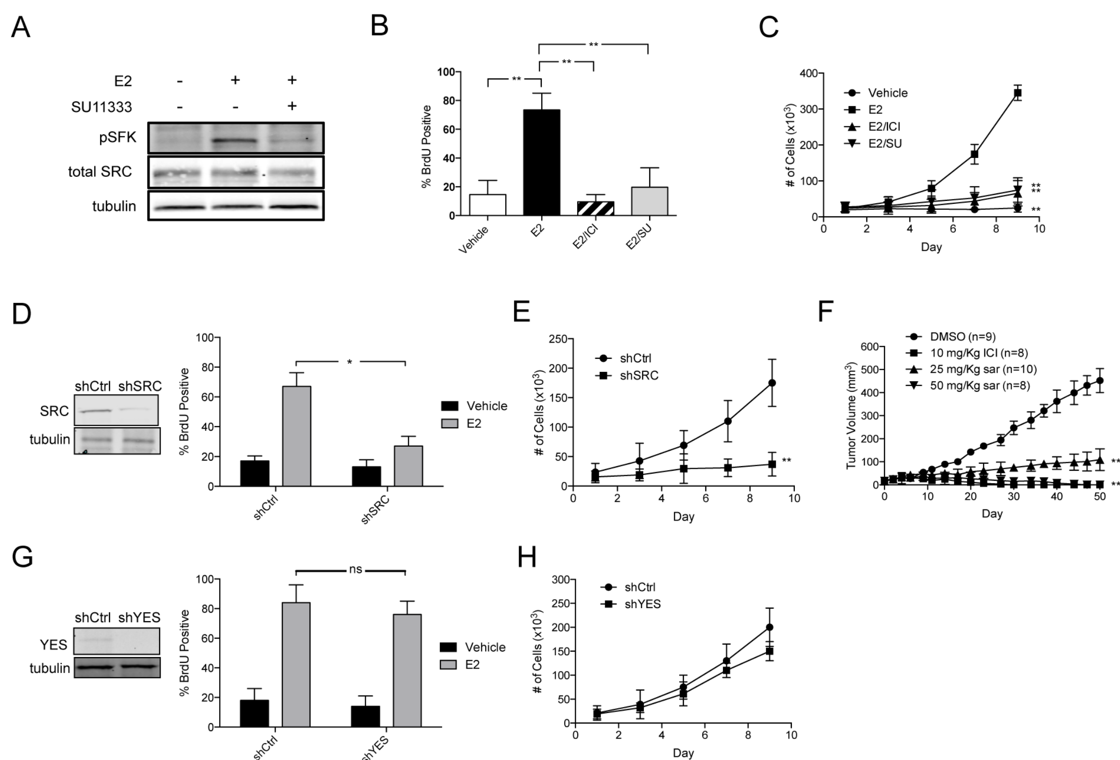

Figure 2.1: SRC, but not YES, is required for E2 stimulated proliferation. (A) Representative immune blot of quiescent MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. (B) BrdU incorporation assay or (C) proliferation assay of quiescent MCF7 cells pretreated with 10 nM ICI182870 or 1 μM SU11333 and treated with vehicle or 5 nM E2 and BrdU. (D) Representative immunoblot analysis of either shCtrl or shSRC stable knockdown MCF7 lines. BrdU incorporation assay or (E) proliferation assay of quiescent shCtrl or shSRC stable knockdown MCF7 lines. (F) Orthotopic MCF7 xenografts in female nude mice. Mice were injected with MCF7 cells at Day 0. Intraperitoneal treatment with DMSO, ICI182870, or saracatinib was performed every three days and tumors measured at the same time. (G) Representative immunoblot analysis of either shCtrl or shYES stable knockdown MCF7 lines. BrdU incorporation assay or (H) proliferation assay of quiescent shCtrl or shYES stable knockdown MCF7 lines. *=$p<0.05$, **=$p<0.01$.

Both ICI182870 and SU11333 blocked E2-dependent BrdU incorporation suggesting that both the E2 stimulus and SFK activity were required to

transition to S phase. To test whether this was an acute cell cycle delay or a sustained cell cycle block, we also performed a proliferation assay. The ICI182870 and SU11333 inhibitor treated cells were unable to proliferate as compared to E2 stimulated cells (Figure 2.1) providing evidence of a long-term persistent block to proliferation.

Because SU11333 inhibits several SFKs (Guan et al., 2004), we wanted to determine whether the cell cycle block was due specifically to SRC or one of the other SFKs. MCF7 cells express SRC at high levels, but also the SFK family member, YES, at low to moderate levels (Figure 2.1). We therefore aimed to test the requirement for these SFKs in cell cycle progression and proliferation independently using shRNA-mediated knockdown (Figure 2.1). In shSRC cells, BrdU incorporation and proliferation were both inhibited relative to shCtrl cells. However, shYES cells showed no statistical difference in BrdU incorporation or proliferation from shCtrl cells (Figure 2.1). These data suggest that SRC is the main SFK required for cell cycle progression and proliferation due to E2 stimulation, and that the kinase activity is required for these functions.

To further test the role of SRC in ER-positive breast cancer proliferation and growth, we next aimed to test whether SRC was required for *in vivo* tumor growth in an orthotopic xenograft model in immunocompromised mice. MCF7 cells have been demonstrated to require sustained E2 stimulation via an implanted E2 pellet to grow as tumors in mice. Using female nude mice,

we first injected the slow-release E2 pellets into the mice one week prior to injection of cells. For cell injection, 1x 10^6 cells were resuspended in PBS and mixed with Matrigel at 1:1 and a final volume of 100 μl was injected into the fourth mammary gland of the mouse. To test the requirement of SRC kinase activity in the mice, we used the clinically available SRC+ inhibitor, saracatinib, although we acknowledge that this inhibitor has additional kinase targets (Green et al., 2009). At a volume of 50 mm^3, we randomized the mice into treatment groups consisting of DMSO vehicle control, 10 mg/Kg ICI182870, and 25 mg/Kg or 50mg/Kg saracatinib (Figure 2.1). Mice were injection intraperitoneally every three days and tumors were measured at the same time. The vehicle treated tumors grew to an average of 400 mm^3 by day 45 (Figure 2.1). As a control for the MCF7 cells requiring E2 for growth *in vivo*, inhibition of the E2 stimulation with ICI182870 caused regression of all treated tumors (Figure 2.1). Treatment of the tumors with the lower dose of saracatinib caused a significant decrease in tumor growth of MCF7 cells. At the higher dose, tumors regressed similar to those treated with the anti-estrogen (Figure 2.1). Overall, our *in vivo* data is consistent with the *in vitro* data that SRC activity is required in MCF7 cells for E2-dependent cellular proliferation and tumor growth. To demonstrate that this was not a cell line specific effect, we also performed similar *in vitro* assays using another ER-positive breast cancer cell line, ZR-75-1, and observed similar effects in BrdU incorporation and proliferation assays using SU11333 (Figure 2.2).

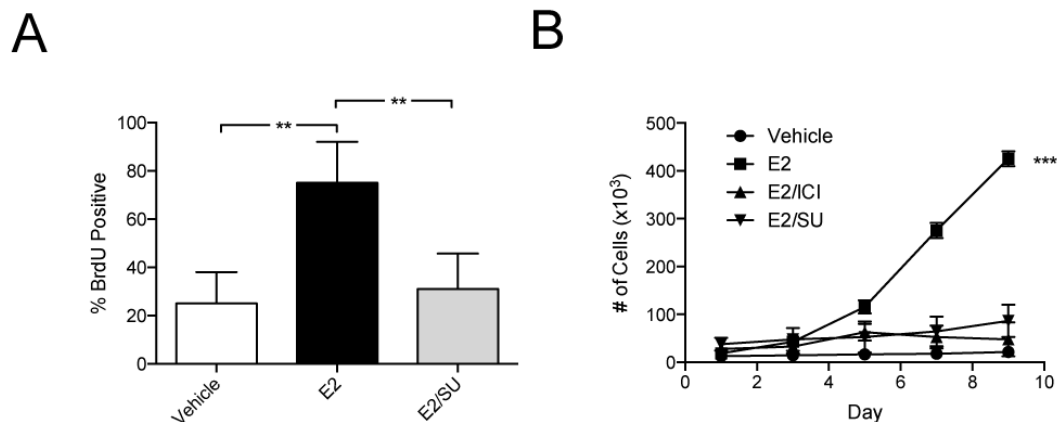

Figure 2.2: ZR-75-1 cells require SFKs for E2-dependent proliferation, *MYC* mRNA accumulation, and *MYC* mRNA stabilization. (A) BrdU incorporation assay or (B) proliferation assay of quiescent ZR-75-1 cells pretreated with 10 nM ICI182870 or 1 μM SU11333 and treated with vehicle or 5 nM E2 and BrdU. **=p<0.01, ***=p<0.001

Estrogen Stimulates SRC-dependent Expression of MYC.

Previous studies had suggested that *MYC* expression is required for E2-dependent proliferation. We wanted to test whether MYC was required for E2-dependent cell cycle progression in the MCF7 cells using shRNA-mediated knockdown of *MYC* mRNA. After achieving an efficient knockdown of *MYC* in the stable cells as analyzed by immunoblot (Figure 2.3), we used these cells and performed BrdU incorporation and proliferation assays. E2 was unable to stimulate the shMYC cells to enter S-phase compared to the control cells (Figure 2.3). As we had implicated a SFK activity for BrdU incorporation, we also wanted to teste whether SFK activity was required for cell cycle progression in shMYC cells. While SU11333 was able to block BrdU

incorporation in shCtrl cells relative to E2-stimulated cells, we did not observe a difference between the E2 stimulated and SU11333-treated shMYC cells (Figure 2.3). This suggested a potential role for SFKs upstream of MYC during G1/S transition. Down-regulation of *MYC* also led to a decrease in proliferation over several days (Figure 2.3).

As both the ER and SRC have been implicated in regulating expression of *MYC* mRNA, we wanted to test whether E2 was able to stimulate *MYC* mRNA expression and if so was SRC required. Indeed, E2 was able to stimulate *MYC* mRNA levels in a concentration dependent manner as well as over time after stimulation with 5 nM E2 relative to the cells treated with vehicle (Figure 2.4). To test whether SRC was required for this increase, we again used either SU11333 or shSRC knockdown cells. *MYC* mRNA levels

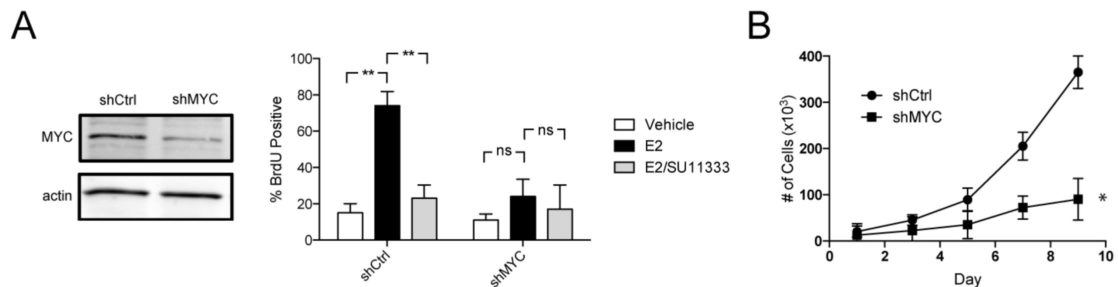

Figure 2.3- MYC is required for E2-dependent proliferation. (A) Representative immunoblot of shCtrl of shMYC MCF7 cells. BrdU incorporation assay of quiescent shCtrl or shMYC stable knockdown MCF7 lines pretreated with 1 μM SU11333 and treated with vehicle or 5 nM E2 and BrdU (B) proliferation assay of quiescent shCtrl or shMYC stable knockdown MCF7 lines treated with vehicle or 5 nM E2. *=p<0.05, **=p<0.01.

were not induced upon any concentration of E2 stimulation or at any time after E2 stimulation in either the inhibitor-treated or the shSRC cells (Figure 2.4). We also observed that SU11333 could block MYC mRNA accumulation in ZR-75-1 cells (Figure 2.4). These data suggest that SRC activity is required for *MYC* mRNA accumulation after E2 stimulation.

SRC Mediates E2-dependent Expression of* MYC *mRNA by Stabilizing Its mRNA Levels.

We next aimed to investigate the possible mechanism of *MYC* mRNA accumulation after E2-stimulation. As E2 has previously been reported to transcriptionally activate *MYC* mRNA (Dubik and Shiu, 1988), we first performed a nuclear run-on assay to test whether *MYC* mRNA transcription was induced in our system (Figure 2.5). We stimulated quiescent cells with E2 and then paused transcription on ice at various time points. We isolated intact nuclei from each time point for analysis. We next added labeled 5-ethynyl Uridine (EU), and placed samples at 37C to allow for elongation of initiated transcripts and incorporation of the EU. The EU was then biotinylated and collected using streptavidin beads to collect only actively transcribed mRNAs. RNA was then isolated, cDNA was generated, and qPCR analysis was performed. The housekeeping gene, *PPIA*, was analyzed as a negative control as E2 should not stimulate its transcription (Figure 2.5). As a positive control, we measured the transcription of *PS2,* a known transcriptional target of the ER

(Brown et al., 1984; Kim et al., 2000), and observed an increase in *PS2* transcription after E2 stimulation (Figure 2.5). However, we did not detect an

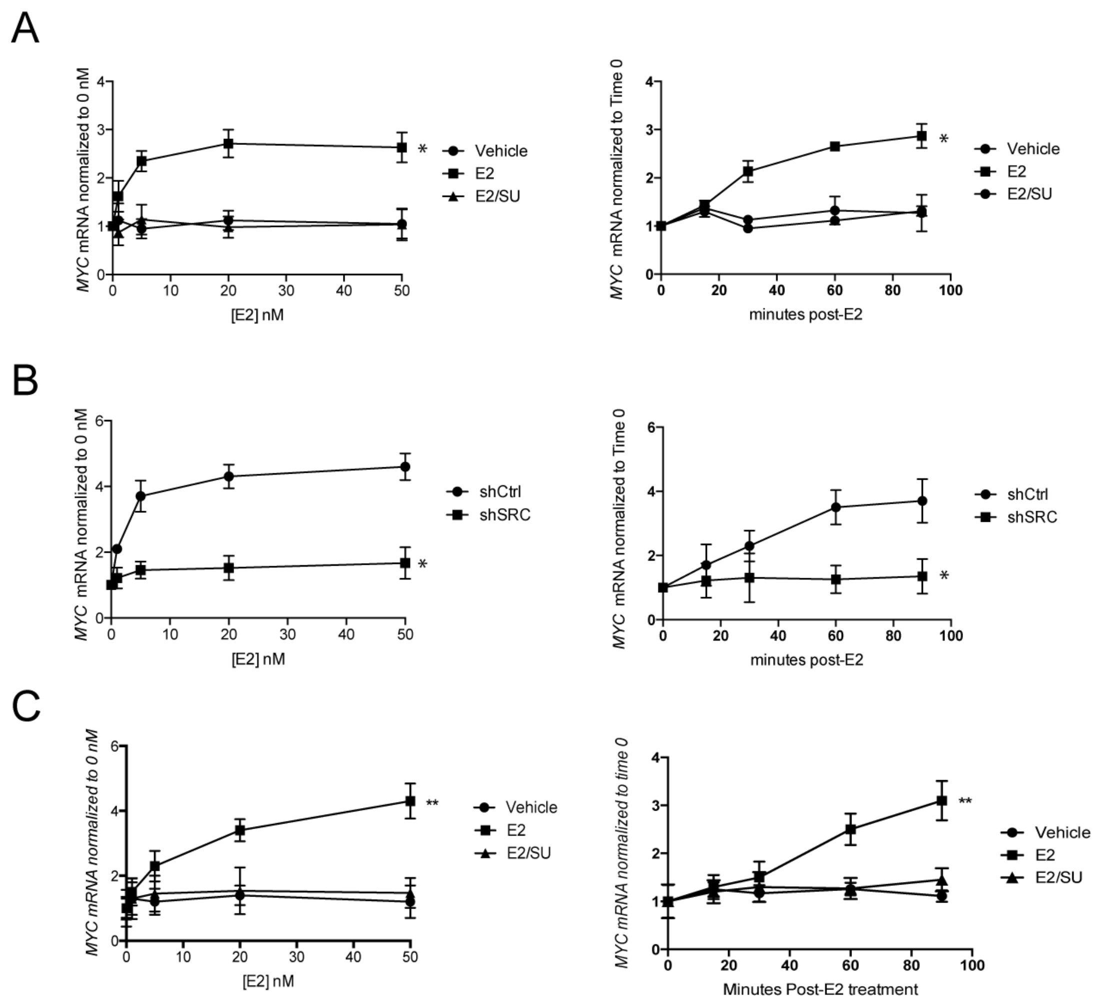

Figure 2.4- E2 induces *MYC* mRNA accumulation dependent on SRC. (A) Left: qPCR analysis of quiescent MCF7 cells that were pretreated with vehicle or 1 μM SU11333 and treated with increasing [E2] or Right: with 5 nM over 90 min. (B) Left: qPCR analysis of quiescent shCtrl or shSRC MCF7 lines that were pretreated with vehicle or 1 μM SU11333 and treated with increasing [E2] or Right: with 5 nM over 90 min. (C) Left: qPCR analysis of quiescent ZR-75-1 cells that were pretreated with vehicle or 1 μM SU11333 and treated with increasing [E2] or Right: with 5 nM over 90 min. *=$p<0.05$

increase in transcription of *MYC* mRNA after E2-stimulation (Figure 2.5). For each time point, we also had collected the cytoplasmic fractions of mRNA. Interestingly in the cytoplasm, we observed an increase in both *PS2* mRNA as well as *MYC* mRNA, but not in *PPIA* mRNA (Figure 2.5). These data taken together suggest that in our system, *MYC* mRNA is not being regulated via transcription of the gene, but rather post-transcriptionally.

As we observed an increase in cytoplasmic levels of *MYC* mRNA, but not in transcription, we next aimed to test whether *MYC* mRNA was being regulated by another mechanism. There is evidence that E2 may be regulating *MYC* mRNA post-transcriptionally by stabilizing the transcript (Santos et al., 1988). We tested this by stimulating cells for sixty minutes with 5 nM E2 to detect increases in *MYC* mRNA. We then treated the cells with Actinomycin D to block transcription and isolated RNA from the cells at various time points after Actinomycin D treatment. Compared to the basal decay of *MYC* mRNA as observed in vehicle treated cells, we detected an increase in stabilization of *MYC* mRNA in E2-stimulated cells (Figure 2.5). This stabilization was not observed with *PS2* mRNA suggesting that upon E2-stimulation (Figure 2.5), these two genes are regulated by different mechanisms. We also observed similar results in the ZR-75-1 cells (Figure 2.5).

We previously published that SRC regulated *MYC* by stabilizing its mRNA in fibroblasts after PDGF stimulation (Bromann et al., 2005) so we

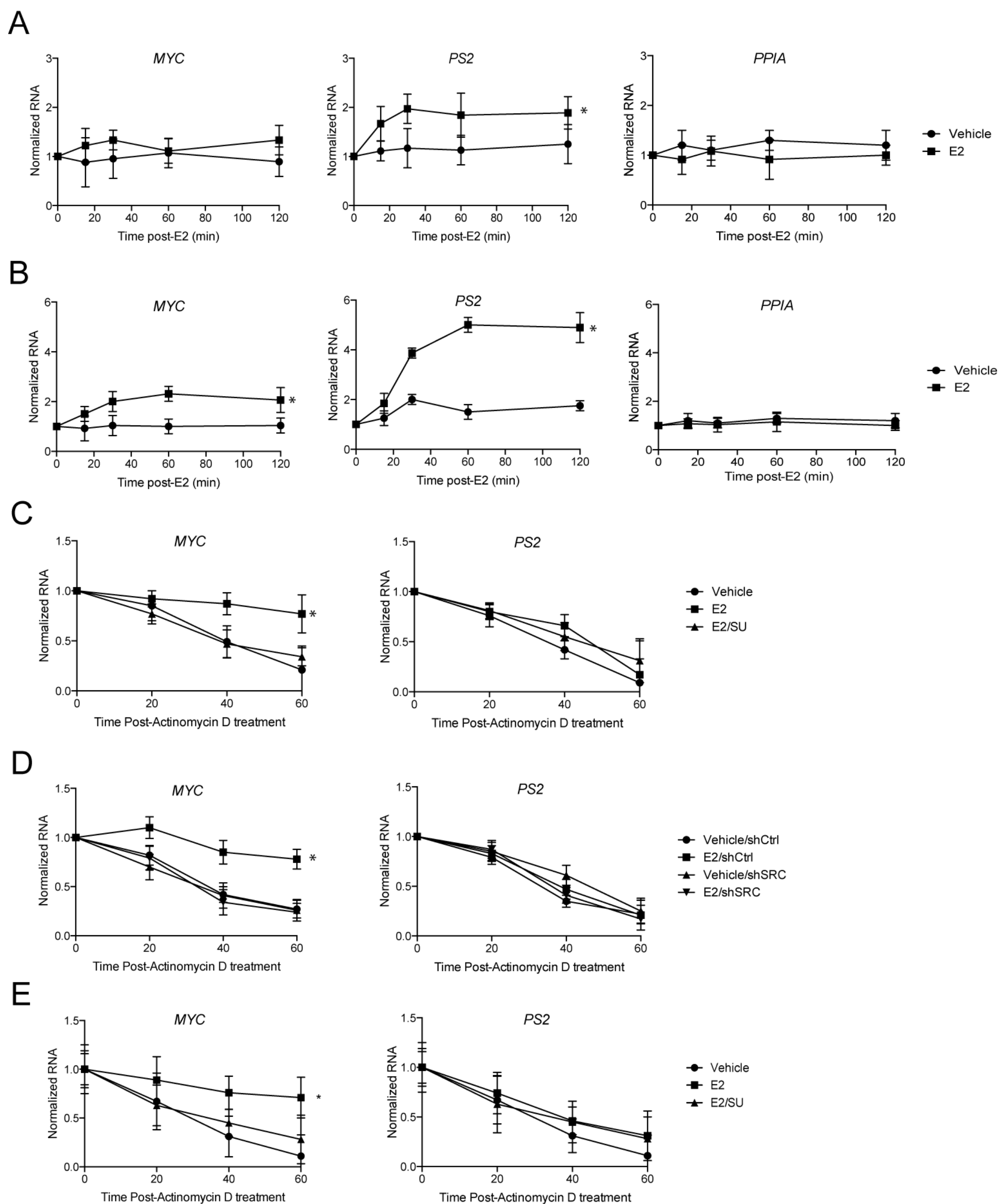

Figure 2.5: E2 stimulation induces *MYC* mRNA stabilization, not *MYC* transcription. (A) Nuclear run-on assay of MCF7 cells treated with E2 over a time course. (B) Cytoplasmic fractions from the same samples as the nuclear run on assay in (A). *MYC* mRNA stability assay in (C) MCF7 cells or (D) shCtrl and shSRC MCF7 cells or (E) ZR-75-1 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. After 60 min, cells were treated with 5 μM Actinomycin D to block transcription. *=p<0.05

hypothesized that E2 was able to stabilize *MYC* mRNA also dependent upon SRC. To test this hypothesis, we again used either SU11333 or shSRC knockdown cells and performed the mRNA stability assay. E2-stimulated *MYC* mRNA stability was abrogated upon inhibition of SFKs by SU11333 or shSRC knockdown (Figure 2.5). Also, SRC inhibition had no effect on *PS2* half-life. SFK inhibition also blocked the *MYC* mRNA stabilization after E2 stimulation in ZR-75-1 cells (Figure 2.5).

ΔN-IMP1, a Truncated Form of the RNA-binding Protein, IMP1, is Required for* MYC *mRNA Stability

To test the mechanism behind *MYC* mRNA stabilization, we first hypothesized that a candidate RNA-binding protein, IMP1, could be involved. IMP1, a developmentally expressed RNA-binding protein, has been shown to be re-expressed in cancer (Ioannidis, 2005; Kim et al., 2017; Köbel et al., 2007). IMP1 has also been demonstrated to stabilize *MYC* mRNA in a variety of cell types (Bell et al., 2013; Fakhraldeen et al., 2015; Kim et al., 2017; Weidensdorfer et al., 2009). We first performed qPCR analysis to test whether IMP1 was expressed at the RNA level in our ER-positive breast cancer lines as well as 293T cells known to express IMP1 (Figure 2.6). We did not detect any expression at the RNA level of IMP1 in our cell lines, although we did in the 293T cells (Figure 2.6). However, a recent report suggested that there was

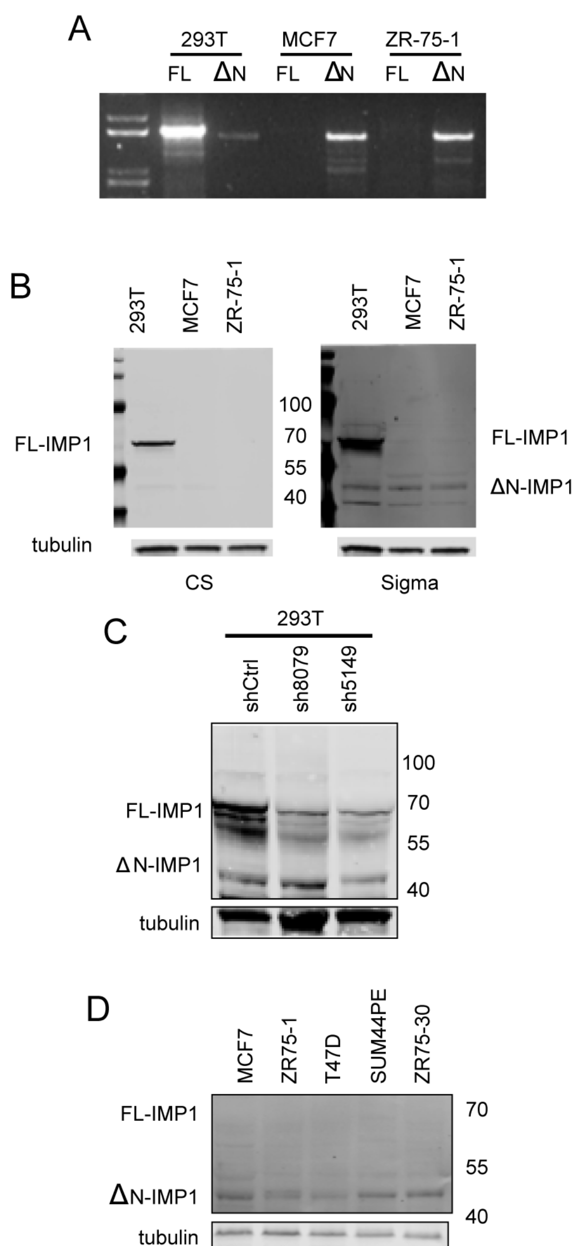

Figure 2.6- ΔN-IMP1 is expressed in ER-positive breast cancer cell lines. (A) RT-PCR analysis of RNA expression in 293T, MCF7 and ZR-75-1. (B) Representative immunoblot analysis of 293T, MCF7, and QR-75-1 cell lysates and probing for FL-IMP1 (CS) or FL-IMP1/ΔIMP1 (Sigma). (C) Validation of shRNA knockdown in 293T of FL-IMP1 (sh8079) or FL-IMP1/ΔIMP1 (sh5149). (D) Representative immunoblot analysis of ER-positive breast cancer cell lines for FL-IMP1/ΔIMP1 expression.

also truncated form of the protein, ΔN-IMP1, that was expressed in some cancer cell lines, including MCF7 (Fakhraldeen et al., 2015). We used primers specific for the truncated form, and indeed ΔN-IMP1 is expressed at the RNA level in both ER-positive cell lines that we tested, MCF7 and ZR-75-1, as well as at low levels in the 293T cells (Figure 2.6). We next analyzed protein lysates from 293T, MCF7, and ZR-75-1 by immunoblot and detecting IMP1 expression using two antibodies. The CS antibody detects an epitope only present in FL-IMP1, but the Sigma antibody can detect both FL-IMP1 as well as ΔN-IMP1. Similar to the qPCR results, we found that the MCF7 and ZR-75-1 cells expressed only the short form, while the 293T cells expressed primarily FL-IMP1, but also ΔN-IMP1 at low levels (Figure 2.4). We next wanted to analyze the expression of IMP1 and ΔN-IMP1 protein in several ER-positive breast cancer cell lines. We were able to detect ΔN-IMP1 in all of the cell lines that we tested while none expressed the full-length form (Figure 2.6). Because we detected multiple bands in some of the immunoblots, we generated stable knockdown in either 293T cells (Figure 2.6) or MCF7 cells (Figure 2.7) using shRNAs that targeting either FL-IMP1 only (sh8079) or both forms of IMP1 (sh5149). Immunoblot analysis demonstrated that indeed the constructs we used were able to specifically knockdown the FL-IMP1 or both forms of IMP1.

We hypothesized that either FL-IMP1 or ΔN-IMP1 could be required for *MYC* mRNA stabilization so we used the MCF7 shCtrl of sh5149 knockdown cells and compared them to scrambled control (shCtrl) cells in the RNA

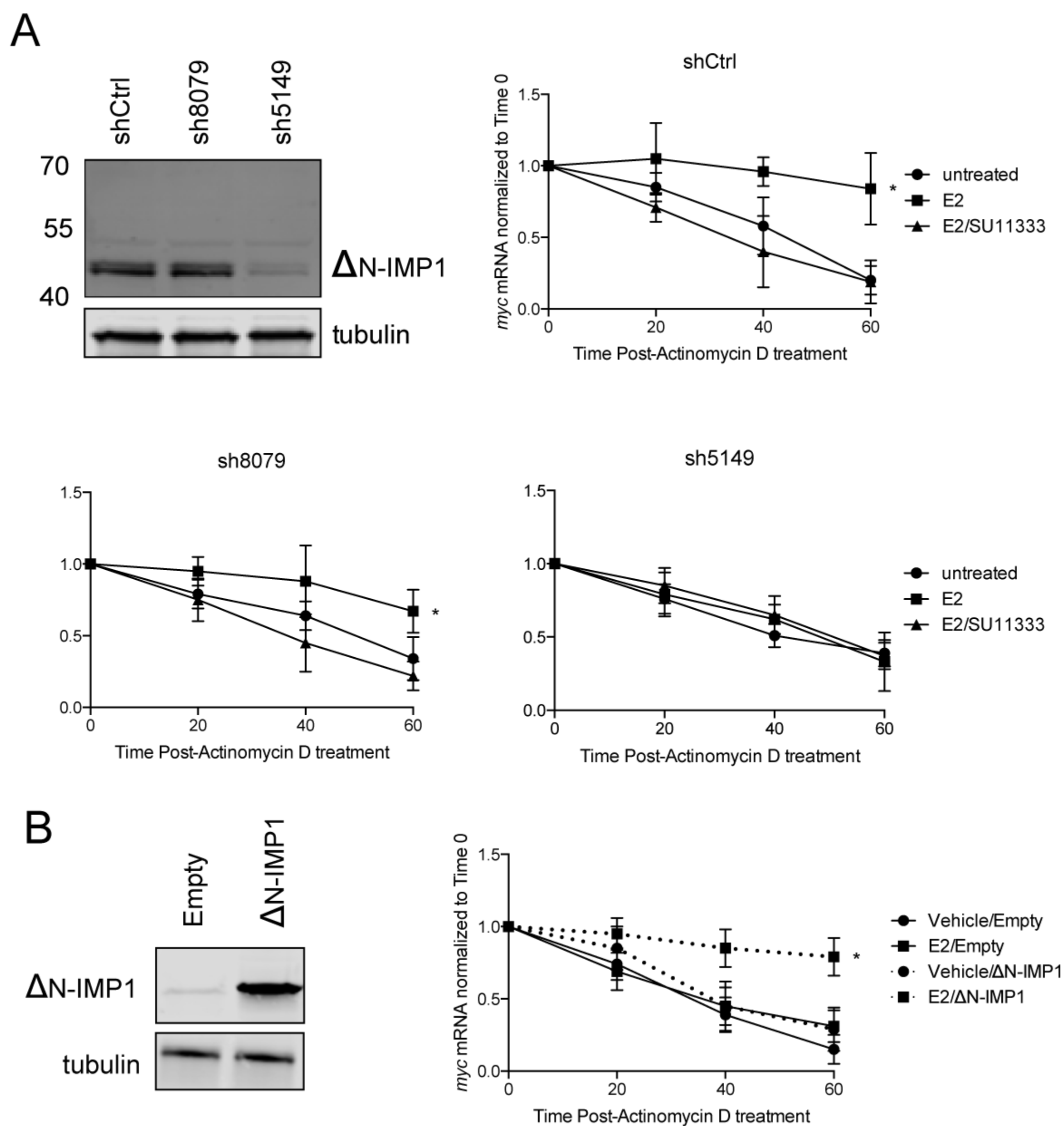

Figure 2.7: ΔN-IMP1 is required for *MYC* mRNA stabilization after E2 stimulation. (A) Representative immunoblot of shCtrl, sh8079 (FL-IMP1), or sh5149(FL-IMP1/ ΔIMP1) knockdown in MCF7 cells. *MYC* mRNA stability assay shCtrl, sh8079, and sh5149 MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. After 60 min, cells were treated with 5 μM Actinomycin D to block transcription. (B) (left) Representative immunoblot of sh5149 knockdown MCF7 cells also expressing either empty vector or ΔIMP1. (right) *MYC* mRNA stability assay empty vector or ΔIMP1 expressing sh5149 knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=$p<0.05$

stability assay and measured *MYC* mRNA half-life (Figure 2.7). The sh8079 cells behaved similar to shCtrl cells in this assay (Figure 2.7). However, sh5149 cells that targeted both forms of IMP1 were unable to stabilize *MYC* mRNA upon E2 stimulation (Figure 2.7). This suggested that one of the forms of IMP1 is required for *MYC* mRNA stabilization. We wanted to test whether ΔN-IMP1 was specifically required for *MYC* mRNA stability due to E2 stimulation. To test this, we "rescued" ΔN-IMP1 by expressing ΔN-IMP1 or an empty vector as a control in the sh5149 cells. We then performed the RNA stability assay with the rescue cell line. ΔN-IMP1 expression was able to rescue the *MYC* mRNA stabilization whereas empty vector-expressing cells were not (Figure 2.7).

We wanted to determine whether the loss of IMP1 also affected cell cycle progression and proliferation. Using the sh8079 and sh5149 and comparing them to shCtrl cells, loss of only FL-IMP1 showed a similar phenotype as shCtrl cells, however, sh5149 cells were unable to enter S phase and also had a proliferative defect (Figure 2.8). These data, taken together with the *MYC* mRNA stability defect, suggest that *MYC* stability may be require for cell cycle progression upon E2-stimulation.

Phosphorylation of Tyrosine 260 of ΔN-IMP1 is Required for* MYC *mRNA Stability.

SRC has previously been shown to regulate FL-IMP1 function through phosphorylation of tyrosine 396 in the context of β-actin mRNA (Hüttelmaier et

al., 2005). Given that our data suggested that SRC kinase activity is required for *MYC* mRNA stability and that ΔN-IMP1 was necessary for stabilization, we hypothesized that phosphorylation of this residue was required for ΔN-IMP1 to

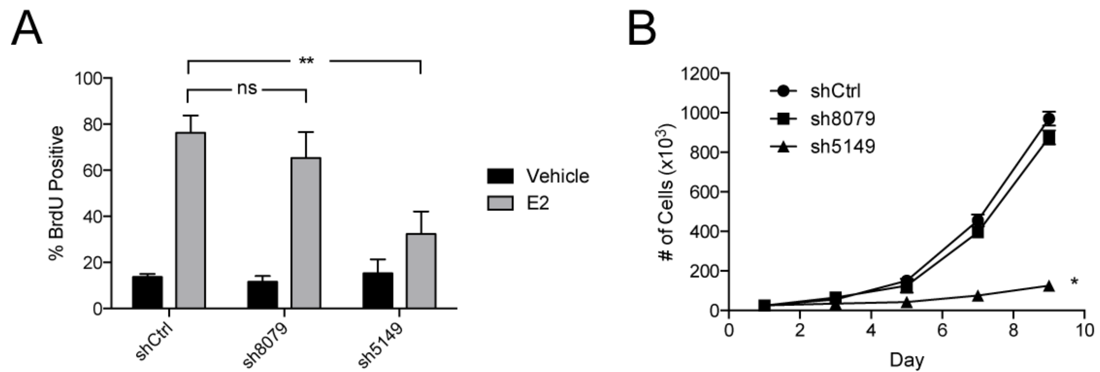

Figure 2.8: Loss of FL-IMP1 and ΔIMP1 results in a proliferation defect. (A) BrdU incorporation assay and (B) proliferation assay in shCtrl, sh8079, sh5149 knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=p<0.05

stabilize *MYC* mRNA. To test this hypothesis, we generated a ΔN-IMP1 mutant with tyrosine 260 (corresponding to the Y396 residue in FL-IMP1) mutated to phenylalanine to mimic a non-phosphorylated residue (Figure 2.9). We then expressed either wild-type ΔN-IMP1 or the Y260 mutant in the sh5149 knockdown cells. Introduction of a wild-type version of ΔN-IMP1 resulted in an increased *MYC* mRNA half-life compared to the empty vector (Figure 2.9). Expression of the ΔN-IMP1-Y260F mutant was unable to rescue stabilization of *MYC* compared to the wild-type ΔN-IMP1, suggesting that phosphorylation of the tyrosine residue at 260 is required for increased *MYC* mRNA stability (Figure 2.9).

The sh5149 knockdown cells had previously shown a defect in cell cycle progression and proliferation, so we wanted to test what effect the Y260F mutant had on these phenotypes. Using the BrdU incorporation assay, our sh5149 knockdown cells were unable to progress in the cell cycle compared to shCtrl cells (Figure 2.9). If we expressed wild-type ΔN-IMP1 in the sh5149 knockdown cells, these cells could rescue the cell cycle and proliferation defects.

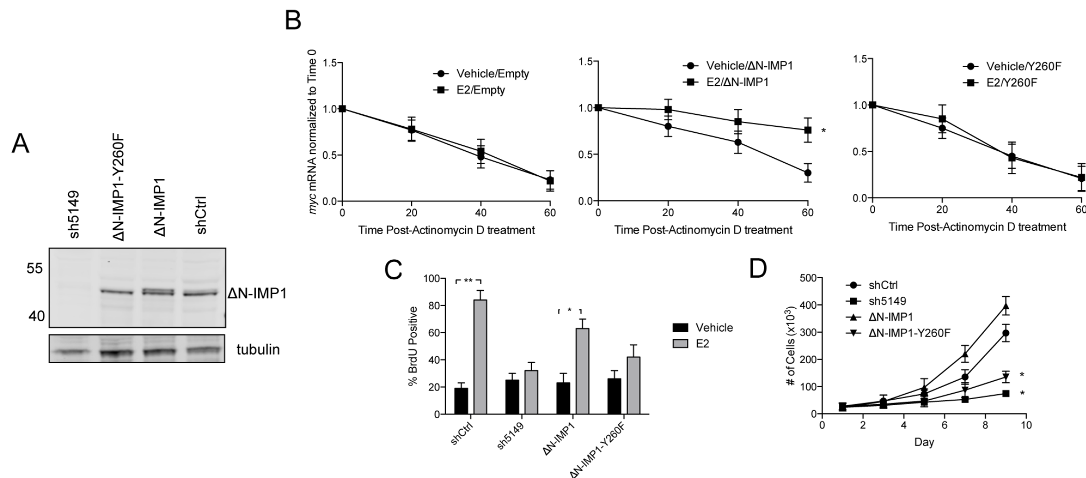

Figure 2.9: Y260F mutant of ΔIMP1 is unable to rescue *MYC* mRNA stabilization in sh5149 knockdown cells. (A) Representative immunoblot of shCtrl, sh5149, ΔIMP1/sh5149, or ΔIMP1-Y260F/sh5149 MCF7 cells. (B) *MYC* mRNA stability assay in shCtrl, sh5149, ΔIMP1, and ΔIMP1-Y260F MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. After 60 min, cells were treated with 5 μM Actinomycin D to block transcription. (C) BrdU incorporation assay and (D) proliferation assay in shCtrl, sh5149, ΔIMP1, and ΔIMP1-Y260F MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=$p<0.05$

However, in the cells where we expressed ΔN-IMP1-Y260F, we were unable to observe cells entering S phase or proliferating compared the wild-type ΔN-IMP1 rescue cells, and they more resembled the sh5149 knockdown cells

(Figure 2.9). These data suggest that the phosphorylation of Y260 is required for MYC mRNA stability, and ultimately, cell cycle progression.

Discussion

The genomic effects after E2 stimulation have been widely studied, however, the non-genomic pathway is more poorly understood. The non-genomic effects are thought to be acting via cytoplasmic signaling pathways potentially involving SRC, MAPK, PKC, and PI3K (Castoria et al., 2001; Lobenhofer et al., 2000). Our lab's focus on SRC kinase has led us to explore the role of SRC kinase during E2-dependent signaling and proliferation in an ER-positive breast cancer model. We hypothesized that SRC was required for E2-dependent proliferation and aimed to further understand its potential role in non-genomic E2 signaling.

We found that SFKs were required for E2-dependent proliferation by using a small-molecule inhibitor of SFKs, SU11333. The MCF7 cells that we used expressed two SFKs, SRC and YES. The literature has suggested that SFKs have redundant roles based on data from SFK knockout mice (Lowell and Soriano, 1996). While *src*$^{-/-}$ mice only displayed a mild osteoclast phenotype, mice in which two or three ubiquitously expressed SFKs (SRC, YES, and FYN) are disrupted display severe phenotypes including embryonic lethality (Klinghoffer et al., 1999; Stein et al., 1994). Using shRNA-mediated SRC or YES knockdown cells, we found that SRC is required for E2-dependent mitogenesis, whereas YES is not required. This is consistent with

data from the MMTV/PyVmT breast cancer mouse models in which full knockout (Guy et al., 1994) or mammary epithelial-specific knockout (Marcotte et al., 2012) of SRC have defects in mammary gland development, E2-dependent cell cycle progression, and development of mammary tumors. Our findings are also consistent with data from the MMTV/PyVmT mice in *yes*$^{-/-}$ backgrounds that do not show any impairment of tumor formation as compared to wild-type mice (Guy et al., 1994). These data taken together suggest non-overlapping roles for SRC and YES in E2-dependent proliferation.

Our data suggest that SRC's role in E2-dependent proliferation is to stabilize *MYC* mRNA levels. This is consistent with other reports suggesting that *MYC* mRNA levels are regulated post-transcriptionally due to E2 or growth factor stimulation (Blanchard et al., 1985; Bromann et al., 2005; Dean et al., 1986; Greenberg and Ziff, 1984; Kelly and Siebenlist, 1986; Kindy and Sonenshein, 1986; Nepveu et al., 1987; Santos et al., 1988). However, this conflicts with several studies that suggest that E2 stimulation induces transcription of *MYC* mRNA (Dubik et al., 1987; Dubik and Shiu, 1988; Shiu et al., 1993; Wang et al., 2011). There are a few explanations that could explain these conflicts. First, the E2 concentrations used for many experiments vary in some cases up to 50-fold from the doses that were used in this study (Dubik et al., 1987; Dubik and Shiu, 1988; Shiu et al., 1993), and increased concentrations of E2 may result in more pharmacological signaling than

physiological signaling. Another possibility arises from the frequent use of MCF7 cell line as a model for E2 signaling. Some publications have described variations in E2 response as well as expression profiles of different sources of MCF7 cells (Nugoli et al., 2003; Osborne et al., 1987). To support our findings for E2-dependent stabilization of *MYC* mRNA, we complemented the analyses in the MCF7 cells with analyses in the ZR-75-1 cells.

We identified ΔN-IMP1 to be expressed in several ER-positive breast cancer cell lines and necessary for *MYC* mRNA stability due to E2 stimulation. We found that ΔN-IMP1 is needed *for* MYC mRNA stability, but the mechanism behind how it is regulating mRNA stability is unknown. Preliminary experiments to immunoprecipitate (IP) ΔN-IMP1 were unsuccessful as the antibody that we used to detect it could not IP the protein (data not shown). This technical limitation has currently prevented us from testing whether SRC directly phosphorylates ΔN-IMP1 as well as performing an RNA-IP to test whether ΔN-IMP1 can interact with *MYC* mRNA.

Little is known about the function of the truncated form since it was first identified (Fakhraldeen et al., 2015), and hints about its function are derived from studies involving the function of full length IMP1. FL-IMP1 contains six RNA binding domains including two RNA recognition motifs and four KH domains (Bell et al., 2013) while ΔN-IMP1 only retains the four KH domains (Fakhraldeen et al., 2015). ΔN-IMP1 also retains the tyrosine residue described to be phosphorylated by SRC and required for release of β*ACTIN*

mRNA (Hüttelmaier et al., 2005). Our data suggests that this residue in ΔN-IMP1 is important for *MYC* mRNA stability. The discrepancy between how SRC regulates mRNA binding to either IMP1 or ΔN-IMP1 may be an intrinsic feature of the target mRNA or differences between the isoforms. Supporting this hypothesis are several studies examining which KH domains are required for specific target mRNA binding. *MYC* mRNA has been found to likely require the first and second KH domains of IMP1 for binding (Barnes et al., 2015), β*ACTIN* mRNA has been suggested to require the third and fourth KH domains for binding (Farina et al., 2003; Nicastro et al., 2017; Patel et al., 2012) and the *CD44* mRNA requires the fourth KH domain for IMP1 binding (Barnes et al., 2015). These data suggest that specific characteristics of the mRNA, possibly the sequence, are important for the function of IMP. IMP1 has been described to regulate a large cassette of target genes (i.e. *MYC,* β*ACTIN, CD44, IGF-II, MITF*) via numerous functions (mRNA stability, mRNA localization, translational repression) (Bell et al., 2013). However, the overlap between target genes and function of full-length IMP1 and ΔN-IMP1 is completely unknown. Additionally, because many of the previous studies have looked at only the full-length form of IMP1, and many of the reagents used for studies can recognize both full-length and truncated IMP1, some of the published results may have results that are confounded by this isoform. While this study has suggested a potential role for ΔN-IMP1, many open research questions remain. Interestingly, we observed ΔN-IMP1 to be expressed in ER-

positive breast cancer cell lines. ER-positive breast cancer cell lines may serve as a good model system for future studies.

In our orthotopic ER-positive breast cancer xenograft model, we found that inhibition of SFKs using saracatinib, a tyrosine kinase inhibitor that is somewhat selective for SFKs and ABL kinase, inhibited growth of tumors in mice. These data suggest that treating ER-positive breast cancer tumors with SRC inhibitors may be an effective strategy. Indeed, several studies have tested this in preclinical and clinical trials. A Phase II clinical trial using the kinase inhibitor, dasatinib (an SFK inhibitor with some selectivity also for KIT, PDGFR, and ABL), found an objective response rate (ORR) of 4% and a disease control rate (DCR) of 16% in hormone receptor (HR)-positive breast cancer patients suggesting limited benefits for patients (Mayer et al., 2011). Another Phase I/II study using dasatinib in combination with zoledronic acid for treatment of breast cancer bone metastasis found that 23% of patients showed partial response (PR) in bone metastases, and these patients all had HR-positive breast cancer (Mitri et al., 2016). Treatment of MMTV/PyVmT mice with bosutinib, a SRC/ABL kinase inhibitor, showed decreased tumor development and inhibition of growth of established tumors (Hebbard et al., 2011). In a clinical trial using bosutinib in patients with locally advanced metastasis or metastatic breast cancer, progression free survival (PFS) at 16 weeks was highest in the HR-positive patients (44 patients, 43%) and 4 HR-positive patients had confirmed PR (Campone et al., 2012).

Increased expression of SRC in ER-positive tumors (Elsberger, 2014; Ottenhoff-Kalff et al., 1992; Verbeek et al., 1996) taken with our data suggesting that both the E2 stimulus and SRC activity are required for proliferation, suggest potential benefits in combined ER and SRC inhibition. Several studies have studied this combination. Combined use of tamoxifen and saracatinib has been shown to synergistically inhibit anchorage independent growth of MCF7 cells (Herynk et al., 2006). SRC has also been suggested to be involved as a mechanism for anti-estrogen resistance. MCF7 cells that were generated to be resistant to tamoxifen showed increased activated SRC levels (Hiscox et al., 2007). In a follow-up to this study, two ER-positive cell lines were treated individually with either tamoxifen or saracatinib and in both cases, the cells acquired resistance to the inhibitors (Hiscox et al., 2009). However, cells that were treated with combined tamoxifen and saracatinib treatment were all dead after 13 weeks and unable to acquire resistance to the inhibitors (Hiscox et al., 2009). Another study combined the aromatase inhibitor, anastrozole, with saracatinib to treat ER-positive xenografts of MCF7 cells engineered to express aromatase (Chen et al., 2009). Saracatinib initially inhibited tumor growth, but tumors acquired resistance during the study. The combined saracatinib and anastrozole treatment was more effective than either inhibitor alone (Chen et al., 2009). Interestingly, the saracatinib-resistant tumors showed upregulation of MAPK and PI3K, two other kinases implicated in the non-genomic signaling pathway.

These data suggest that the non-genomic E2 signaling pathway play a role in resistance to anti-estrogen treatment. Further research into the effects of combined therapies in ER-positive breast cancer models is needed.

Methods

Cell Culture

MCF7 cells (ATCC) were grown in IMEM supplemented with 10% FBS. ZR-75-1 (ATCC), T47D (ATCC), ZR-75-30, and SUM44PE (gift from Joe Gray) cells were grown in RPMI supplemented with 10% FBS. 293T cells were grown in DMEM supplemented with 10% FBS. For starvation prior to estrogen stimulation in the assays described below, cells were washed 1X in PBS and starved for 48 hours in starvation medium [phenol red-free IMEM, 0.5% charcoal, dextran stripped FBS (Hyclone)]. For pre-treatment with inhibitors, inhibitors were diluted into starvation medium and incubated for 2 hours prior to stimulation. All experiments were performed in the absence of antibiotics.

Plasmids

RNAi knockdown was performed using the pLKO.1 or pLKO.1-TetOn shRNA expression vectors. The RNAi consortium clone numbers for each of the shRNA constructs are: SRC (TRCN0000195339), YES (TRCN0000001609), IMP1 (TRCN0000075149), IMP1 (TRCN0000218079). Overexpression was performed by expressing the following cDNAs in the pCDH lentiviral vector: ΔN-IMP1.

Chemicals

ICI182780 (Tocris Biosciences), Actinomycin D (Sigma-Aldrich), saracatinib (APExBio), SU11333 (Sanford|Burnham|Prebys Medical Research Institute), were used in pre-treatment of cells during starvation as described above.

Bromodeoxyuridine (BrdU) Incorporation Assay

Cells were plated at 60% confluency on glass coverslips in normal growth medium and allowed to grow overnight. The next morning cells were washed once with PBS and then placed in starvation medium. Cells were pretreated for 2 hours with 1 µM SU11333 or DMSO vehicle prior to stimulation. Cells were then treated with either ethanol or 5 nM estrogen (E2) with 5 µM BrdU for 18 hours. After treatment, cells were washed twice with cold PBS and fixed with ice-cold 1:1 methanol:acetone. Coverslips were then rehydrated in PBS for 15 minutes. Cells were permeabilized in 0.1% TX-100 in PBS for 10 minutes. Cells were then treated with 2N hydrochloric acid at 37C for 15 minutes. Coverslips were then washed 3x in PBS with rocking. Coverslips were blocked with 1% goat serum in PBS for 1 hour with rocking. Anti-BrdU (Millipore) antibody was used to detect incorporated BrdU (Millipore). Anti-mouse 488 secondary was used to visualize staining via immunofluorescence analysis. Vectashield containing DAPI was used to mount the coverslips and stain nuclei.

Proliferation Assays

Cells were plated at 25 x 10^3 and viable cells were counted every 2 days using the Countess II Automated Cell Counter (Thermo Fisher Scientific).

Reagents and Antibodies for Immunoblotting

The following commercial antibodies were used: anti-SRC [pY418] antibody (Invitrogen), anti-SRC (327) antibody (Abcam), anti-YES, anti-MYC (Y69) antibody (Abcam), anti-IMP1 antibody (Cell Signaling Technology), anti-IGF2BP1 antibody (Sigma-Aldrich), anti-γ–tubulin antibody (Sigma-Aldrich), anti-actin (C-74) antibody (Sigma), and anti-BrdU (Millipore). For secondary antibodies, Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen) or IR800 (Rockland Immunochemicals) were used for immunoblotting.

Immunoblotting

Cell lysates were prepared by washing cells twice with cold Tris-buffered saline (TBS) containing 100 μM Na_3VO_4 and then lysed in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% Triton X-100, 50 mM NaF, 100 μM Na3VO4 and 1mM EDTA lysis buffer containing a dissolved complete Mini protease inhibitor tab (Roche Diagnostics). Supernatant of cell lysates was assayed for total protein content using the BCA protein assay (Thermo Fisher Scientific), and 50 μg of total protein per sample was separated in a polyacrylamide gel (Invitrogen). Membranes were scanned using an infrared imaging system (Odyssey; LI-COR Biosciences).

RNA Isolation and cDNA generation

Trizol extraction of RNA was performed per manufacturer's instructions. cDNA was generated using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

MYC mRNA Accumulation Assay

Cells were starved and stimulated as described above. Post-E2 stimulation, RNA was isolated at the indicated times, and cDNA was generated. To quantify the RNA accumulation, qPCR analysis was performed. For each sample, *MYC* mRNA was normalized independently to two different housekeeping genes (*PPIA or RPLP0*). Primers for analysis were used as previously described (Weidensdorfer et al., 2009). Time points for each condition were all normalized to time 0 for each condition. Representative experiments are shown.

Transcriptional Run-on Assay

To label transcripts, the Click-iT Nascent RNA Capture Kit (Invitrogen) was used and adapted for this assay. Cells were starved and stimulated as described above. Transcription was then paused by placing samples on ice at various time points post-stimulation. Intact nuclei were isolated at each time point for analysis. Labelled 5-ethyl Uridine (EU) were added; and samples were incubated at 37C to allow for elongation of initiated transcripts. EU will only be incorporated into these initiated transcripts. The EU is then biotinylated

and then collected using streptavidin beads. RNA is isolated off the beads and cDNA generated for qPCR analysis.

RNA Stability Assay

Cells were starved and stimulated as described above. Sixty minutes post E2- stimulation, cells were treated with 5 μM Actinomycin D to block transcription. At the indicated time points, RNA was isolated and cDNA generated for each condition. For each sample, *MYC* mRNA was normalized independently to two different housekeeping genes (*PPIA or RPLP0*). These genes were selected so that Actinomycin D treatment did not alter their expression. Time points for each condition were all normalized to time 0 for each condition. *MYC* mRNA half-life was then calculated for each of the conditions. To determine significance, one -way ANOVA and Tukey's post-hoc tests were performed on the *MYC* mRNA half-life at the endpoint of the experiment to determine statistical significance.

Orthotopic Mouse Xenograft Experiments

Female, athymic nude mice were purchased from Jackson Laboratory. Mice were injected with a slow-release 17β-estradiol pellet (0.72 mg/pellet, 60-day release from Innovative Research of America) by using a trochar. One week later, mammary fat pad injections in the number 4 mammary gland were carried out without clearing of the fat pad. Briefly, cells were harvested by trypsinization and resuspended in PBS (Invitrogen). For each cell line, the

mice were injected in a non-cleared mammary fat pad with $1x10^6$ cells per animal in a volume of 100 µl [1:1 ratio with Matrigel (BD Biosciences)], and tumors were allowed to form with a diameter of up to 1 cm. Tumor onset was determined by physical palpation. Tumor growth was measured every 3-4 days using calipers; both the longest (L) and shortest (S) measurements were recorded. Using these values, tumor volumes were calculated as follows: $(L \times S^2) \times 0.5$, and expressed as mean volume ± SEM. Mice were sacrificed when the tumors reached a diameter of 1 cm, according to the Animal Care and Use Policy of OHSU. These experiments were repeated at least 3 times, using 5–10 mice per tumor group. One-way ANOVA, student's t test, or Tukey's post-hoc tests were performed on the tumor volume at the endpoint of the experiment to determine statistical significance as appropriate.

For inhibitor studies, once mice had measurable tumors of 50 mm^3, treatment with 50 µl vehicle, ICI182870 (10 mg/Kg), or saracatinib (25 or 50 mg/Kg) was injected intraperitoneally and tumor volume measured every three days.

Statistical Analyses

Statistical significance was determined by calculating the p-value (P) using the paired Student's t test. $P<0.05$ was considered to be statistically significant. The numbers of samples (n) are indicated in each figure legend. For 2D, 3D, and tumor growth curves, area under the curve analysis was performed on the individual growth curves using the Area Under the Curve

(AUC) function in the GraphPad Prism software. Means and SEM were then calculated and Student's t test or Tukey's post-hoc test was used to determine significance.

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Chapter III:

SRC Relieves a p53-dependent Cell Cycle Block

Abstract

The estrogen receptor (ER)-positive breast cancer subtype frequently expresses wild-type p53 unlike most tumor types. We hypothesized that the protein tyrosine kinase SRC could be inhibiting p53 in ER-positive breast cancer cells in a non-genomic E2 signaling pathway. We demonstrated that SRC is not required for cell cycle progression or proliferation in cells lacking p53 *in vitro*. In an MCF7 orthotopic xenograft model expressing SRC shRNA cells, we demonstrated that SRC is critical for initiation of xenografts independent of p53 expression. In established tumors, SRC inhibition by RNAi caused tumor regression in control tumors, but only decreased growth in p53-knockdown tumors. However, in saracatinib treated xenografts, tumors responded regardless of p53 status. The p53 knockdown tumors, as well as *in vitro* cell lines showed a role for p53 in repressing expression of *MYC*. Upon knockdown of p53, we observed increased basal half-life of *MYC* without any changes in transcription. We also observed an increase in ∆N-IMP1 expression, which we previously showed to be required for stabilization of *MYC* mRNA. Lastly using RNAi, we implicated MDM2 as a downstream effector of SRC signaling to inhibit p53. Mutation of SRC target tyrosines on MDM2 caused decreased proliferation. These data suggest a pathway where SRC inhibits p53 via MDM2, and p53 represses *MYC* mRNA leading to a cell cycle block.

Introduction

Loss of p53 function is one of the most prevalent alterations in cancer. It has been suggested through its loss-of-function and gain-of-function effects, that it can be contributing for the majority, if not all, of Hanahan and Weinberg's Hallmarks of Cancer (Hanahan and Weinberg, 2011). However, in some cancer types, as in estrogen receptor-positive (ER+) breast cancer (BC), wild-type p53 is often retained (Caleffi et al., 1994; Dumay et al., 2013). Because this loss of function seems to be a critical event in cancer development, it is hypothesized that cancer cells that express wild-type p53 have some other mechanism of inhibiting p53 function. In some cases, increased expression or activating mutations of the p53 inhibitor, MDM2, can also result in loss of p53 function.

In addition to retaining wild-type p53, ER-positive breast cancer also expresses the protein tyrosine kinase, SRC, at high levels (Elsberger, 2014; Ottenhoff-Kalff et al., 1992; Verbeek et al., 1996). Several pieces of evidence hint at SRC playing a role in inhibiting p53 function. From our previous work, we have demonstrated that in fibroblasts and MEFs lacking functional p53, a requirement for SRC in cell cycle progression is abolished (Broome and Courtneidge, 2000). This suggests that SRC is potentially acting as an upstream inhibitor of p53 in response to growth factor stimulation. Despite these data, little research to our knowledge has been focused on the mechanism by which SRC could inhibit p53 activity.

The most widely studied p53 inhibitor is the E3-ubiquitin ligase, MDM2. MDM2 ubiquitinates p53 which leads to sequestration from the nucleus and ultimately proteasomal degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kruse and Gu, 2009; Kubbutat et al., 1997; Wu et al., 1993). MDM2 can also be phosphorylated by SRC and be converted into a NEDDylating enzyme that can inhibit p53 transcriptional activity (Batuello et al., 2015; Xirodimas et al., 2004). MDM2 is, itself, a p53 target gene, thus, negatively regulating its own expression (Wu et al., 1993). Another MDM family member, MDMX, has also been described as a p53 inhibitor functioning through direct binding to either p53 or MDM2 (Huang et al., 2011; Linares et al., 2003; Marine and Jochemsen, 2005; Waning et al., 2011). MDM2 and MDMX have been shown to be critical, non-redundant inhibitors of p53 evidenced by both MDM2 (Jones et al., 1995; Montes de Oca Luna et al., 1995) and MDMX (Parant et al., 2001) knockout mice presenting with embryonic lethality that can be rescued by concomitant loss of p53. While most function of MDM2 and MDMX has been studied during the DNA damage response, some evidence exists that they play roles in cell cycle inhibition (Gilkes et al., 2008). Both MDM2 and MDMX have been shown to be regulated by ABL kinase to regulate p53 although studies have shown both activation and inhibition of p53 downstream of ABL (Goldberg et al., 2002; Sionov et al., 2001; Sionov et al., 1999; Waning et al., 2011; Zuckerman et al., 2009). Interestingly, ABL has also been shown to be downstream of SRC and required to overcome a cell cycle block during

mitogenesis (Furstoss et al., 2002). Taken together these studies suggest a potential role for MDM2 and MDMX in mitogenesis.

Given that in fibroblasts, SRC may be inhibiting a p53 cell cycle block, and that *MYC* expression can overcome a SRC inhibition-mediated cell cycle block, it is interesting that a few studies suggest that p53 may be able to repress *MYC* mRNA expression. One study suggests that p53 is able to bind to the *MYC* promoter and represses *MYC* expression (Ho et al., 2005). Another study suggests that p53 may repress *MYC* via transcriptional regulation of *miR-145* which directly targets *MYC* mRNA (Sachdeva et al., 2009). Also in vascular smooth muscle cells, podosome formation is regulated via a SRC-p53-*miR-145* pathway providing additional evidence for SRC inhibition of p53 (Quintavalle et al., 2010).

ER-positive breast cancer provides a good model system for examining the role of SRC and p53 during cell cycle regulation. Insights from these studies are not only potentially applicable to therapeutic strategies, they are likely essential to understanding normal cell cycle regulation as well. Here, we examine the role of SRC overcoming a cell cycle block in ER-positive breast cancer.

Results

SRC is Required to Relieve a p53 Cell Cycle Block After E2 Stimulation.

We previously demonstrated that SRC was required for cell cycle progression, and we wanted to test whether this was mediated by relieving a

p53 cell cycle block similar to our published results in fibroblasts (Broome and Courtneidge, 2000). To test this using shRNA-mediated knockdown, we generated stable p53 (shTP53) and scrambled control (shCtrl) knockdown in MCF7 cells and then performed a BrdU incorporation assay after stimulating quiesced MCF7 cells with E2 (Figure 3.1). In shCtrl cells, after E2 stimulation, cells entered S phase and incorporated BrdU, and cell cycle entry was blocked in cells pre-treated with SU11333. However, in shTP53 knockdown cells, SU11333 was unable to block E2-dependent BrdU incorporation (Figure 3.1). Additionally, we performed a proliferation assay to assess the long-term effects of SFK inhibition in shTP53 cells (Figure 3.1). Here, the vehicle treated shCtrl and shTP53 cells grew at nearly the same rate. Pretreatment with SU11333, blocked proliferation in the shCtrl cells, however, in the shTP53 cells, SU11333 was unable to block proliferation. To complement the SFK inhibitor experiment, we also generated individual shSRC or shTP53 knockdown cells or double shSRC/shTP53 knockdown cells and assessed both BrdU incorporation and proliferation (Figure 3.1). Consistent with the inhibitor experiments, targeted knockdown of SRC was able to block S phase entry and proliferation, but was unable to block BrdU incorporation or proliferation in shTP53 cells. Taken together, the inhibitor and shRNA experiments suggested a role for SRC kinase activity to overcome a p53 block to S phase entry and proliferation due to E2.

Because we used shRNA that potentially could have off-target effects, we wanted to further test the specific role of p53 by introducing mouse p53 in our shTP53 cells to test whether this was able to restore the cell cycle block upon SU11333 treatment (Figure 3.2). Indeed, in the shTP53 cells, SU11333

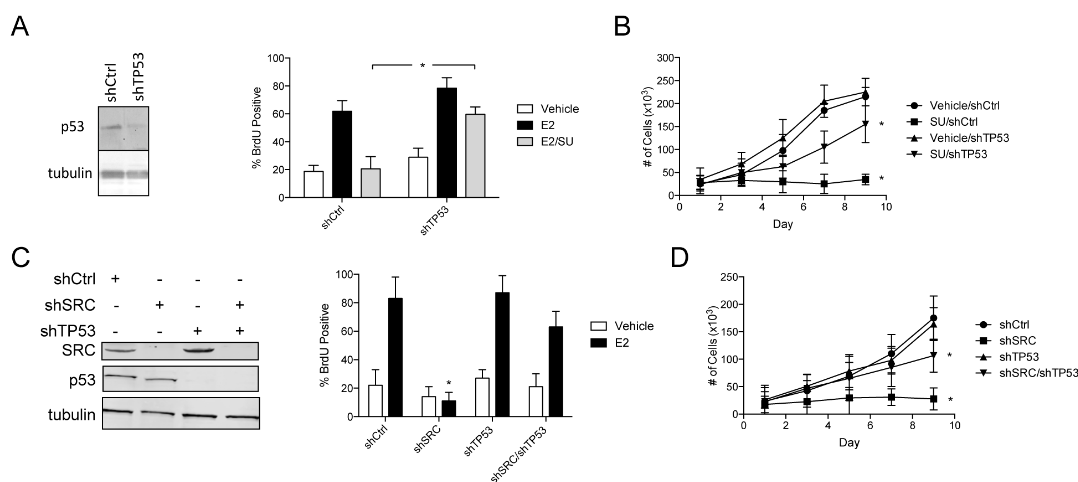

Figure 3.1-Knockdown of p53 abolishes a requirement for SRC in E2-dependent proliferation. (A) (left) Representative immunoblot of shCtrl and shp53 cell lysates. (right) BrdU incorporation assay and (B) proliferation assay in shCtrl and shp53 knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. (C) Representative immunoblot analysis of shCtrl, shSRC, shTP53, and shSRC/shTP53 double knockdown MCF7 cells. BrdU incorporation assay and (D) proliferation assay in shCtrl, shSRC, shTP53, and shSRC/shTP53 double knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=$p<0.05$

was unable to block cell cycle progression. However, in the cells that also expressed mouse p53, we were able to block cell cycle progression, and ultimately proliferation, with SU11333 (Figure 3.2). These experiments suggest that p53 is necessary and required for the cell cycle block due to SRC inhibition *in vitro*.

Saracatinib Inhibits Tumor Growth of MCF7 Xenografts Regardless of Expression of p53.

Our *in vitro* data suggested that SRC was required for E2-dependent cell cycle progression and proliferation in cells which expressed p53. As we previously demonstrated that inhibition of SRC activity with the SRC+ inhibitor, saracatinib, slowed or inhibited growth of MCF7 xenografts (Figure 2.1), we next wanted to test whether this also required p53 to be expressed in the tumor *in vivo*. Using nude mice, we implanted E2 pellets one week prior to injection of cells. For the cell preparation, we injected 100 μl containing a 1:1 mixture of Matrigel and PBS resuspension of 1 x 10^6 of either shCtrl or shTP53 cells. Upon reaching 50 mm^3, mice were randomized into either DMSO, ICI182870, or saracatinib treatment groups. We measured tumors every three days for several weeks. In the shCtrl cells, ICI182870-treated tumors regressed completely whereas saracatinib-treated mice showed a decrease in tumor growth relative to the DMSO treated tumors (Figure 3.3). Similar to the shCtrl tumors, the shTP53 tumors regressed upon treatment with ICI182870 (Figure 3.3) suggesting that E2 was required for tumor growth regardless of p53 expression in the tumor. Surprisingly, in the shTP53 cells

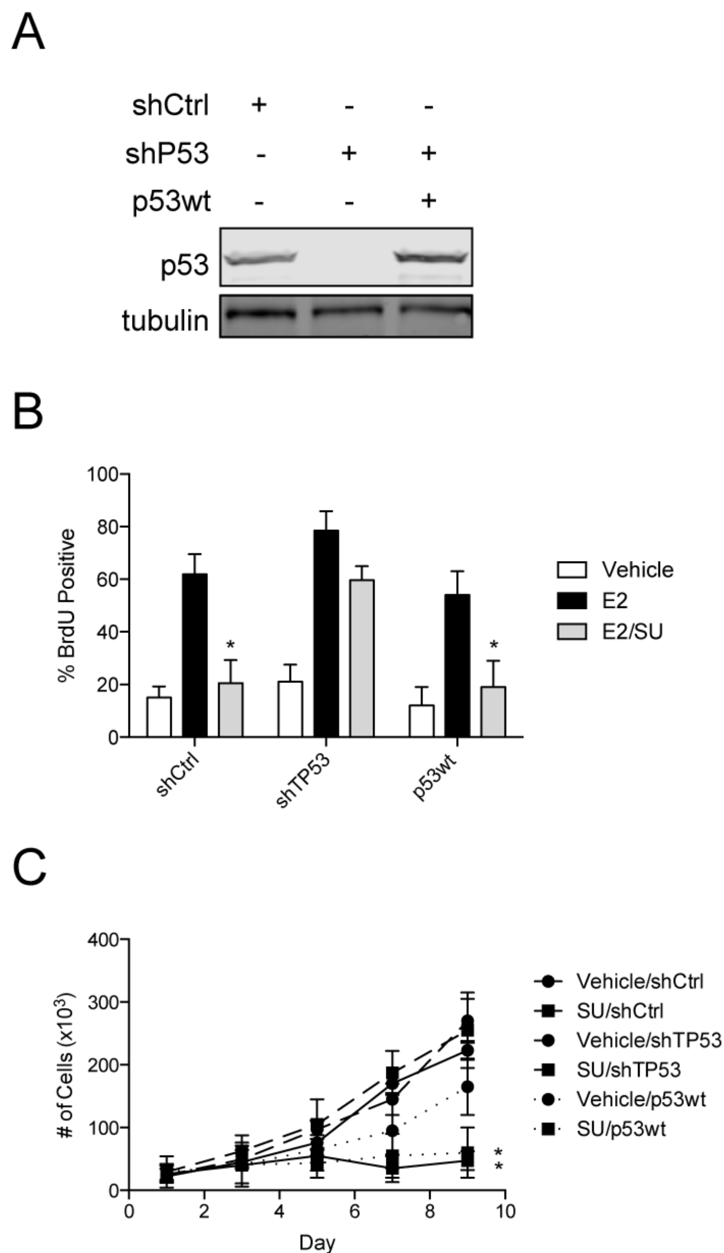

Figure 3.2: Re-expression of p53 in shTP53 knockdown cells restores a SFK-dependent cell cycle and proliferation block. (A) Representative immunoblot analysis of shCtrl, shTP53, and p53wt (rescue in shTP53) MCF7 cells. (B) BrdU incorporation assay and (C) proliferation assay in shCtrl, shTP53, and p53wt MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=p<0.05.

treated with saracatinib, we observed a decrease in tumor growth similar to what we observed in the shCtrl cells (Figure 3.3). We hypothesize that this discrepancy may be due to using a SRC inhibitor suited for *in vivo* use (saracatinib) versus using the more selective inhibitor, SU11333, as in our *in vitro* studies. Several other targets in addition to SRC of saracatinib may confound the effects we observed *in vivo* (Green et al., 2009). Because our *in vitro* inhibitor studies were complemented with shRNA-mediate knockdown experiments, we aimed to test whether we could use stable knockdown cells to test our hypothesis in the orthotopic xenograft model.

MCF7 Cells Require SRC Kinase Expression for Tumor Initiation in the Mouse Mammary Fat Pad.

Using the orthotopic MCF7 xenograft model in nude mice to test the requirement for SRC in shTP53 knockdown cells using shRNA-mediated knockdown. Rather than using inhibitors, we injected either shCtrl cells, individual shSRC cells, individual shTP53 cells, or double shSRC/shTP53 knockdown cells into the mouse mammary fat pad. We then measured tumors every three days. Compared to the shCtrl cells, the shTP53 cells grew at nearly the same rate generating large tumors during the course of the experiment (Figure 3.4). Mice injected with the shSRC cells did not develop tumors at any point during the experiment. Only 2/7 mice with the double shSRC/shTP53 knockdown tumors developed any tumors albeit very small around day 35 and only reach an average of 36 mm^3 (Figure 3.4). We also

performed immunoblot analysis on some of the tumors (none of the shSRC tumors) to test that the knockdown persisted in these tumors (Figure 3.4). From these data, we concluded that SRC is likely required for MCF7 grafting of the orthotopic tumors. This role for SRC in tumor grafting appears to be independent of p53 expression in the tumor cells. All of our *in vitro* studies were performed using quiescent cells that were stimulated with E2. We hypothesized that this pathway is important in cells entering the cell cycle from a quiescent state. We performed the same experiment with the shCtrl, shSRC, shTP53, and shSRC/shTP53 cells, however, this time before resuspending them in PBS for injection, we serum starved them for two days. We again measured tumors every 3 days. No tumors were found in any mice until day 14 (Figure 3.4). At this point, again the shCtrl and shTP53 cells grew at about the same rate for the duration of the experiment (Figure 3.4). Although there was a delay in tumor development, the rate of growth was similar to the previous experiment with non-quiesced cells Figure 3.4). Neither the shSRC or shSRC/shTP53 cells developed into tumors in any of the mice for the duration of the experiment (Figure 3.4).

SRC Kinase is Required for Tumor Growth in Established MCF7 Xenografts.

Because we were unable to observe tumors in the shSRC or double shSRC/shTP53 injected mice, we aimed to overcome the grafting defect of the xenografts by using a doxycycline-inducible shSRC construct. We generated

stable cell lines doxycycline (Dox)-inducible (Tet-on) TOshCtrl or TOshSRC and combined them with stable shCtrl or shTP53 constructs (TOshCtrl/shCtrl,

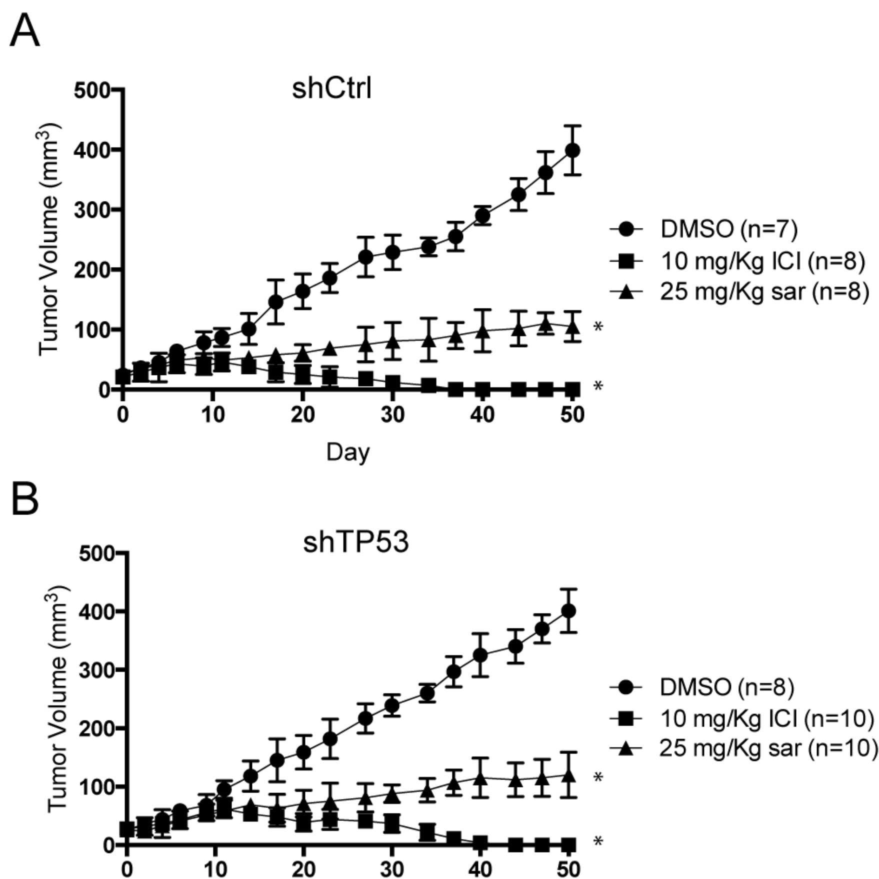

Figure 3.3- ICI182870 and saracatinib treatment inhibits growth of MCF7 orthotopic xenografts. Mice were injected with (A) shCtrl or (B) shTP53 knockdown MCF7 cells at Day 0. Once tumor reached 50 mm^3, intraperitoneal treatment with DMSO, ICI182870, or saracatinib was performed every three days and tumors measured at the same time. *=$p<0.05$

TOshSRC/shCtrl, TOshCtrl/shTP53, or TOshSRC/shTP53). To test the inducible knockdown in the cell lines, we first performed an *in vitro* time course by treating the cells with Dox. At 24 hours after treatment, we could still observe some expression of SRC, however, by 48 hours and persisting at 72

hours, we observed efficient knock down of SRC (Figure 3.5). After confirming the inducible knockdown of SRC, we implanted mice with E2 pellets and after a week injected the mice with the various combinations of inducible and stable shRNA expressing cell lines. We allowed the cells to grow until they reached 100 mm^3 and then randomized the mice into (-) Dox or (+) Dox chow. As a control for the inducible cell lines, we did not treat some mice with Dox (Figure 3.5). All groups of mice in the (-) Dox group grew at the same rate suggesting that the inducible shRNA vectors had no basal “leaky” expression. The remainder of the mice were treated with Dox. Similar to what we observed in our study with constitutive double knockdown experiments, the TOshCtrl/shCtrl and the TOshCtrl/shTP53 cells grew at nearly the same rate (Figure 3.5). Upon doxycycline treatment TOshSRC/shCtrl cells began to regress until no measurable tumors were present (Figure 3.5). Interestingly, the TOshSRC/shTP53 tumors did not grow, but also did not regress as did the TOshSRC/shCtrl cells (Figure 3.5). In this inducible knockdown xenograft experiment, we observed that with loss of SRC in tumors that expressed p53 lead to regression, but in tumors without p53, the tumors only have a growth defect.

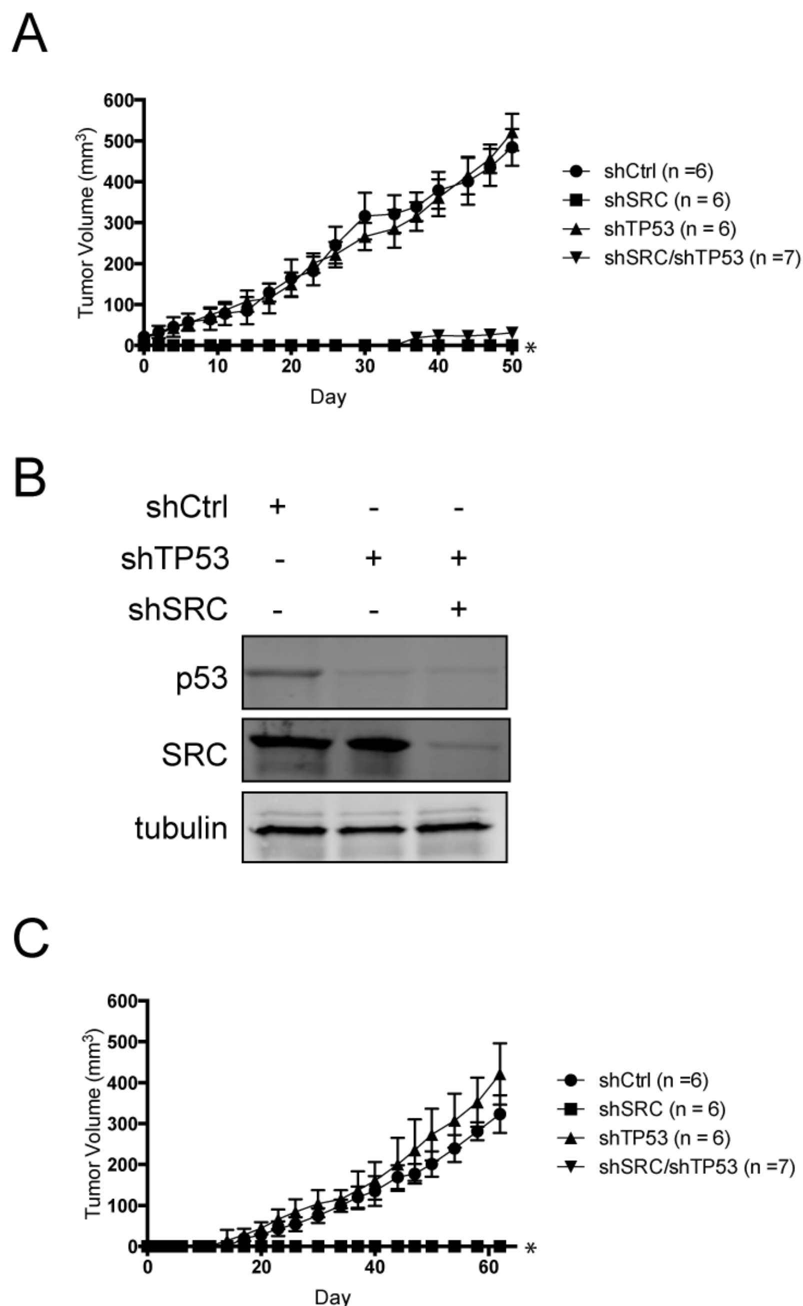

Figure 3.4: Knockdown of SRC impairs development of MCF7 orthoptic xenografts. (A) Mice were injected with shCtrl, shSRC, shTP53, or shSRC/shTP53 double knockdown MCF7 cells at Day 0. Tumors were measured every three days. (B) Representative immunoblot of shCtrl, shTP53, and shSRC/shTP53 tumor lysates to validate knockdown in tumors. (C) The same experiment as (A) except cells were quiesced for 2 days before injection in the fat pad. *=$p<0.05$.

We previously described a role for SRC to induce *MYC* mRNA which leads to proliferation so we wanted to test whether this would correlate with the differences in tumor growth (Figure 2.3). We wanted to test whether *MYC* mRNA levels were altered in these tumors due to SRC inhibition, but also whether p53 expression influences *MYC* mRNA levels. We collected the tumors and isolated RNA in Trizol. We then performed qPCR analysis to detect *MYC* mRNA levels in the tumors. Compared to TOshCtrl/shCtrl tumors, TOshSRC/shCtrl tumors expressed significantly lower amounts of *MYC* mRNA (Figure 3.5). Interestingly, TOshCtrl/shTP53 tumors had significantly more *MYC* mRNA than in the control tumors (Figure 3.5). Lastly, the TOshSRC/shTP53 tumors had levels of *MYC* mRNA comparable to the control cells. The TOshCtrl/shCtrl tumors and the TOshCtrl/shP53 tumors grew at similar rates despite high levels of *MYC* mRNA expressed in the TOshCtrl/shTP53 tumors. Also, this implicates p53 as a potential inhibitor of *MYC*. Conversely, the TOshSRC/shTP53 tumors had similar levels of *MYC* mRNA to the TOshCtrl/shCtrl tumors, but had a significant growth defect. These data taken together suggest that *MYC* mRNA levels are not correlated with tumor growth and that other factors are contributing to tumor growth.

p53 Inhibits MYC mRNA Expression.

The xenograft experiments suggested a potential role for p53 in inhibiting *MYC* mRNA levels. Our previous results demonstrated a requirement for *MYC* in proliferation downstream of E2 and SRC (Figure 2.3),

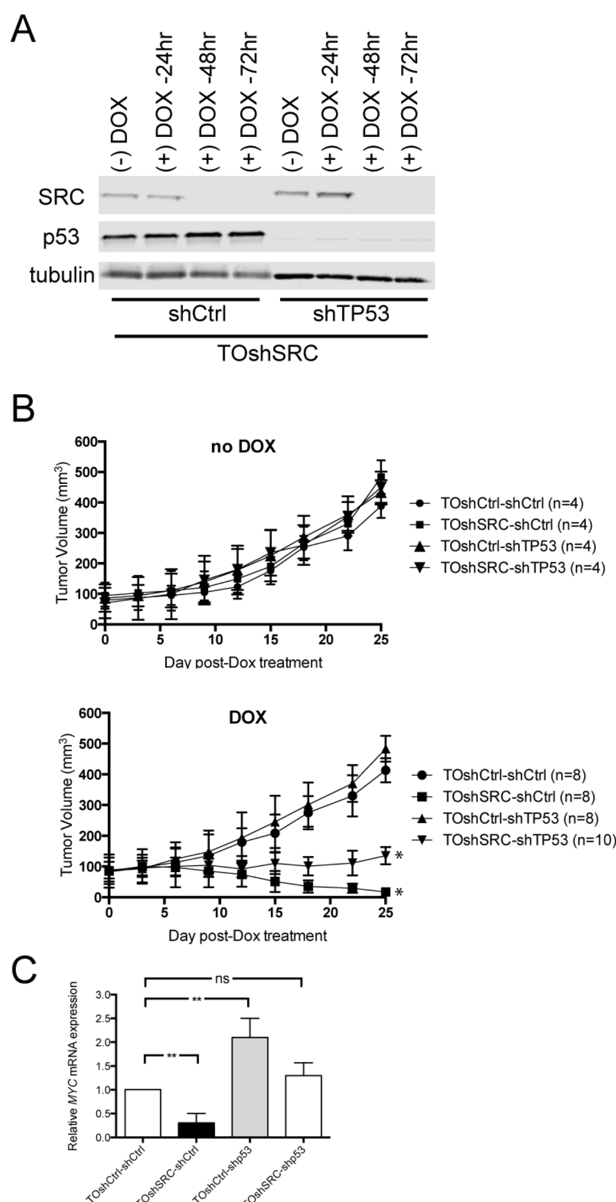

Figure 3.5: Inhibition of SRC in established orthotopic xenografts leads to tumor regression in shCtrl cells and tumor stasis in shp53 cells. (A) Representative immunoblot analysis of TOshSRC/shCtrl and TOshSRC/shp53 double knockdown MCF7 cells after *in* vitro treatment with doxycycline. (B) Mice were injected with TOshCtrl/shCtrl, TPOshCtrl/shTP53, TOshSRC/shCtrl and TOshSRC/shp53 double knockdown MCF7 cells. Once tumor reached 100 mm^3mice were randomly selected for the (top) no Dox or (bottom) Dox was performed every three days and tumors measured at the same time. (C) qPCR analyisis was performed on RNA from TOshCtrl/shCtrl, TPOshCtrl/shTP53, TOshSRC/shCtrl and TOshSRC/shp53 double knockdown MCF7 cells. *=p<0.05.

and we wanted to test whether p53 was also part of this pathway regulating *MYC* mRNA expression. We first analyzed expression of *MYC* mRNA levels in the shTP53 cells. Consistent with the xenograft experiment, compared to shCtrl cells, we observed an increase in *MYC* mRNA (Figure 3.6). We next tested whether there was an increase in MYC protein as well as ∆N-IMP1 (Figure 3.6), the RNA-binding protein that we found to be required to stabilize *MYC* mRNA (Figure 2.7). We found that both MYC and ∆N-IMP1 expression was increased in upon p53 knockdown (Figure 3.6). We next tested whether E2 stimulation of *MYC* mRNA was affected by p53 knockdown. Compared to shCtrl cells, we observed no differences in *MYC* mRNA accumulation in shTP53 cells with increasing concentrations of E2 or over time after stimulation with 5 nM E2 (Figure 3.6). We performed the same time course, but pretreated the cells with SU11333 to test whether the requirement for SRC in *MYC* mRNA accumulation was abrogated in cells lacking p53. SU11333 blocked *MYC* mRNA induction compared to vehicle treated cells (Figure 3.6). However, comparing shTP53 cells to shCtrl cells, treatment with SU11333 was unable to block *MYC* mRNA accumulation (Figure 3.6). These data suggested that p53 likely inhibits *MYC* mRNA expression downstream of SRC.

We previously described a role for SRC in regulating *MYC* mRNA stability, not *MYC* transcription, upon E2 stimulation (Figure 2.5). We next tested whether p53 knockdown affected the transcription of *MYC*. We performed a transcriptional run-on assay in the shTP53 cells and assayed

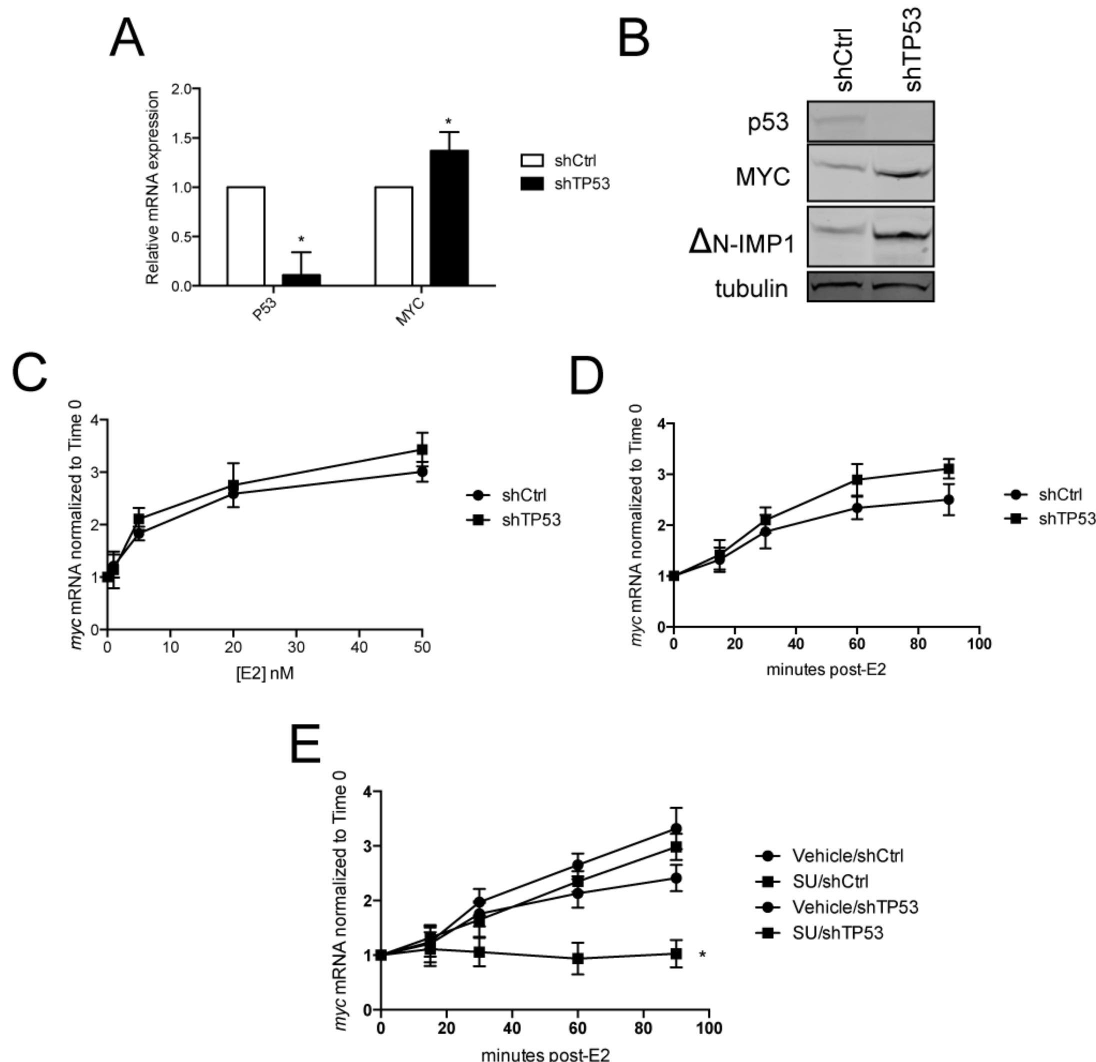

Figure 3.6: Loss of p53 leads to increases in *MYC* mRNA and ΔIMP1 independent of SFK activity. (A)qPCR analysis and (B) Representative immunoblot analysis of shCtrl and shTP53 cells. qPCR analysis of quiescent shCtrl or shTP53 knockdown MCF7 lines that were treated (C) with increasing [E2] or (D) with 5 nM over 90 min. (E) qPCR analysis of quiescent shCtrl or shTP53 knockdown MCF7 lines that were pretreated with vehicle or 1 μM SU11333 and treated with 5 nM over 90 min. *=$p<0.05$.

transcription of *MYC*, *PS2*, and *PPIA*, as well as measured the cytoplasmic levels of these mRNAs at the coinciding time points (Figure 3.7). At the

transcriptional level, we observed an increase in transcription of *PS2,* but no change in either *MYC* or *PPIA* Figure 3.7). However, in looking at the cytoplasmic mRNA levels, we observed an increase in *MYC* mRNA levels in shTP53 cells as compared to shCtrl cells consistent with our previous experiments (Figure 3.7).

Observing no increase in E2-dependent transcription of *MYC* mRNA in shTP53 cells, we next tested whether *MYC* mRNA stability was affected upon p53 knockdown. We stimulated cells with E2 and after one hour of stimulation, treated the cells with Actinomycin D to block transcription. We then quantified the *MYC* mRNA levels by qPCR after stopping transcription to calculate the half-life of *MYC* mRNA. In the shCtrl cells we observed a low basal half-life whereas the E2 stimulated cells showed significant increase in half-life that was abrogated upon pretreatment with SU11333 (Figure 3.7). Interestingly, in the shTP53 cells, we were unable to see an increase in half-life due to E2 as compared to the shCtrl cells (Figure 3.7). However, we do see an increased basal half-life of *MYC* mRNA as compared to the shCtrl cells, and a similar half-life is observed in the SU11333 treated cells (Figure 3.7). Upon knockdown of p53, it appears that the cells lose the ability to stabilize the *MYC* mRNA upon E2 stimulation, but instead maintain a higher basal stabilization of *MYC* mRNA. We could hypothesize that this may be due to the increased

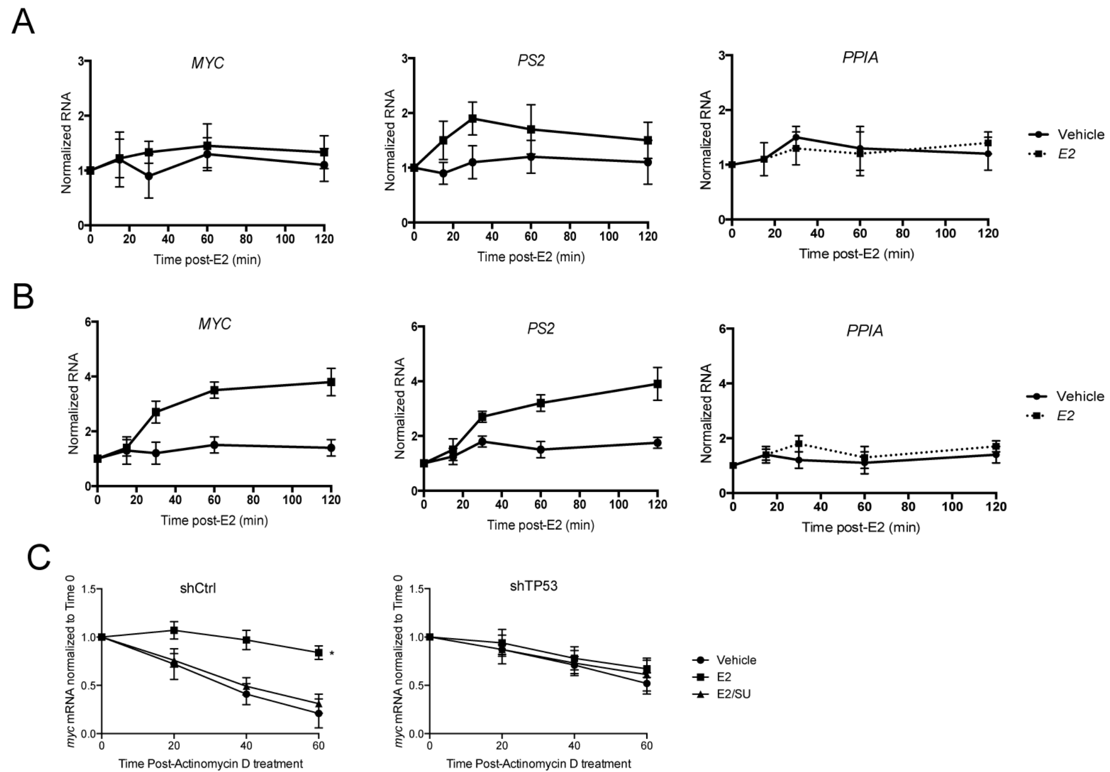

Figure 3.7: Loss of p53 does not affect *MYC* transcription, but increases the stabilization of *MYC* mRNA. (A) Nuclear run-on assay of MCF7 cells treated with E2 over a time course. (B) Cytoplasmic fractions from the same samples as the nuclear run on assay in (A). (C) *MYC* mRNA stability assay in shCtrl and shp53 knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. After 60 min, cells were treated with 5 μM Actinomycin D to block transcription. *=p<0.05.

ΔN-IMP1 expression observed in the p53 knockdown cells. These data are consistent with a model that increased expression of *MYC* mRNA is due to an increase in the basal half-life of *MYC* mRNA.

Loss of MDM2 is Lethal in MCF7 Cells Expressing Wild-type p53.

Much of the data presented here is consistent with SRC acting as an upstream inhibitor of a p53 cell cycle block. However, it is unknown how SRC inhibits p53. A likely candidate to act downstream of SRC is MDM2, a known

inhibitor of p53. To test this hypothesis, we generated MDM2 knockdown cells to test whether MDM2 was required for E2-dependent cell cycle progression and proliferation. Upon lentiviral transduction of the shMDM2 construct, we observed massive cell death, killing all cells within 48 hours. We attempted to conduct some experiments within this time frame. We tested the knockdown of MDM2 using immunoblot analysis and showed efficient knockdown 24 hours after transduction. We then performed a BrdU assay to test whether MDM2 was required for cell cycle progression (Figure 3.8). While the shCtrl cells entered S phase, shMDM2 cells were significantly blunted in their ability to enter S phase. Interestingly, if we pretreated the cells with SU11333, there was no further decrease in cells incorporating BrdU suggesting that they may be involved in the same pathway (Figure 3.8). However, in this assay many of the cells were clearly stressed. This finding that we had difficulty generating MDM2 knockdown lines in consistent with previous studies where MDM2 knockdown was attempted in cells expressing wild-type p53. Previous studies have demonstrated that in cells expressing wild-type p53, inhibition of MDM2 often results in stabilization of p53 and activation of an apoptotic response (Jones et al., 1995; Montes de Oca Luna et al., 1995). To test whether this may be why our cells were dying, using lentivirus we generated stable shCtrl cells, shMDM2 cells, shTP53 cells, and double shMDM2/shTP53 cells, and assessed the efficiency of the knockdown (Figure 3.8).

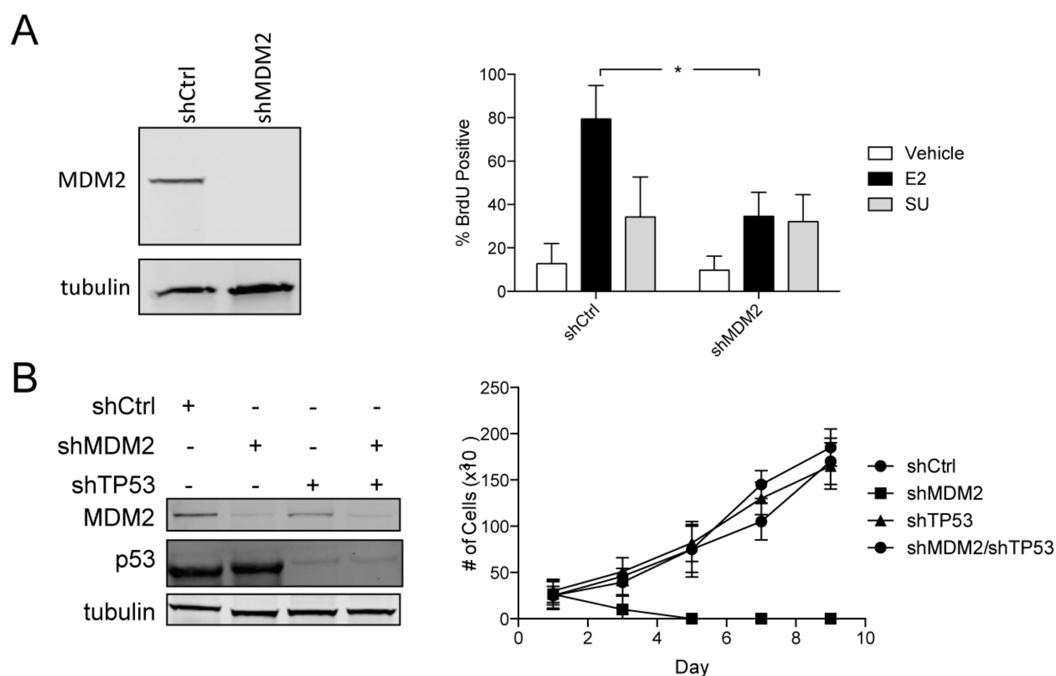

Figure 3.8: Loss of MDM2 causes cell death unless combined with loss of p53. (A) (left) Representative immunoblot analysis of shCtrl and shMDM2 knockdown MCF7 cells. (right) BrdU incorporation assay in shCtrl and shMDM2 knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. (B) (left)Representative immunoblot analysis of shCtrl, shMDM2, shTP53, shMDM2/shTP53. (right) Proliferation assay in shCtrl, shSRC, shTP53, and shSRC/shTP53 double knockdown MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=p<0.05.

We then conducted a proliferation assay with these stable knockdown lines. Similar to what we observed before, the shCtrl and shTP53 cell lines grow at similar rates. The shMDM2 cells decreased in number rapidly following transduction (Figure 3.8). However, the shMDM2/shTP53 knockdown cells grew at the same rate as the shCtrl cells (Figure 3.8). This suggested that loss of p53 in this pathway rescues cells from the proliferative defect or induction of apoptosis due to loss of MDM2.

Phosphorylation of Y281 and Y302 on MDM2 Are Required for Proliferation in MCF7 Cells.

A recent report linked SRC phosphorylation of MDM2 to inhibition of p53's transcriptional activity (Batuello et al., 2015). There are 5 tyrosine phosphorylation sites on MDM2 that have been demonstrated to be phosphorylated by either SRC (2 sites, Y281, Y302) or the tyrosine kinase ABL (3 sites, Y276, Y394, Y405). We focused on these sites because both SRC and ABL have been implicated in inhibition of p53 in the context of fibroblast mitogenesis (Broome and Courtneidge, 2000; Furstoss et al., 2002). Because we could not generate stable knockdown lines to then rescue expression with the phospho-mutants, we opted to overexpress several phospho-mutants in MCF7 cells expressing endogenous MDM2. We generated constructs corresponding to wild-type MDM2 (wt), the 2 SRC phosphotyrosines (2YF), the 3 ABL phosphotyrosines (3YF), and all 5 of these tyrosines (5YF). We recognize that overexpression of these mutants in cells with endogenous MDM2 may confound some of our results, but given the technical limitations of using the MDM2 knockdown cells, we may gain insights from these experiments. Upon transfection of these constructs, within 24 hours all of the 3YF and 5YF transfected cells were dead, and we, therefore, could not conduct any functional analyses on these cells (data not shown). This suggested that the 3 ABL phosphotyrosines are may be important for suppression of apoptosis. We performed immunoblot analysis on the empty

vector, wt, and 2YF overexpressing cells. Compared to the empty vector expressing cells, the wild-type expressing cells had a slightly decreased expression of MDM2 (Figure 3.9). This could possibly be due to a highly regulated negative feedback loop between MDM2 and p53. In the 2YF mutant, we observed increases in both MDM2 and p53 protein (Figure 3.9) This suggested that the 2YF mutant may have decreased ability to regulate p53 levels.

We next wanted to test the mutants in functional assays. We first tested the effects of the mutants in the BrdU incorporation assay. There is a significant decrease in BrdU incorporation in the MDM2-wt overexpressing cells compared to the empty vector control (Figure 3.9). However, the 2YF mutant shows no change in BrdU incorporation relative to overexpressing MDM2-wt (Figure 3.9). We next looked at longer term proliferation, and observe that the MDM2-wt overexpressing cells grow relatively similar to the empty vector control. Unlike the BrdU assay, the 2YF cells proliferate slower than the empty vector or MDM2-wt overexpressing cells (Figure 3.9). These data taken together suggest that the two tyrosine residues suggested to be phosphorylated by SRC may be required for long term proliferative effects, but not acute S phase entry that we have observed upon SRC inhibition. This suggested that SRC is likely regulating other factors needed for proliferation, such as SRC phosphorylation of ∆N-IMP1. While not exhaustive, these

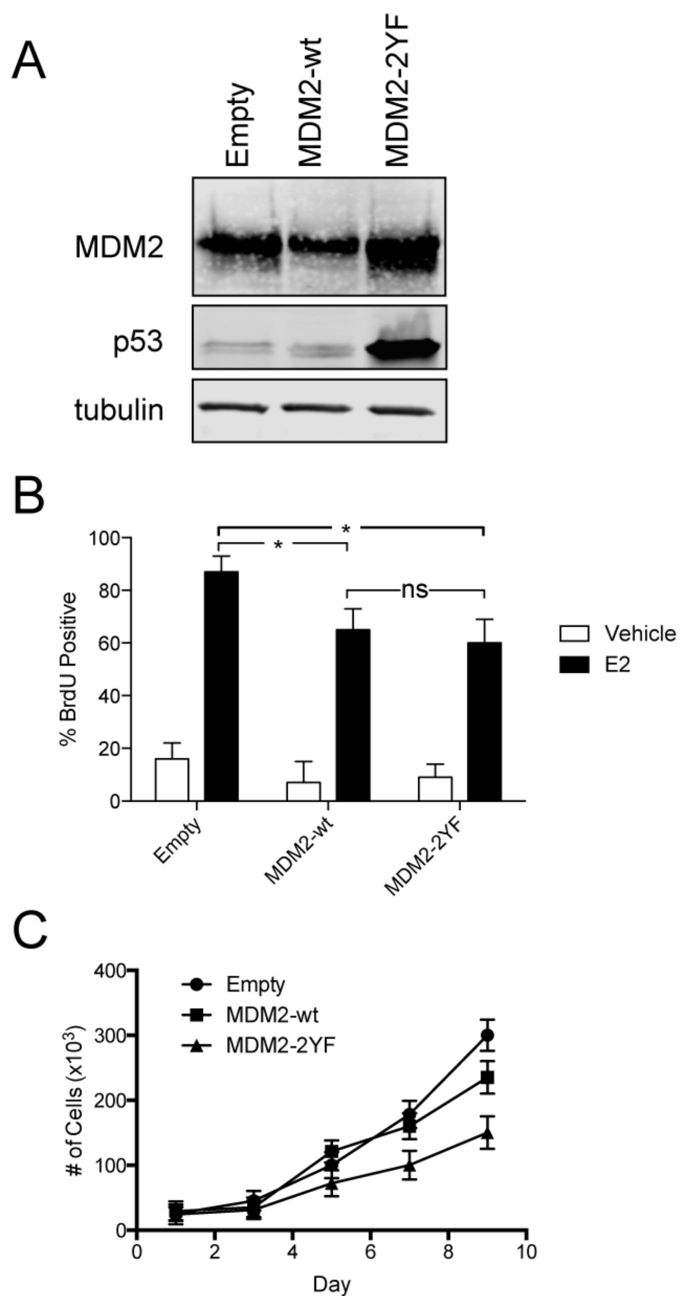

Figure 3.9: Overexpression of MDM2 Y281/302F mutant leads to increased p53 expression and a decrease in proliferation. (A) Representative immunoblot of empty vector, MDM2-wt, and MDM2-2YF. (B) BrdU incorporation assay and (C) proliferation assay in empty vector, MDM2-wt, and MDM2-2YF overexpressing MCF7 cells pretreated with vehicle or 1 μM SU11333 and stimulated with vehicle or 5nM E2. *=$p<0.05$.

experiments offer some hints that MDM2 may act as a potential mediator of a SRC and p53 signaling axis.

Discussion

In this study, we aimed to characterize a potential role for SRC to inhibit p53 in the context of ER-positive breast cancer. Knockdown of p53 abolished the requirement for SRC in cell cycle progression and proliferation *in vitro* after E2 stimulation, and we also demonstrated that overexpression of mouse p53 could rescue the cell cycle block. Studies from our lab and others have suggested that growth factor signaling inhibits a p53 cell cycle block (Broome and Courtneidge, 2000; Lei et al., 2011; Leri et al., 1999; Quintavalle et al., 2010; Ries et al., 2000; Shaulian et al., 1997), and the current study is, to our knowledge, the first that suggests this is also true after E2 stimulation. This is particularly interesting in the context of ER-positive breast cancer because unlike most cancer types, p53 is rarely inactivated by mutation in ER-positive breast cancer (Caleffi et al., 1994; Dumay et al., 2013). This study suggests that activation of SRC is a mechanism to inactivate p53.

While our *in vitro* data suggests that SRC is not required for proliferation in cells lacking p53, the *in vivo* xenografts studies are a bit more complex. MCF7 cells expressing constitutive knockdown of SRC are unable to form tumors at an orthotopic site in nude mice regardless of p53 expression in the cells. This suggests that SRC is required for initial grafting of the tumor

cells in the mammary fat pad. These data are consistent with several mouse models where SRC is disrupted. An observed defect in ER signaling in the *src*$^{-/-}$ mouse is consisted with our finding (Kim et al., 2005). MCF7 cells have been shown to require supplemental E2 when being grown as xenografts in mice. Disruption of SRC either genetically, as in the knockout mouse, or via shRNA-mediated knockdown is effectively inhibiting the E2 signaling pathway required for MCF7 tumor xenografts to form. Our data are also consistent with a role for SRC in the MMTV/PyVmT breast cancer model crossed with a germline SRC knockout (Guy et al., 1994) where it has been shown to inhibit tumor development as compared to the wild-type mice. The xenografts studies where we used inducible SRC knockdown to test whether SRC was required for established xenograft growth showed that in cells lacking p53, SRC knockdown seemed to suppress growth of the tumor whereas in cells expressing p53, SRC knockdown caused regression of tumors. This finding suggests that tumors that express p53 may respond better to SRC inhibition than tumors lacking p53 or expressing inactivated mutant p53.

While no clinical trials have previously utilized p53 status as a biomarker for responsiveness to SRC inhibitors, several preclinical and clinical trials in breast cancer have suggested that multi-targeted kinase inhibitors that inhibit SRC, including dasatinib (Mayer et al., 2011; Mitri et al., 2016) and bosutinib (Campone et al., 2012; Hebbard et al., 2011) may be effective in hormone receptor (HR)-positive breast cancer patients. While correlative, HR-

positive breast tumors typically express wild-type p53 (Caleffi et al., 1994; Dumay et al., 2013). Most of the clinically approved kinase inhibitors while "selective" for SRC, often inhibit a number of other kinases. The off-target effects of inhibitors can potentially confound experimental results.

While the SRC knockdown experiments are consistent with the rest of our *in vitro* experiments and published findings, the results of the experiment where we used saracatinib as an inhibitor of SRC activity in tumors with or without p53 conflict with most of our other findings. One explanation for this discrepancy could be due to off-target effects of saracatinib. In addition to SFKs, saracatinib can also potentially inhibit ABL, EGFR, KIT at IC_{50} values near to those of SFKs (Green et al., 2009). Of these kinases, ABL has been suggested to both inhibit (Carr et al., 2016; Waning et al., 2011) and activate p53 (Sionov et al., 2001; Sionov et al., 1999; Zuckerman et al., 2009) via interactions with MDM2 and MDMX. ABL has also been described to be activated by SRC after PDGF stimulation in fibroblasts (Furstoss et al., 2002), however, it is unclear what downstream effectors SRC and ABL may have in common. Thus, dual inhibition may have a confounding effect as compared to individual inhibition of either kinase alone. An additional explanation could be due to the systemic effects of saracatinib in the mouse and on the tumor microenvironment in the mammary fat pad. We chose not to use the SU11333 inhibitor used in the *in vitro* studies in the mouse studies for two reasons. First, the pharmacokinetics and pharmacodynamics of the inhibitor are unknown in

mice. Second, we opted to use a clinically available inhibitor to be more relevant as a preclinical model. While the *in vivo* inhibitor study was somewhat confounding, the knockdown studies demonstrated a clear role for SRC in inhibiting p53 function.

Our finding that inhibition of p53 led to increases in *MYC* mRNA suggests a pathway wherein p53 is repressing *MYC* mRNA expression. Our analyses suggest that this is by increasing the basal half-life of *MYC* mRNA, rather than inducing transcription. Several possible mechanisms for the increased basal stability of *MYC* mRNA due to p53 exist. First, we showed that p53 is also repressing ∆N-IMP1 which we previously showed to be required to stabilize *MYC* mRNA. A second RNA-binding protein that has been involved in *MYC* mRNA destabilization, tristetraprolin (Marderosian et al., 2006; Rounbehler et al., 2012), has also been shown to be induced by p53 (Lee et al., 2013). Another potential mechanism is via p53 regulation of miRNAs (Feng et al., 2011). One study has suggested that *miR-145* is regulated by p53 and *MYC* has been demonstrated to be a direct target of *miR-145* (Sachdeva et al., 2009). Work from our lab has also found that *miR-145* is also regulated downstream of SRC and p53 in vascular smooth muscle regulation of podosomes (Quintavalle et al., 2010). Some evidence also exists that p53 can also bind to the *MYC* promoter and repress its translation (Ho et al., 2005). While we did not observe increased transcription in p53 knockdown cells, our

finding is consistent with p53 repressing *MYC* via destabilization of the *MYC* mRNA

While we can potentially implicate MDM2 as an inhibitor of p53 downstream of SRC for cell cycle progression, many questions remain surrounding this pathway. Technical limitations involving induced cell death upon MDM2 knockdown complicate many of our experiments. While concomitant knockdown of MDM2 and p53 may allow cells to grow, they do not allow us to cleanly test our hypothesis. Inhibition of MDM2 in cells which express wild-type p53 often lead to apoptosis making it difficult to observe cell cycle effects. Introduction of the SRC and ABL tyrosine mutants of MDM2 suggest that ABL phosphorylation is important in suppressing cell death. The SRC phospho-mutants proliferate slower than control cells, but because these mutants are expressed in cells with endogenous MDM2 the phenotypes of the mutants may be shielded. Because modulation of MDM2 itself seems to be detrimental to cell survival, this hypothesis may need to be tested using downstream effectors of MDM2. As SRC has been found to convert MDM2 from a ubiquitin ligase to a neddylating enzyme (Batuello et al., 2015), additional studies may conduct mutational analysis of the putative p53 neddylation sites to test whether the mutations affect cell cycle progression. Additionally, CRISPR deletion of one allele of MDM2 or substitution of one wild-type allele for a mutant allele may be a way to observe dose dependent responses to MDM2 modulation without killing the cells. The role of MDMX

also needs to be further studied in the context of SRC and cell cycle inhibition. Because MDM2 and MDMX are both critical inhibitors of p53 (Huang et al., 2011; Kruse and Gu, 2009; Kubbutat et al., 1997; Marine and Jochemsen, 2005; Wang et al., 2011), however, they have been shown to have non-redundant roles as MDMX lacks E2 ligase activity. Additional studies need to further explore these two p53 inhibitors as mediators of SRC-dependent p53 inhibition.

Methods

Cell Culture

MCF7 cells (ATCC) were grown in IMEM supplemented with 10% FBS. For starvation prior to estrogen stimulation in the assays described below, cells were washed 1X in PBS and starved for 48 hours in starvation medium [phenol red-free IMEM, 0.5% charcoal, dextran stripped FBS (Hyclone)]. For pretreatment with inhibitors, inhibitors were diluted into starvation medium and incubated for 2 hours prior to stimulation. All experiments were performed in the absence of antibiotics.

Plasmids

RNAi knockdown was performed using the pLKO.1 or pLKO.1-TetOn shRNA expression vectors. The RNAi consortium clone numbers for each of the shRNA constructs are: SRC (TRCN0000195339), TP53 (TRCN0000003753), MDM2 (TRCN0000355726). Overexpression was

performed by expressing the following cDNAs in the pCDH lentiviral vector: ΔN-IMP1; MDM2.

Chemicals

Estrogen, (Sigma-Aldrich), ICI182780 (Tocris Biosciences), Actinomycin D (Sigma-Aldrich), saracatinib (APExBio), SU11333 (Sanford|Burnham|Prebys Medicinal Chemistry Core), were used as described above.

Bromodeoxyuridine (BrdU) Incorporation Assay

Cells were plated at 60% confluency on glass coverslips in normal growth medium and allowed to grow overnight. The next morning cells were washed once with PBS and then placed in starvation medium. Cells were pretreated for 2 hours with 1 μM SU11333 or DMSO vehicle prior to stimulation. Cells were then treated with either ethanol or 5 nM estrogen (E2) with 5 μM BrdU for 18 hours. After treatment, cells were washed twice with cold PBS and fixed with ice-cold 1:1 methanol:acetone. Coverslips were then rehydrated in PBS for 15 minutes. Cells were permeabilized in 0.1% TX-100 in PBS for 10 minutes. Cells were then treated with 2N hydrochloric acid at 37C for 15 minutes. Coverslips were then washed 3x in PBS with rocking. Coverslips were blocked with 1% goat serum in PBS for 1 hour with rocking. Anti-BrdU (Millipore) antibody was used to detect incorporated BrdU (Millipore). Anti-mouse 488 secondary (was used to visualize staining via

immunofluorescence analysis. Vectashield containing DAPI was used to mount the coverslips and stain nuclei.

Proliferation Assays

Cells were plated at 25 x 10^3 and viable cells were counted every 2 days using the Countess II Automated Cell Counter (Thermo Fisher Scientific).

Reagents and Antibodies for Immunoblotting

The following commercial antibodies were used: anti-SRC (327) antibody (Abcam), anti-MYC (Y69) antibody (Abcam), anti-IGF2BP1 antibody (Sigma-Aldrich), anti-p53 (DO-1) antibody (Santa Cruz Biotechnology), anti-MDM2 (SMP14) antibody (Santa Cruz Biotechnology), anti-MDM2 (C-18) antibody (Santa Cruz Biotechnology), anti-γ–tubulin antibody (Sigma-Aldrich), anti-actin (C-74) antibody (Sigma), and anti-BrdU (Millipore). For secondary antibodies, Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen) or IR800 (Rockland Immunochemicals) were used for immunoblotting.

Immunoblotting

Cell lysates were prepared by washing cells twice with cold Tris-buffered saline (TBS) containing 100 μM Na3VO4 and then lysed in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% Triton X-100, 50 mM NaF, 100 μM Na3VO4 and 1mM EDTA lysis buffer containing a dissolved complete Mini protease inhibitor tab (Roche Diagnostics). Supernatant of cell lysates was assayed for total protein content using the BCA protein assay (Thermo Fisher

Scientific), and 50 µg of total protein per sample was separated in a polyacrylamide gel (Invitrogen). Membranes were scanned using an infrared imaging system (Odyssey; LI-COR Biosciences).

RNA Isolation and cDNA generation

Trizol extraction of RNA was performed per manufacturer's instructions. cDNA was generated using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

MYC mRNA Accumulation Assay

Cells were starved and stimulated as described above. Post-E2 stimulation, RNA was isolated at the indicated times, and cDNA was generated. To quantify the RNA accumulation, qPCR analysis was performed. For each sample, *MYC* mRNA was normalized independently to two different housekeeping genes (*PPIA or RPLP0*). Primers for analysis were used as previously described (Weidensdorfer et al., 2009). Time points for each condition were all normalized to time 0 for each condition.

Transcriptional Run-on Assay

To label transcripts, the Click-iT Nascent RNA Capture Kit (Invitrogen) was used and adapted for this assay. Cells were starved and stimulated as described above. Transcription was then paused by placing samples on ice at various time points post-stimulation. Intact nuclei were isolated at each time point for analysis. Labelled 5-ethyl Uridine (EU) and placed samples at 37C to

allow for elongation of initiated transcripts. EU will only be incorporated into these initiated transcripts. The EU is then biotinylated and then collected using streptavidin beads. RNA is isolated off the beads and cDNA generated for qPCR analysis.

RNA Stability Assay

Cells were starved and stimulated as described above. Sixty minutes post E2- stimulation, cells were treated with 5 μM Actinomycin D to block transcription. At the indicated time points, RNA was isolated and cDNA generated for each condition. For each sample, *MYC* mRNA was normalized independently to two different housekeeping genes (*PPIA or RPLP0*). These genes were selected so that Actinomycin D treatment did not alter their expression. Time points for each condition were all normalized to time 0 for each condition. *MYC* mRNA half-life was then calculated for each of the conditions. To determine significance, one -way ANOVA and Tukey's post-hoc tests were performed on the *MYC* mRNA half-life at the endpoint of the experiment to determine statistical significance.

Orthotopic Mouse Xenograft Experiments

Female, athymic nude mice were purchased from Jackson Laboratory. Mice were injected with a slow-release 17β-estradiol pellet (0.72 mg/pellet, 60-day release from Innovative Research of America) by using a trochar. One week later, mammary fat pad injections in the number 4 mammary gland were

carried out without the clearing of the fat pad. Briefly, cells were harvested by trypsinization and resuspended in PBS (Invitrogen). For each cell line, the mice were injected in a non-cleared mammary fat pad with $1x10^6$ cells per animal in a volume of 100 µl [1:1 ratio with Matrigel (BD Biosciences)], and tumors were allowed to form with a diameter of up to 1 cm. Tumor onset was determined by physical palpation. Tumor growth was measured every 2–3 days using calipers; both the longest (L) and shortest (S) measurements were recorded. Using these values, tumor volumes were calculated as follows: $(L \times S^2) \times 0.5$, and expressed as mean volume ± SEM. Mice were sacrificed when the tumors reached a diameter of 1 cm, according to the Animal Care and Use Policy of OHSU. These experiments were repeated at least 3 times, using 5–10 mice per tumor group. One-way ANOVA, student's t test, or Tukey's post-hoc tests were performed on the tumor volume at the endpoint of the experiment to determine statistical significance as appropriate.

For inhibitor studies, once mice had measurable tumors of 50 mm^3, treatment with 50 µl vehicle, ICI182870 (10 mg/Kg), or saracatinib (25 mg/Kg) was injected intraperitoneally and tumor volume measured every three days.

For the doxycycline-inducible shRNA-expressing cells, tumors were allowed to either 100 or 200 mm^3 before being placed on chow with 0.2% (2000 ppm) doxycycline (Test Diet). Tumors were then measured as described above until control animals reached a diameter of 1 cm.

Statistical Analyses

Statistical significance was determined by calculating the p-value (P) using the paired Student's t test. P<0.05 was considered to be statistically significant. The numbers of samples (n) are indicated in each figure legend. For 2D, 3D, and tumor growth curves, area under the curve analysis was performed on the individual growth curves using the Area Under the Curve (AUC) function in the GraphPad Prism software. Means and SEM were then calculated and Student's t test and Tukey's post-hoc test were used to determine significance.

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Chapter IV:

Conclusions and Perspectives

New insights into non-genomic E2 signaling are beginning to show the many complexities of E2 signaling. As has been discussed, much of the research on E2 signaling has focused on the intrinsic DNA-binding and transcriptional role of the estrogen receptor (ER). However, our data demonstrated a role for SRC in mediating E2-dependent proliferation independent of transcription. SRC stabilized *MYC* mRNA upon E2 stimulation. Global analysis of E2-dependent gene expression should be conducted to uncover which E2 genes are being regulated by transcription versus mRNA stabilization. Our lab has previously looked at gene expression in quiescent fibroblasts that were pretreated with different kinase inhibitors, and then stimulated with PDGF. Using microarrays, we observed several different pathways acting downstream of PDGF, with distinct gene signatures that were being regulated by SRC, MAPK, or PI3K (Bromann et al., 2005). It would be interesting to test whether these pathways that have also been implicated in non-genomic E2 signaling pathways regulate similar target genes.

We found that SRC stabilized *MYC* mRNA via ΔN-IMP1. The mutagenesis experiments suggest that this is via phosphorylation of Y260, however it remains to be tested whether it is phosphorylated directly by SRC. We are not sure how generalizable this mechanism is for potential SRC stabilization of other mRNAs and how dependent the stabilization is on ΔN-IMP1. Additional studies are needed to test what genes are being regulated by both SRC and ΔN-IMP1.

This study suggested one function for ΔN-IMP1, however, little is known about this RNA-binding protein outside of inferences from studies of the full-length protein. ΔN-IMP1 lacks the first two RNA binding domains (RNA recognition motifs, RRMs) that are found in IMP1, however, the 4 KH domains are present (Fakhraldeen et al., 2015). This lack of RNA-binding domains presumably translates to changes in the specificity of target mRNAs that ΔN-IMP1 can bind relative to IMP1. Interestingly, our data suggested that ΔN-IMP1 likely retains the ability to bind *MYC* mRNA, however, the antibody used to detect ΔN-IMP1 is unable to immunoprecipitate the protein to test for a direct interaction.

The mechanisms of regulation of ΔN-IMP1 are unknown, however several experiments from this study suggest some possibilities. We determined that SRC phosphorylation of ΔN-IMP1 promotes *MYC* mRNA stability which has not been shown previously. A more exhaustive analysis of how SRC phosphorylation potentially regulates other FL- or ΔN-IMP1 targets is needed. The expression pattern of ∆N-IMP1 in tissues is also largely unknown. The full-length form of this protein had been suggested to have an oncofetal expression pattern, so further expression analysis of ΔN-IMP1 expression is needed and currently limited by lack of a specific antibody to that isoform. The current reagents used to study IMP1 will detect either only the full-length form or both forms, thus, the truncated form is likely being misreported. Currently, RNA expression or immunoblotting studies are the

only option for differentiating between the two forms. Several immunohistochemical expression studies will need to be revisited to see which isoforms are being expressed and which isoform are necessary for previous findings (Ioannidis et al., 2004; Ioannidis et al., 2003; Ioannidis et al., 2005; Ioannidis et al., 2001; Köbel et al., 2007).

This study also suggests a potential mechanism of regulation of the gene. We also demonstrated that upon p53 loss, there was increased expression of ΔN-IMP1. ΔN-IMP1 is expressed from a novel promoter found in the second intron of the *IGF2BP1* gene. This may implicate p53 as a transcriptional repressor of ∆N-IMP1. We also observed that loss of p53 increased basal *MYC* mRNA stability, and we hypothesize that this may be due to the increased expression of ∆N-IMP1. From our data, we suggest that increased expression is also at the post-transcriptional level. As stated in previous chapters, p53 appears to suppress *MYC* mRNA stabilizers (ΔN-IMP1, TTP) which could lead to decreases in *MYC* mRNA. An intriguing thought is that p53 regulating a cassette of miRNAs regulating global RNA stability. Previous studies have found that p53 also inhibits *MYC* mRNA via regulation of *miR-145* which directly inhibits *MYC* mRNA stability (Sachdeva et al., 2009), Work from our lab has shown that *miR-145* is also suppressed by SRC inhibition of p53 (Quintavalle et al., 2010). While SRC may be mediating acute *MYC* mRNA levels via ∆N-IMP1, p53 may be regulating basal expression of *MYC* mRNA via miRNA control. SRC suppression of p53 function could

lead to long-term miRNA deregulation lead to a “pro-tumorigenic” signaling environment.

Suppression of wild-type p53 function by SRC is an important finding. First, studies should be conducted in other cancer types that commonly harbor wild-type p53 to see if SRC is upregulated in those tumors as well, and if SRC inhibits p53 in those tumors. The data in this study suggest that SRC inhibitors should be targeted towards patients that retain wild-type p53, such as ER-positive breast cancer patients. Our studies have primarily shown SRC inhibition in cells with p53 to be cytostatic. While this would be beneficial to patients, additional therapies may want to be combined because cancer clinical success is often measured by regression of tumors, not stasis of tumors. For example, radiation or chemotherapy, which trigger the DNA damage response, may be beneficial to combine with SRC inhibitors. Upon SRC inhibition, p53 will be more likely to be active and activate apoptotic pathways.

Inhibition of MDM2 in our cells that harbor wild-type p53 led to cell death likely due to apoptosis from active p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). In the experiments where we overexpressed mutated SRC phosphorylated tyrosines (Batuello et al., 2015) on MDM2 to be “unphosphorylated”, we showed a decreased growth rate, suggesting that the phosphosites are important for proliferation. To test the hypothesis that SRC functions via MDM2 to inhibit p53, experiments will have to be performed as to

not induce apoptosis. As was stated earlier in this thesis, decreasing the dose of MDM2 and possibly MDMX is required for cell survival, but may increase the likelihood of manipulating either MDM2 or MDMX to test for cell cycle regulators of p53. These studies are important to define a role for MDM2 in cell cycle regulation.

Lastly, these studies were conducted in the ER-positive breast cancer cell lines, MCF7 and ZR-75-1, which have been almost exclusively used for the study of ER-positive breast cancer for years. These cell lines have provided invaluable insight into E2 signaling both in normal and cancerous cells, however, there are some questions about how generalizable are the findings in these cell lines. Several reports have also suggested that different sources of MCF7 cells have different responses to E2 as well as overall gene expression profiles (Nugoli et al., 2003; Osborne et al., 1987).

New models of ER-positive breast cancer would greatly benefit the field, and could be used to confirm some of the findings in the MCF7 and ZR-75-1 cells. Several studies have suggested that injection of ER-positive cells intraductally leads to tumors that better mimic the human disease, including ductal carcinoma *in situ* (DCIS) (Behbod et al., 2009; Valdez et al., 2011). For example, MCF7 cells transplanted into the mammary fat pad undergo gene expression changes that make the tumors more basal-like as compared to MCF7 cells injected intraductally which results in maintenance of a more luminal phenotype (Sflomos et al., 2016).

While the intraductal injections often still use established breast cancer cell lines, many groups are developing ER-positive patient derived xenografts (Kanaya et al., 2016; Matthews and Sartorius, 2017). These xenografts typically involve dissecting a biopsy sample and dissociating the cells. The cells are then grow in an immunocompromised mouse, and upon reaching a certain size, dissociated again and passaged into a new mouse. These models are attractive as new models of ER-positive breast cancer, however, the ER-positive xenografts tend not to graft as well as triple negative breast cancers.

Genetically engineered mouse (GEM) models of breast cancer are incredibly useful tools to study many of the biological processes involved in cancer progression. Germline knockout mice can offer insight into initiation of spontaneous models of cancer. A variety of new conditional models are becoming available so that disruption of a gene can occur in both a tissue and time specific manner to study a variety of questions. While the mouse mammary tumor virus (MMTV)/Polyoma virus middle T antigen (PyVmT) breast cancer model is useful for studying SRC driven cancers, ER-positive tumors do not develop. Unfortunately for the ER-positive breast cancer research community, very few if any mouse models of breast cancer accurately recapitulate the human disease. Mouse models of ER-positive breast cancer that do exist, often have mutated or inactivated p53, express the ER at relatively low frequency, and typically have very long (10-24 months)

latency period before tumor development (Dabydeen and Furth, 2014; Mohibi et al., 2011).

Better ER-positive model systems are required for use in future preclinical trials, particularly for testing the efficacy of SRC inhibitors. Several preclinical and clinical trials in breast cancer have suggested that inhibition of SRC using kinase inhibitors including dasatinib (Mayer et al., 2011; Mitri et al., 2016) and bosutinib (Campone et al., 2012; Hebbard et al., 2011) resulted in stable disease in a fraction of the hormone receptor (HR)-positive cohort. We postulate that these patients expressed wild-type p53, however, expression was not analyzed in these studies. Based on this, we suggest that therapeutic inhibition of SRC may be effective in early stage ER-positive breast cancer expressing wild-type p53, and more generally, to cancers that retain wild-type p53. In addition to selecting patients for SRC inhibitor therapy based on p53 status, we also suggest that the multi-targeted kinase inhibitors used to target SRC in the clinic may not have an appropriate selectivity for SRC. From this study, genetic inhibition of SRC in tumors that lack p53 did not regress, however, treatment of tumors with saracatinib led to regression of the tumors regardless of p53 status. This suggests that “off-target” effects of the inhibitor may complicate targeted treatment based on p53 status. SRC inhibition as a treatment strategy for patients will likely require the use of p53 status as a biomarker, and potentially, the generation of more selective SRC inhibitors for use in the clinic.

In conclusion, this study furthered our understanding of the non-genomic E2 signaling pathway in ER-positive breast cancer. We found a mechanism by which SRC regulates gene expression by stabilization of mRNA rather than transcription after E2 stimulation. We also found a role for SRC in inhibiting p53 function and some suggestion that MDM2 may be mediating this function. In addition to understanding this mechanism, the E2-SRC-p53 axis may also inform therapeutic decisions in the clinic. Our data suggest that patients who are ER-positive and retain wild-type p53 may benefit the most from SRC inhibitors.

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Part II

Chapter V:

Foreword to Part II

In addition to my biomedical sciences-based thesis work, I also had the opportunity to conduct biology education research (BER). I have an interest in education and my long-term career goals involved teaching and running a research program at a primarily undergraduate institution. To this end I conducted BER research under the supervision of Dr. Ella Tour. Part II consists of two published manuscripts detailing this work where we used primary literature articles in a Master's level course to test the effects on students' science process skills, perceptions, and perceived difficulties with reading primary literature.

Chapter VI:

Critical Analysis of Primary Literature in a Master's-Level Class: Effects on Self-Efficacy and Science-Process Skills

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Article

Critical Analysis of Primary Literature in a Master's-Level Class: Effects on Self-Efficacy and Science-Process Skills

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The ability to think analytically and creatively is crucial for success in the modern workforce, particularly for graduate students, who often aim to become physicians or researchers. Analysis of the primary literature provides an excellent opportunity to practice these skills. We describe a course that includes a structured analysis of four research papers from diverse fields of biology and group exercises in proposing experiments that would follow up on these papers. To facilitate a critical approach to primary literature, we included a paper with questionable data interpretation and two papers investigating the same biological question yet reaching opposite conclusions. We report a significant increase in students' self-efficacy in analyzing data from research papers, evaluating authors' conclusions, and designing experiments. Using our science-process skills test, we observe a statistically significant increase in students' ability to propose an experiment that matches the goal of investigation. We also detect gains in interpretation of controls and quantitative analysis of data. No statistically significant changes were observed in questions that tested the skills of interpretation, inference, and evaluation.

INTRODUCTION

Rapid technological and scientific advances of the past few decades have generated demands for a workforce that possesses the skills associated with critical and creative scientific thinking: analysis and evaluation of data, problem solving, and generation of new concepts and ideas (Autor *et al.*, 2003; Autor and Price, 2013). These skills are often referred to as science-process skills (Coil *et al.*, 2010). Calls for an increased emphasis on teaching science-process skills have been issued by several major educational bodies in the recent past: the National Research Council (NRC, 2009), the American Association of Medical Colleges and Howard Hughes Medical Institute (AAMC-HHMI, 2009), and the American Association for the Advancement of Science (AAAS, 2011). For example, the *Vision and Change in Undergraduate Education: A Call for Action* report proposes that the ability to apply the process of science, described as "posing problems, generating hypotheses, designing experiments, observing nature, testing hypotheses, interpreting and evaluating data, and determining how to follow up on the findings," constitutes the first of the six fundamental core competencies that need to be developed by all undergraduate students (AAAS, 2011, p. 14).

Several studies have shown that despite overwhelming agreement that critical-thinking and science-process skills are very important instructional goals, very few college faculty members explicitly teach and assess these skills (Paul *et al.*, 1997; Coil *et al.*, 2010). Among the identified barriers to teaching these skills in biology classrooms are time constraints, the need to cover content, and the lack of validated, biology-specific assessments of critical thinking (Bissell and Lemons, 2006; Coil *et al.*, 2010). However, successful approaches to teaching critical-thinking and

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science-process skills have been reported (e.g., Kitchen *et al.*, 2003; Dirks and Cunningham, 2006; Hoskins *et al.*, 2007; Coil *et al.*, 2010; Gottesman and Hoskins, 2013). The common theme in these studies is the implementation of a variety of active-learning approaches that include frequent practice of science-process skills inside and outside the classroom. However, such educational approaches are not common. Arguably, college-level biology education remains centered primarily around instructor-mediated transfer of facts (Alberts, 2009).

The need for critical-thinking and science-process instruction is even more acute in graduate education. Individuals with graduate degrees in biology tend to seek jobs that require routine use of higher-order thinking skills (e.g., physicians, researchers in academia and industry, educators). The need for physicians well-trained in problem solving, evaluating "competing claims in the medical literature and by those in medical industries" and capable of "application of scientific knowledge and scientific reasoning based on evidence" was articulated in the report *Scientific Foundations for Future Physicians* (AAMC-HHMI, 2009, pp. 4–5). Furthermore, individuals equipped with such skills face better job prospects. While the contribution of routine manual and routine cognitive skills (cognitive skills that can be replaced by computers, such as bookkeeping, clerical work) to the U.S. labor market has declined in the past 50 yr, the contribution of the nonroutine cognitive tasks (those that require critical-thinking and science-process skills) has been on the rise (Autor *et al.*, 2003; Autor and Price, 2013).

To assess students' science-process skills, we first need to define the different components of this complex set of skills. Bloom's taxonomy of educational objectives (Bloom *et al.*, 1956) provides a framework frequently used by educators for identifying the different components of science-process skills, designing activities, and creating assessments to evaluate these skills (Bissell and Lemons, 2006; Crowe *et al.*, 2008). Bloom's taxonomy identifies six categories of learning: knowledge, comprehension, application, analysis, synthesis, and evaluation (Bloom *et al.*, 1956). The first two categories are aligned with lower-order cognitive skills (LOCS), while the last three categories require higher-order cognitive skills (HOCS)—skills overlapping with critical thinking and science process (Zoller *et al.*, 1995; Crowe *et al.*, 2008; Coil *et al.*, 2010). The third category, application, is considered to be transitional between LOCS and HOCS. Another perspective for classifying the critical-thinking component of science-process skills is provided by the Delphi report of the American Philosophical Association (Facione, 1990). This report describes the consensus core critical-thinking skills, as determined by experts in the critical-thinking field, primarily from philosophy, social sciences, and education. According to the Delphi report, the core critical-thinking skills consist of interpretation, analysis, evaluation, inference, explanation, and self-regulation (Facione, 1990). Importantly, the Delphi report also notes the overlap between the different categories of critical thinking, suggesting that "creating arbitrary differentiation simply to force each and every subskill to become conceptually discrete from all others is neither necessary nor useful" (Facione, 1990, p. 6). For example, to evaluate a hypothesis, one needs to analyze the data on which the hypothesis is based and draw one's own conclusions from these data. The frameworks of Bloom's taxonomy and the Delphi report provide useful complementary perspectives for classification of science-process skills. For example, such an important science-process skill as experimental design is not included in the core consensus critical-thinking skills defined by the Delphi report (Facione, 1990), while in Bloom's taxonomy it is categorized as synthesis, one of the HOCS (Bloom *et al.*, 1956; Crowe *et al.*, 2008).

In classroom settings, discussion of primary literature provides an excellent opportunity to practice science-process skills: analyzing the data presented, drawing independent conclusions, evaluating the authors' conclusions, synthesizing new hypotheses, and designing new experiments to test them. Several studies have reported that undergraduate courses that focus on analysis of primary literature have positive effects on students' science-process and critical-thinking skills (Hoskins *et al.*, 2007; Gottesman and Hoskins, 2013; Segura-Totten and Dalman, 2013). For example, the CREATE (Consider, Read, Elucidate hypothesis, Analyze and interpret the data, and Think of the next Experiment) approach, which offers structured engagement with linked sequences of articles from the same lab, was associated with a statistically significant increase in students' skills in data analysis and drawing logical conclusions in an upper-division undergraduate class (Hoskins *et al.*, 2007) and significantly improved students' performance in the Critical Thinking Assessment Test (CAT; Stein *et al.*, 2012) in a freshmen-level class (Gottesman and Hoskins, 2013). However, the effects of other primary literature–centered approaches on the development of science-process skills, in particular among graduate students, remain unexplored.

We report here on the development and assessment of a primary literature–based course designed for students enrolled in the contiguous BS/MS program in biology at the University of California, San Diego (UCSD). In this research-based master's program, biology undergraduates can extend the research they perform in their senior undergraduate year to obtain a master's degree. As graduate students, they are routinely expected to use various science-process skills: interpreting primary literature, contributing to experimental design, analyzing results, and, finally, writing and defending a substantial research thesis within 1 or 2 yr after graduating with a bachelor's degree. However, as we will demonstrate here, our master's students often feel unprepared for these tasks. One of our goals was to design a course that can improve their skills of critical analysis of primary literature and experimental design and increase their sense of self-efficacy in their ability to perform these tasks.

To achieve these goals, the course described here incorporated structured group and individual activities in which students practiced skills required to understand and analyze four papers from diverse fields of biology. Students' evaluations point to significant perceived gains in science-process skills in the context of primary literature. However, using a science-process skills test, we detected a statistically significant increase in students' ability to propose an experiment that matches the goals of investigation, interpretation of controls, and quantitative analysis of data, but we did not see a similar increase in responses to questions assessing the skills of inference and evaluation.

METHODS

Students' Demographics and Career Aspirations

The elective course described in this study was designed and offered specifically to the students enrolled in the contiguous BS/MS program of the Division of Biological Sciences at the UCSD. Only UCSD biology undergraduates can enter this program during their senior year. Altogether, they complete at least six quarters of research (typically, three quarters as undergraduates, followed by at least three quarters of graduate research in the same lab) and defend a research-based thesis.

The data on students' demographics, major, career aspirations, and experience with primary literature were collected via anonymous surveys. Figure 1 presents the data collected in Fall 2013 and Winter 2014 from 28 students who completed the beginning-of-the-quarter survey. The majority of students participating in this study were recent undergraduates, primarily in their first (59%) or second (25%) quarter of the master's program (Figure 1A). Forty-five percent of our students were Asian, another 45% were non-Hispanic white, and 3% were Hispanic or Latino (Figure 1B). The students represented all UCSD biology undergraduate majors, except for bioinformatics (Figure 1C). Medicine was the most frequently considered career aspiration, followed by biotechnology and teaching (Figure 1D).

Selection of Scientific Papers

The course described in this study, BGGN 211: Recent Advances and Experimental Approaches in Modern Biology, was taught by one of the authors (E.T.). Because this is the only course that is specifically geared toward master's students, it was designed to have an appeal to master's students working in a variety of subfields of biology. With this goal in mind, we selected the course papers to increase the variety of experimental approaches and to train students in critical analysis of papers outside their areas of expertise (Supplemental Table S1). The papers were also chosen to achieve our educational goal (Muench, 2000), namely, to enhance the skills of critical analysis of scientific literature (Figure 2A). The first paper used relatively straightforward experimental techniques and, importantly, contained drawbacks in experimental design and occasional flaws in data interpretation that did not require expert knowledge to detect. The second paper was selected by the students (via online voting) out of a group of articles from different fields in biology that were suggested by local biology faculty members as well-designed and important recent publications in their areas of research. The third and fourth papers were research articles that addressed the same experimental question but reached opposite conclusions (Figure 2A). These conflicting papers were included to prompt students to critically

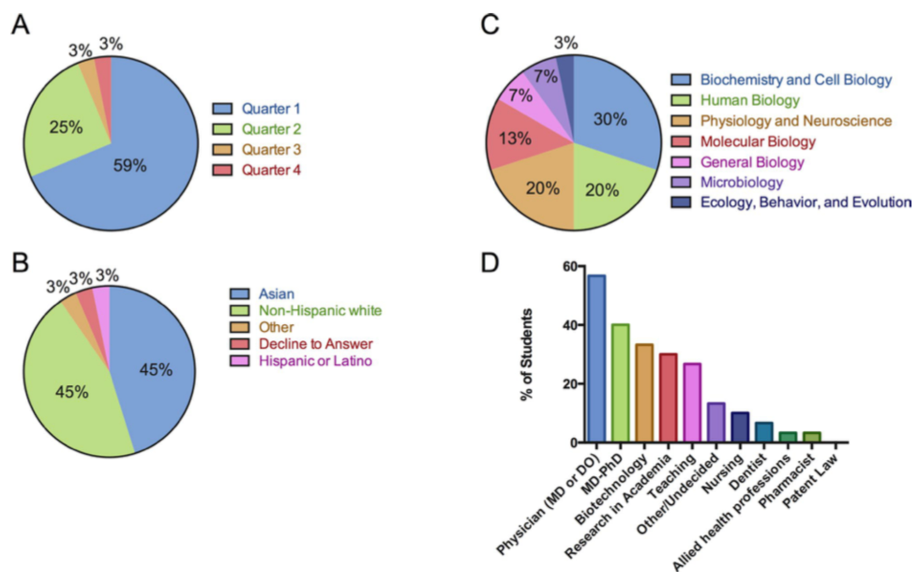

Figure 1. Students' demographics and career aspirations, based on an anonymous precourse survey. $n = 28$ students. (A) Quarter in the master's program. (B) Students' ethnic background. (C) UCSD biology major affiliation as undergraduate. (D) Students' current career aspirations. Students could select all career options they are currently considering from a list of options, so the sum of responses exceeds the total number of students.

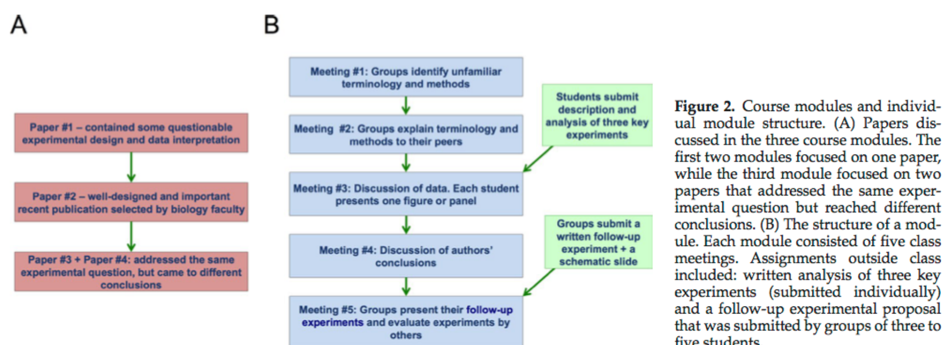

Figure 2. Course modules and individual module structure. (A) Papers discussed in the three course modules. The first two modules focused on one paper, while the third module focused on two papers that addressed the same experimental question but reached different conclusions. (B) The structure of a module. Each module consisted of five class meetings. Assignments outside class included: written analysis of three key experiments (submitted individually) and a follow-up experimental proposal that was submitted by groups of three to five students.

examine every aspect of the two papers to try to determine which group of authors had a stronger scientific argument.

Course Activities

The course met for 80 min twice a week over a 10-wk quarter. It had three modules, each module centered on the focus paper(s), with five meetings in each module (Figure 2B). We designed the modules to provide a stepwise, structured approach to a paper. Anonymous surveys from previous quarters indicated that unfamiliar background and unfamiliar experimental techniques were among the most difficult aspects of scientific papers for our students. To provide students with practice in overcoming these difficulties, during the first meeting of each module, we had student work in small groups to identify the important methods, terminology, and background they would need to know to understand the paper. Each group was assigned one or two of the identified techniques and background items, which they presented to the class in the second meeting of the module (Figure 2B).

Before the third meeting, students read the entire assigned paper and wrote individual analyses of three key experiments (as selected by the student). Guidelines for this assignment prompted the students to provide a detailed description of experimental setups and their own analyses of these experiments (Appendix A in the Supplemental Material). During the third meeting of the module, most students were asked to present at least one experiment from the paper, focusing on clearly describing how the experiment was performed and on their own interpretation of the data. Each figure presentation was followed by a brief question-and-answer session. The entire class was encouraged to participate in both asking and answering questions. The instructor also asked probing questions, prompting students to question authors' interpretations and conclusions, evaluate authors' experimental design, and decide whether additional controls were needed. Asking a thoughtful question and, especially, providing an answer to a question earned the students participation points. Each student had to accumulate at least 20 participation points to earn a perfect score in the "participation" category (accounting for 20% of the overall grade). These participation points seemed to provide students with an additional motivation to participate in the discussion, especially in the beginning of the course. In the second half of the course, participation in discussions became habitual for most of the students.

The fourth class meeting was dedicated to the discussion of the authors' conclusions and identification of questions that remain to be answered. The groups also worked on designing an experiment that would follow up on the paper. Before the last meeting of the module, each group of students submitted a written one-page proposal of the follow-up experiment. The guidelines for this assignment are provided in Appendix B in the Supplemental Material. Briefly, the assignment included articulating the experimental question and its importance, providing a detailed experimental design, including the controls, and then predicting the expected outcomes. Each group also provided a slide that contained a schematic of its experiment. During the last class meeting of each module, the groups presented their experimental proposals and evaluated proposals presented by other groups, acting as "grant panels," an activity described in the CREATE approach (Hoskins *et al.*, 2007; Hoskins and Stevens, 2009). Each experimental design presentation was followed by a brief question-and-answer session.

Science-Process Test

To assess the progress of our students in science-process skills, we developed a test in which students were asked to interpret data from two experiments, evaluate hypotheses based on these experiments, and propose their own experimental designs (Appendices C and D in the Supplemental Material). Because scientists use more than one approach to investigate a question, our test included two related experiments, one being a follow-up to the other. The experimental approaches and data presentation in this test were selected to minimize any specialized background knowledge required to understand the questions. The experimental condition utilized RNA interference–mediated knockdown of a target gene. While this technique may not be familiar to all the students, descriptions of the technique and its effects on expression of a gene were provided in the prompt. The students

were asked to consider two pieces of data that reflected the effects of a down-regulation of a hypothetical gene (gene X or Y) on cell numbers and programmed cell death (apoptosis). In the first part of the test (questions 1-1 through 1-3, Appendices C and D in the Supplemental Material), the students were asked to interpret data, draw conclusions, and evaluate a hypothesis based on the first piece of data. In the second part of the test (questions 2-1 through 4-1), the second piece of data was presented. Questions 2-3, 3-1, and 4-1 then prompted students to evaluate a hypothesis (question 2-3) and propose a new hypothesis based on both pieces of data (questions 3-1 and 4-1). Finally, in the third part of the test (question 4-2), the students were asked to design an experiment to test a hypothesis that a particular mutation in gene X (or gene Y) contributes to cancer development. Importantly, at this point, students could use the experimental approaches they saw in part 1 and 2 of the test as the basis for their own experimental design, thus minimizing the need for specialized knowledge in how cells can be manipulated. On the other hand, the students were also free to choose a completely different experimental approach.

Two isomorphic versions of the test were generated (Appendices C and D in the Supplemental Material). Development of the test was an iterative process, wherein the authors used comments from biology PhD students who took the test and data from the two quarters in which the test was piloted (Fall 2012 and Winter 2013) to revise and clarify the questions. The version used here (Fall 2013 and Winter 2014) was reviewed by three experts in biology education (who also had PhDs in biology) from three different institutions and was revised based on their comments. Finally, the alignment of the individual questions in our science-process skills test with the consensus critical-thinking skills (Facione, 1990) was conducted by nine biology faculty members at three different institutions and three postdocs and three graduate students who are members of the UCSD STEM-Education and Diversity Discussion group. The validators were provided with brief descriptions of each of the consensus critical-thinking skills as defined in the Delphi report and with the text that contained the full consensus descriptions of core critical-thinking skills and subskills (Facione, 1990). The validators were asked to select all core critical-thinking skills that were required to answer each of question in our test. The results of this survey for all questions, except for the experimental design question (Q4-2), are summarized in Supplemental Table S2. For each question, the core critical-thinking skill that received the most votes was designated as the primary skill, while the skill that received 50% or more votes was designated as the secondary skill.

Although not considered to be one of the consensus core critical-thinking skills in the Delphi report (Facione, 1990), experimental design is one of the core science-process skills and it aligns with the Bloom's category of synthesis. The experimental design score was based on seven components that included appropriateness (the match between the hypothesis and the proposed experiment), identification of experimental system, treatment, control group, assay, quantity measured, and expected outcomes (Supplemental Table S3). To decrease the probability of students unintentionally omitting aspects of experimental design, we included these elements in the prompt of the experimental design question. During the writing of this article, a paper by Dasgupta and colleagues was published that described a comprehensive review of the difficulties in experimental design that had been described in the K–12 and college education literature and that also provided a rubric of experimental design (RED) that targeted these identified difficulties (Dasgupta *et al.*, 2014). In Supplemental Table S3, we match the experimental design categories scored in our test with the experimental design difficulties identified by Dasgupta and colleagues and the difficulties assessed in the RED (Dasgupta *et al.*, 2014).

Science-Process Test Administration and Scoring

The tests were administered in class during week 1 (pretest) and week 10 (posttest) in a counterbalanced design, such that half of the students were randomly assigned to take version A as a pretest and version B as the posttest, and vice versa. Thirty-three students took both pre- and posttests in Fall 2013 and Winter 2014 quarters. The tests were deidentified, and each test received a randomly generated number. The tests were then evaluated by three of the authors, who are biology faculty members or graduate students in biology (C.A., E.T., and R.L.). The raters were blind to both students' identities and to the pre/post status of the test. A random sample of approximately 10 tests of each version was selected as a training set, and the scoring rubric was developed based on this sample. The three raters scored the entire training set together, discussing each score (Appendix E in the Supplemental Material). Each rater then scored the remaining tests independently. In cases in which the score for a particular question differed by 50% or more among the raters, all three raters re-examined the student's response and discussed the ratings. The goal of these face-to-face discussions was to make sure that the student's handwritten response was correctly read and a consensus interpretation was achieved. Each of the raters articulated his or her reasoning for giving the response a particular rating. In some cases, but not in all, these discussions lead the raters to revise their scores. Interrater reliability was high after the revision process (Cronbach alpha > 0.90 across 27 items). The tests were then reidentified, and pre- and posttests were matched. Dependent-measures t tests were used to assess changes in student performance between the pre- and posttest.

Students' Anonymous Surveys

Students' anonymous surveys were administered online via SurveyMonkey (www.surveymonkey.com), during the first and the last week of a 10-wk quarter. Both pre- and postversions of the surveys assessed students self-efficacy in skills related to critical analysis of primary literature (for the full list of self-efficacy questions, see Supplemental Table S2). The preinstruction survey also contained questions about students' demographics and career aspirations (Figure 1). The students received small course credit for completing the surveys. To allow matching between the beginning- and the end-of-the-quarter surveys while preserving the anonymity of responses, we asked students to provide the same five-digit number in both surveys. Twenty-eight students completed both pre- and postsurveys in the Fall 2013 and Winter 2014 quarters. Wilcoxon signed-rank tests were used to analyze the changes in students' self-efficacy ratings.

Institutional Review Board

Protocols used in this study were approved by UCSD Human Research Protections Program (project 111351SX).

RESULTS

Students' Self-Efficacy

To gain insight into whether students perceived any change in their science-process skills as a result of taking this course, we administered an anonymous online survey at the beginning and the end of the quarter. Using a five-point Likert scale ranging from "poor" to "excellent," we asked the students to rate their current skills in interpretation and inference (interpreting data from a paper and independently drawing conclusions), evaluation (critically evaluating authors' conclusions), and experimental design (proposing an experiment with the appropriate controls as follow-up on a paper). Thus, our survey provided us with a readout of students' self-efficacy: "the construct of perceived confidence in executing a given behavior" (Baldwin *et al.*, 1999). Because students' self-efficacy could depend on whether or not the paper was from the area of their master's research, we asked the students to evaluate their skills for a paper within and outside their research areas separately (Table 1). In Figure 3, we present the combined results (skills within and outside students' areas of expertise) of 28 pairs of students' responses, grouped into categories of interpretation and inference, evaluation, and experimental design. Supplemental Table S4 contains students' self-efficacy ratings for all individual questions.

At the beginning of the quarter, only 58% of the students rated their skills in interpretation and inference as good (32%), very good (19%), or excellent (7%; Figure 3). A substantial positive shift was observed at the end of the quarter, with 87% of the students rating their interpretation and inference skills as good (29%), very good (37%), or excellent (21%; Figure 3). The difference in students' self-efficacy ratings in analysis was statistically significant (Wilcoxon signed-rank test, $S = 89.50$, $p < 0.0001$, Cohen's $d = 1.11$, $n = 28$). In the categories of evaluation and experimental design, the students' ratings in the presurvey were lower than in the category of interpretation and inference. In the category of evaluation, 53% of the students rated their skills low in the beginning of the quarter, with 20% of the students rating their skills as poor and 33% as adequate (Figure 3). A statistically significant increase in students' self-efficacy in this category was observed at the end of the quarter ($S = 105.00$, $p < 0.0001$, Cohen's $d = 1.27$), at which time none of the students rated their skills as poor, and only 18% rated them as adequate (Figure 3). Similar trends were observed in students' self-efficacy ratings in experimental design: at the beginning of the quarter, 59% of the students rated their skills either as poor (29%) or adequate (30%; Figure 3). A significant shift ($S = 120.00$, $p < 0.0001$, Cohen's $d = 1.19$) in students' self-efficacy occurred at the end of the quarter: only 20% rated their skills as poor (2%) or adequate (18%; Figure 3).

Students rated their skills higher when asked about a paper within their areas of research, as opposed to a paper outside their areas of research (Supplemental Figure S1). For example, in the beginning of the quarter, when asked about their self-efficacy in proposing an experiment following up on a paper *outside* their areas of research, none of the students evaluated their skills as very good or excellent (Supplemental Figure S1A). At the same time, 28% of students evaluated their skills of proposing an experiment that would follow up on a paper *within* their areas of research as either very good (21%) or excellent (7%; Supplemental Figure S1A). At the end of the quarter, a statistically significant increase in

Table 1. List of questions used to assess students' self-efficacy in science-process skills in the context of scientific papers[a]

Interpretation and inference
Interpreting data in a paper *within* your area of research
Interpreting data in a paper *outside* your area of research
Independently drawing conclusions from data presented in a paper *in* your area of research
Independently drawing conclusions from data presented in a paper *outside* your area of research
Evaluation
Critically evaluating authors' conclusions in a paper *in* your area of research
Critically evaluating authors' conclusions in a paper *outside* your area of research
Experimental design
Proposing an experiment, with the appropriate controls, that would follow up on a paper *in* your area of research
Proposing an experiment, with the appropriate controls, that would follow up on a paper *outside* your area of research

[a]Questions were grouped in the categories of interpretation and inference, evaluation, or experimental design, as indicated.

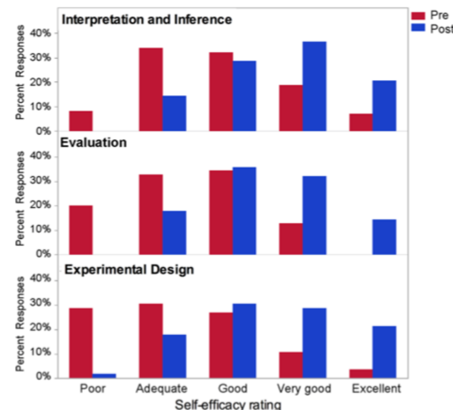

Figure 3. Students' self-efficacy in science-process skills in the context of primary literature. Twenty-eight pairs of matched responses from anonymous surveys given at the beginning (Pre) and end (Post) of the quarter were analyzed. A list of survey questions that were grouped into the categories of interpretation and inference, evaluation, and experimental design is provided in Table 1. "Percent responses" refers to the frequency of a specific rating (poor, adequate, etc.) among all responses to the questions that were grouped into the same category (interpretation and inference, evaluation, or experimental design).

self-efficacy in proposing follow-up experiments to papers both within and outside of students' areas of research was observed (within: $S = 79.00$, $p < 0.0001$; outside: $S = 99.50$, $p < 0.0001$). After instruction, 36% of students evaluated their self-efficacy in proposing an experiment that would follow up on a paper *outside* their areas of research either as very good (29%) or excellent (7%; Supplemental Figure S1A). When asked about proposing experiments *in their own* fields, 64% rated their skills at the synthesis level as either very good (29%) or excellent (36%; Supplemental Figure S1A).

Analysis of Science-Process Skills Test

Students' surveys pointed to high gains in their *perceived* level of science-process skills in the context of analysis of scientific papers; however, we wished to examine whether we could also detect measurable changes in student performance in science-process skills in the context of biological experiments. To that end, we designed two isomorphic versions of a test in which students were presented with a sequence of two experiments that examined the same experimental system (Supplemental Material, Appendices C and D). In the test, students were asked to analyze data, draw conclusions, and evaluate and propose hypotheses based first on one piece of data and then on both pieces of data, and to design a follow-up experiment (see Supplemental Table S2 for alignment of the questions with the consensus core critical-thinking skills and Supplemental Table S3 for the components of the experimental design score). The data in the test were presented and described in such a way as to minimize the demands for a specialized subject and technical knowledge in any specific area of biology. Thirty-three paired (pre- and postinstruction) tests were rated by three raters blind to both the identity of the students and the pre/post status of the test (the scoring rubric is provided in Appendix E in the Supplemental Material). Dependent-measures t tests were used to assess whether preinstruction and postinstruction scores in each of the categories were statistically different. No statistically significant changes in posttests were observed in the categories of interpretation, inference, and evaluation (Figure 4A). Statistically significant gains were detected in the experimental design category ($p = 0.039$, Figure 4A). More detailed analysis of student performance in each category is presented below.

In the interpretation category, students scored very high in both the pre- and posttest (89.9% and 94.1%, respectively), indicating the data presented in the test were accessible to the vast majority of the students (Figure 4A). The inference category questions probed for two types of skills: drawing conclusions from two pieces of experimental data presented in the test and proposing hypotheses (Appendix E in the Supplemental Material). The average pre- and postscores in the "drawing conclusions" subcategory were higher than the corresponding scores in the "proposing hypothesis" subcategory, implying that students found the latter skill more challenging (Supplemental Figure S2A). Positive but not statistically significant trends were observed in student performance postcourse in both subcategories (Supplemental Figure S2A).

Questions aligned with the evaluation skill asked students to evaluate a hypothesis, based first on one piece of data and then on two pieces of data. Not surprisingly, the latter task was more challenging to the students: in the pretest, the average score for the evaluation question based on one piece of data was 81%, while the average score for the evaluation question based on two pieces of data was 66.3%, a 14.7% difference (Supplemental Figure S2B). At the end of the course, the difference decreased to only 2.4% (Supplemental Figure S2B); however, this change was not statistically significant ($p = 0.191$).

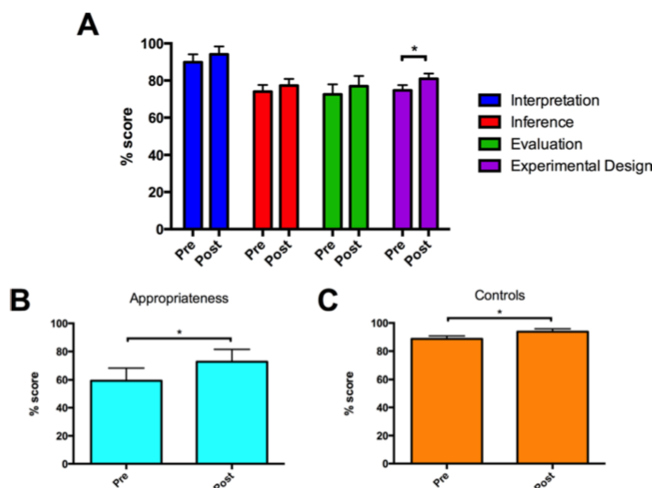

Figure 4. Analysis of science-process tests. Thirty-three pairs of matched tests from the beginning (Pre) and end (Post) of the quarter were evaluated. (A) Student performance in the categories of interpretation, inference, evaluation, and experimental design, before and after instruction. Small but statistically significant gains were observed in the experimental design category ($p = 0.039$, Cohen's $d = 0.379$). (B) Average pre- and posttest score in the category of appropriateness. The increase in the posttest scores was statistically significant ($p = 0.005$, Cohen's $d = 0.526$). (C) Average pre- and posttest score in the category of interpretation of controls. The increase in the posttest scores was statistically significant ($p = 0.049$, Cohen's $d = 0.37$).

A statistically significant increase was observed in students' ability to propose an experiment based on a given hypothesis ($p = 0.039$, Cohen's $d = 0.379$). A similar statistically significant increase in experimental design ability was observed in the two quarters in which this test was piloted (Fall 2012 and Winter 2013): 61.8% for the mean pretest score and 72.2% for the mean posttest score ($p = 0.011$, $n = 42$; Supplemental Figure S4A). In the 2012–2013 version of the experimental design question, the follow-up experiment was not constrained to a specific hypothesis: students were asked to propose any experiment that would follow up on the experiments presented in the test.

Experimental Design Ability

In the experimental design part of our science-process test, students were given a scenario that described a large family with high incidence of certain cancer. Genomic sequencing of the family members revealed a correlation between the presence of a particular mutation in the gene interrogated in the first part of the test and the development of brain cancer (version A) or colon cancer (version B). Students were asked to design an experiment that would test the hypothesis that this mutation contributes to cancer development. As in a real scientific investigation, more than one appropriate experiment could be proposed. The experimental design score consisted of seven components: appropriateness to the goal of investigation, identification of an experimental system, treatment, assay, quantity measured, identification of controls, and statement of anticipated outcomes (see Supplemental Table S3 for the alignment of these components with the previously described difficulties in experimental design identified in Dasgupta *et al.*, 2014). The experimental design component in which the students had the lowest pretest mean score (59.5%) was appropriateness of the proposed experiment to the goal of investigation (Figure 4B and Supplemental Figure S3). Examples of quotes taken from students' responses relevant to the appropriateness of the experimental design are shown in Table 2. Experiments that appropriately addressed the given hypothesis (a specific mutation in gene X or Y contributes to brain or colon cancer development) ranged from introducing this mutation into animals and looking for tumor development (example 1272) to transfecting the gene with the mutation into brain or colon cells and looking for increased cell proliferation (example 8529). Partially correct or incorrect responses proposed

Table 2. Example quotes of students' responses relevant to the "appropriateness" of the proposed experimental design to the goal of investigation[a]

Test number	Student response	Quality of response/ rater's comments	Score (out of 2)
1272	"In vivo assay testing the proliferation effects of Gene Y mutation on tumor formation. Gene Y mutation containing colon cancer cells will be injected subcutaneously *in different amounts* into immunodeficient mice and monitored biweekly for tumor formation. Controls: inject healthy colon cells without gene Y mutation into mice at some place."	Appropriate experiment. Note that the italicized text indicates a problem with combinatorial reasoning (Dasgupta *et al.*, 2014): different numbers of cells are injected in the treatment but not in the control condition. This aspect of the experimental design is evaluated in the "treatment/independent variable" category.	2
8529	"The assay would be whether or not the rats develop colon cancer … We would be observing the appearance of colon cancer in rats that are just past middle aged. Controls would include: healthy rats with no gene Y mutation; gene Y mutation rats."	Appropriate experiment. Note that the student considers the treatment condition to be one of the controls.	1.67
6582	"Use cultured cells to introduce the same Gene Y mutation as seen in humans … perform a proliferation assay to measure the number of proliferated cells."	Partially correct response. The response describes an appropriate experiment; however, it does not address the colon cancer aspect of the prompt.	1.5
3064	"I would perform an experiment on cultured cells to overexpress Gene X and look at its effects by transfecting cells with an expression vector that contains Gene X and a strong promoter … A proliferation assay would be performed on all the controls and the Gene X overexpression cells."	Inappropriate experiment for the given hypothesis (does *the specific mutation* in gene X contribute to cancer). The experiment proposed by the student addresses a related question: Does overexpression of gene X contribute to cancer?	1
1524	"Transfection of Gene Y into non-Gene Y expressing cells to look for increased proliferation."	Inappropriate experiment for the given hypothesis. The experiment proposed here tests a related question: Does the expression of Gene Y contribute to proliferation?	0.5
7803	"Clinical study w/human patients to see if there are any individuals w/o gene X mutant, but w/cancer."	Inappropriate experiment for the given hypothesis. The experiment proposed here tests a different question: Can cancer develop without a mutation in gene X?	0

[a]The scores are the average scores of three raters. The complete student responses quoted here are shown in Supplemental Table S5.

experiments that aimed to answer different, although related, questions, such as:

Example 3064: Does overexpression of gene X increase cell proliferation? (note that the effect of the specific mutation is not tested here)
Example 1524: Does expression of gene Y increase cell proliferation?
Example 7803: Can a person have cancer without having a mutation in gene X?

The subcategory of appropriateness also showed the largest and the only statistically significant increase in the posttest mean score (to 72.5%, $p = 0.005$, Cohen's $d = 0.526$; Figure 4B). Components of experimental design in which students performed very well in the pretest and did not show statistically significant change in the posttest were clear identification of experimental system ("experimental subject" in Dasgupta *et al.*, 2014) and inclusion of an appropriate control group (Supplemental Figure S3). No significant changes were observed in such components of experimental design as "independent variable/treatment," "quantity measured," and "expected outcomes" (Supplemental Figure S3). This lack of change could be due, in part, to the fact that the experimental design question prompted the students to include the experimental system, the assay (treatment), what will be measured, and the controls. A very similar science-process test used in Fall 2012–Winter 2013 quarters in this course did not specify which components of experimental design should be included, instead prompting the students to "include all relevant components of experimental design in your experiment." Importantly, the students were free to propose any experiment that would follow up on the data given in the test. Forty-one pairs of pre- and postquarter tests were scored by three raters who were blind to students' identities and the pre- or postcourse status of the test. Statistically significant gains were observed in "experimental system," "independent variable/treatment," "assay," and "quantity measured" components of experimental design (Supplemental Figure S5). The precourse scores in most components of experimental design were also substantially lower in this version of the test in comparison with the later version (compare Supplemental Figures S3 and S5).

Gains in Interpretation of Controls and Quantitative Analysis of the Data

In the analysis of the test questions aligned with interpretation, questions pertaining to identification and interpretation of controls were analyzed separately. Students' tests were scored based on their ability to correctly identify controls in the two experiments described in the test and explain why these controls were included (Figure 4C). The students performed very well in this category in the pretest: the average score in this category was 88.6%. A small but statistically significant increase was observed in this category in the posttest: the mean score was 93.8% ($p = 0.0491$, Cohen's $d = 0.37$; Figure 4C).

Analysis of the science-process tests also revealed statistically significant increases in students' average scores for quantitative analysis of the data (Figure 5). The score in the "quantitative data analysis" category was determined based on the presence and correctness of the comparison between experimental and control data in quantitative terms (e.g., percentages, fold difference; see Appendix E in the Supplemental Material). Throughout the course, students were encouraged to use quantitative terms both in their written paper analyses, where quantifications of differences between experiment and controls were part of the grade, and in the in-class discussions. We observed more than a twofold increase in the average postinstruction score (mean pretest score: 26.7%, mean posttest score: 60.0%, $p = 0.0002$, Cohen's $d = 0.728$). A similar increase was observed in the two quarters in which this test was piloted (Fall 2012 and Winter 2013, $p = 0.015$, Cohen's $d = 0.556$, $n = 41$; Supplementary Figure S4B).

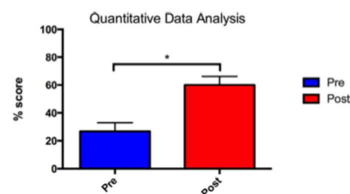

Figure 5. Average pre- and posttest scores in the quantitative data analysis category. The increase in the posttest scores was statistically significant ($p = 0.002$, Cohen's $d = 0.728$, $n = 33$ pairs of pre- and posttests).

DISCUSSION

The course described here utilized four primary research papers from diverse topics in biology, selected with the goal of providing students with opportunities to practice science-process skills. The first paper had drawbacks in experimental design and data interpretation. The second paper was an exemplary scientific investigation. The third and the fourth papers investigated the same experimental question but came to different conclusions. This instructional approach correlated with highly significant increases in students' self-efficacy in a variety of science-process skills: drawing conclusions from data presented in scientific papers, critically evaluating authors' conclusions, and designing an experiment that would follow up on a paper. Using a science-process test that we developed, we detected a statistically significant increase in students' ability to propose an experiment appropriate to the goal of investigation, describe data in quantitative terms, and interpret controls. However, we did not detect statistically significant increases in students' performance on questions aligned with data interpretation, inference, and evaluation.

Gains in Proposing an Experiment Appropriate to the Goal of Investigation

We detected a statistically significant increase in students' ability to propose an experiment that matched the given hypothesis. Analysis of students' responses indicated that students often came up with an experimental design that was testing a related but different hypothesis. Designing

an experiment in which the treatment or the outcomes appropriately address a specific research question represent known areas of difficulty (Dasgupta *et al.*, 2014). We did not detect statistically significant gains in other components of experimental design, such as identification of experimental system, treatment, assay, quantity measured, identification of controls, and statement of anticipated outcomes. A possible contributor to this lack of observed increase is the fact that the experimental design prompt in the test directed the students to provide these components. The purpose of this inclusion was to uncover any ideas students might have about these components; however, this prevented us from assessing what components of experimental design students would provide without being prompted and may have limited our ability to detect changes in experimental design skills. Indeed, when an earlier version of this test that did not provide such prompts was used in this class (Fall 2012 and Winter 2013), statistically significant increases in "experimental system," "independent variable/treatment," and "quantity measured components" were detected. Our ability to compare between the two versions of the test is limited, however, by the fact that the earlier version prompted students to propose any experiment to follow up on the data described in the test, whereas in the later version, students were asked to design an experiment that would test a given hypothesis.

Several potential confounding factors could influence the gains we saw in the appropriateness of the proposed experiments. These include:

1. If students analyzed or designed similar experiments during this course, they might be expected to perform better in the posttest.
2. Students whose major was outside the cell/molecular biology field (e.g., ecology, behavior, and evolution majors who comprised 3% of our students) would be expected to gain knowledge in this field after taking this course and therefore perform better in the posttest.
3. Students' own lab research, as well as other courses they were taking during the same quarter, could positively contribute to their performance in the posttest.

While we acknowledge a possible and likely contribution of the second and the third confounders, we believe that the first confounder is unlikely to explain the gains we observed. None of the papers that we examined in this class involved cancer, which was the topic of the questions in our science-process test, and follow-up experiments proposed by the students during the course were therefore different from those proposed by students in the test. We sought to minimize the effects of the second confounder, subject knowledge gain, by selecting experimental approaches and data presentation used in the test to minimize any specialized background knowledge in biology needed to understand the experiments (the students were expected to know, however, that it is possible to isolate or generate a mutant version of a gene and introduce it into a cell). The effect of familiarity with specific techniques in cell and molecular biology was minimized by the fact that students could use the experimental techniques described in the first part of the test (transfection of cells, cell counts, or apoptosis assays) and adopt them to their experimental design (this strategy was used in only two responses). Finally, the specific component of experimental design that significantly increased after instruction was the match between the proposed experiment and the experimental hypothesis provided. Increase in this category cannot be easily explained by increase in subject knowledge or familiarity with a particular experimental technique.

We could not directly compare our study with other studies that quantified the effects of interventions aimed to enhance the skills of experimental design, because these studies were conducted in different levels of classes (introductory and nonmajor) and utilized different assessment methods (Sirum and Humburg, 2011; Gottesman and Hoskins, 2013; Brownell *et al.*, 2014). Sirum and Humburg (2011) and Gottesman and Hoskins (2013) used the Experimental Design Ability Test (EDAT), an open-response test that measures students' ability to design an experiment testing a claim about the effectiveness of an herbal supplement (Sirum and Humburg, 2011). A student's response in EDAT is scored based on parameters incorporating the fundamental elements of experimental design (in contrast to our test, the EDAT prompt does not include directions as to which experimental design elements should be included). The Expanded EDAT (E-EDAT) uses a similar prompt, with modifications that include asking students to provide justification for their responses (Brownell *et al.*, 2014).

While EDAT and E-EDAT assess general, not subject-specific, experimental design skills, our test probed students' ability to design an experiment using discipline-specific knowledge expected from a graduate with a BS in biology. As we will argue below, the ability to apply discipline-specific knowledge and its "methodological principles" (Facione, 1990, p. 5) is essential to critical thinking within the discipline (Facione, 1990; Bailin *et al.*, 1999; Willingham, 2008). Such discipline-specific experimental design skills as identifying and explaining the purpose of controls in the context of complex biological experiments were the focus of the investigation by Shi and colleagues, who demonstrated that an online tutorial and seven in-class exercises resulted in significant gains in these skills in a sophomore-level cell biology lab course (Shi *et al.*, 2011).

Our assessment did not include such important elements of EDAT or E-EDAT as understanding that experiments have to be repeated, evaluation of the sample size, and knowledge that one can never unambiguously prove a hypothesis (Sirum and Humburg, 2011; Brownell *et al.*, 2014). However, our test included an assessment of the appropriateness of the proposed experiment to the hypothesis being tested, identification of an appropriate control group, and statement of outcomes that would support the hypothesis. In future studies, it will be interesting to compare students' performance in EDAT or E-EDAT and our revised test (in which the prompts of experimental design components will be removed).

Recently, two new tools have become available to assess experimental design abilities at more advanced levels (Dasgupta *et al.*, 2014; University of British Columbia, 2014). The RED, which uses open-response answers, can be used to examine the salient features of both content-specific and content-independent experimental design questions (Dasgupta *et al.*, 2014). The Experimental Design (Third/Fourth Year Undergraduate Level) Concept Inventory is a validated tool that allows examination of students' knowledge of

experimental design in a multiple-choice format (University of British Columbia, 2014).

Skills of Interpretation, Inference, and Evaluation

Our students reported high gains in self-efficacy with respect to data analysis, drawing independent conclusions, and evaluating authors' conclusions from papers within or outside their areas of research. However, we observed no statistically significant gains in the postcourse scores in questions assessing the skills of interpretation, inference, and evaluation of data. Possible interpretations of the observed lack of gain in these skills include: limitations of our science-process test, the teaching format in which these skills were practiced, and insufficient amount of time to cause a measurable increase in these skills, as outlined below.

One limitation of our science-process test is that, while it was designed to be accessible to all the students, it was not challenging enough: in most categories of the test, students' mean scores were already high (above 70%) in the pretest. Interestingly, the only component of experimental design that increased significantly, appropriateness of the proposed experiment, was also the most challenging to the students, with the lowest precourse mean score (59.5%). Increasing the difficulty level of our test might increase its sensitivity for measuring these changes over a term. These shortcomings can be addressed in future versions of the science-process skills test.

If the observed difference between students' gains in experimental design but not in interpretation, inference, and evaluation is not due to the limitations of our test, it might be due to the different ways in which these skills were practiced in this course. Students worked in groups to propose three follow-up experiments. Active group participation was encouraged by the use of peer evaluation. On the other hand, the practice of the skills of data interpretation, inference, and evaluation of authors' conclusions was more individualized. Students submitted analyses of three written experiments, typically presented one experiment in front of the class, and listened to their peers presenting the rest of the experiments. Questions to student presenters were encouraged, but typically only several students asked questions after a presentation of each experiment, partly because of time limitations. We believe that it will be of interest in the future to test the effects of additional group activities specifically targeting interpretation, inference, and evaluation skills.

How do our results compare with similar published interventions? Semester-long courses that utilized the CREATE approach to introducing primary literature have been reported to result in statistically significant gains in analyzing data and drawing logical conclusions, as assessed by pre/postcourse tests in an upper-division seminar class (Hoskins *et al.*, 2007), as well as in a freshman-level class, when assessed by the CAT (Gottesman and Hoskins, 2013). It is difficult to draw direct comparison between these studies because of the multiple ways in which they differ: the duration of the course (semester vs. quarter, in our study), the number of papers examined (fewer in our study), the specific format of the course (a sequence of papers from the same lab in CREATE vs. papers from different labs or different fields in our study), and the level of the students (undergraduate vs. master's in this study). Additionally, we designed our test to measure biology content–specific science-process skills, whereas the CAT test (Stein *et al.*, 2012) is not subject specific. Currently, the use of validated critical-thinking tests, such as the CAT (Stein *et al.*, 2012) or the California Critical Thinking Skills Test (Facione and Facione, 1998) is associated with the investment of monetary and faculty time resources (CAT) that were not accessible to the authors. An additional drawback of these tests is that they are not biology specific and therefore do not examine discipline-specific science-process skills.

Are critical-thinking skills discipline specific? We would argue that they have a significant discipline-specific component. Many experts in the critical-thinking field agree that critical thinking within a discipline is deeply connected to discipline-specific knowledge and ways of reasoning (Facione, 1990; Bailin *et al.*, 1999; Willingham, 2008; reviewed in Lai, 2011). For example, in his summary of the Delphi report, Facione states:

> Although the identification and analysis of critical thinking skills transcend, in significant ways, specific subjects or disciplines, learning and applying these skills in many contexts requires domain-specific knowledge. This domain-specific knowledge includes understanding methodological principles and competence to engage in norm-regulated practices that are at the core of reasonable judgments in those specific contexts … Too much of value is lost if critical thinking is conceived of simply as a list of logical operations and domain-specific knowledge is conceived of simply as an aggregation of information. (Facione, 1990, p. 5, quoted in Lai, 2011)

While we think that our test is a useful step in the direction of testing biology-specific critical-thinking and science-process skills, there is a great need for a free, robust, and validated instrument to measure these skills in biology.

Increases in Quantitative Analysis of the Data

We observed a more than twofold increase in the "quantitative data analysis" score, which reflected the frequency and the correctness of quantitative comparisons the students used when describing the differences between experimental and control conditions in the science-process tests. This suggests that the interventions implemented in this course, such as encouraging the use of quantitative description both in written and in oral analyses of experiments, increased students' quantitative literacy, the ability to apply basic quantitative skills to independently analyze data and evaluate claims, an important component of scientific reasoning (National Council on Education and the Disciplines, 2001).

Summary and the Implications of the Increase in Students' Self-Efficacy

How successful was this course in increasing science-process skills of our students? The results we reported point to a mixed success. Using our science-process skills test, we detected statistically significant increases in the use of quantitative terms in data analysis, understanding of controls, and designing an experiment appropriate to the goal of investigation. However, we failed to detect a statistically significant

increase in students' performance in questions that required data interpretation, inference, and evaluation of hypotheses. We are currently unable to distinguish between the influences of the course format, the duration of the instruction, and a possible lack of discrimination power of our test as potential interpretations of these findings. A validated and freely accessible instrument that measures students' science-process skills in a biological context will be extremely valuable in assessing the efficacy of different instructional approaches in fostering these skills.

Finally, the instructional approaches described here resulted in significant increases in students' self-efficacy in a variety of science-process skills associated with thoughtful engagement with primary literature, such as independently drawing conclusions from papers' data, evaluating authors' conclusions, and designing a follow-up experiment. The discrepancy between students' self-efficacy and actual academic performance has been previously described in multiple studies (Boud and Falchikov, 1989; Falchikov and Boud, 1989; Dunning *et al.*, 2003, Lawson *et al.*, 2007). Overestimation of academic skills tends to be more pronounced among novices and lower-performing students (Dunning *et al.*, 2003). Because of their graduate status and demonstrated good academic achievement (a requirement for admission into the master's program), our students would be predicted to provide a more accurate assessment of their self-efficacy. Indeed, in the beginning of the course, our students rated their self-efficacy in science-process skills quite low. After instruction, students reported high gains in their self-efficacy, but only limited increases in science-process skills were observed using our test. In future studies, it will be interesting to compare students' self-efficacy and their objective gains in science-process skills using a more robust and validated test.

Collaborative learning and question-and-answer activities, frequently used in this course, are likely contributors to students' gains in self-efficacy. A significant positive effect of these active-learning approaches has been observed in introductory physics classes for nonmajors (Fencl and Scheel, 2005). Personal successful performance of a task is one of the most significant contributors to self-efficacy (Bandura, 1977); therefore, repeated practice of critical analysis of scientific papers in written assignments and oral presentations and the group experimental design activities that were part of this course are also very likely contributors to the observed gains in students' self-efficacy. Observing successful performance of the assessed skills by peers (vicarious experience) is another important contributor to self-efficacy (Bandura, 1977); therefore, multiple student presentations in this course could also have contributed to the overall increase in students' self-efficacy.

The increased self-efficacy is likely to empower students to read more scientific papers and to do so with thoughtful and critical attitudes. It can also have broader effects: a large body of research in psychology documents a positive relationship between academic self-efficacy and students' persistence, performance, and career aspirations (reviewed in Bong and Skaalvik, 2003). For example, Lent and colleagues (1986) showed that undergraduate students who reported higher academic self-efficacy were more likely, 1 yr later, to take courses in science and technology, achieve higher grades in these courses, and consider a wider range of career options in science and technology (this correlation was independent of past academic achievement and interest in the subject). The fact that a 10-wk-long structured engagement with primary literature can produce significant shifts in students' self-efficacy is very encouraging. Further research is required to determine whether the observed increases in self-efficacy correlate with higher performance in following graduate courses, higher number of publications by the students, and metrics of career success.

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Chapter VII:

Perceived challenges in primary literature in a Master's class: effects of experience and instruction

Perceived Challenges in Primary Literature in a Master's Class: Effects of Experience and Instruction

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ABSTRACT

Primary literature offers rich opportunities to teach students how to "think like a scientist," but the challenges students face when they attempt to read research articles are not well understood. Here, we present an analysis of what master's students perceive as the most challenging aspects of engaging with primary literature. We examined 69 pairs of pre- and postcourse responses from students enrolled in a master's-level course that offered a structured analysis of primary literature. On the basis of these responses, we identified six categories of challenges. Before instruction, "techniques" and "experimental data" were the most frequently identified categories of challenges. The majority of difficulties students perceived in the primary literature corresponded to Bloom's lower-order cognitive skills. After instruction, "conclusions" were identified as the most difficult aspect of primary literature, and the frequency of challenges that corresponded to higher-order cognitive skills increased significantly among students who reported less experience with primary literature. These changes are consistent with a more competent perception of the primary literature, in which these students increasingly focus on challenges requiring critical thinking. Students' difficulties identified here can inform the design of instructional approaches aimed to teach students how to critically read scientific papers.

INTRODUCTION

Primary literature provides educators with an excellent opportunity to train students how to "think like a scientist": analyze data, draw conclusions, and identify follow-up directions to a scientific investigation. In their report *Scientific Foundations for Future Physicians*, the American Association of Medical Colleges and Howard Hughes Medical (AAMC-HHMI) Institute listed the ability of critical reading and evaluation of scientific papers as one of the competencies that students should possess before entering medical school (AAMC-HHMI, 2009, p. 26). The *Vision and Change* report by the American Association for the Advancement of Science (AAAS) highlighted C.R.E.A.T.E. (Consider, Read, Elucidate hypotheses, Analyze and interpret data, and Think of the next Experiment), a structured approach in which students analyze four papers from the same lab (Hoskins *et al.*, 2007), as an important way to introduce scientific research in nonlab courses (AAAS, 2011, pp. 32–33).

Many studies have described the use of primary literature in the classroom (e.g., Janick-Buckner, 1997; Muench, 2000; Kozeracki *et al.*, 2006; Hoskins *et al.*, 2007; Krontiris-Litowitz, 2013; Round and Campbell, 2013; Sato *et al.*, 2014; Van Lacum *et al.*, 2014; Abdullah *et al.*, 2015). Analysis of primary literature has frequently been associated with gains in multiple aspects of students' academic self-efficacy, such as being able to understand scientific articles, science literacy, and their sense of belonging to the scientific community (Kozeracki *et al.*, 2006; Hoskins *et al.*, 2011; Gottesman and Hoskins, 2013; Van Lacum *et al.*, 2014; Abdullah *et al.*, 2015).

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Some studies have also reported the positive effects of the use of primary literature on students' science literacy skills (Krontiris-Litowitz, 2013), ability to critically read research articles (Van Lacum *et al.*, 2014), and science process/critical -thinking skills (Hoskins *et al.*, 2007; Gottesman and Hoskins, 2013; Segura-Totten and Dalman, 2013; Sato *et al.*, 2014; Abdullah *et al.*, 2015).

The benefits of reading primary literature notwithstanding, instructors have detected that students struggle with some of its aspects (Gehring and Eastman, 2008; Krontiris-Litowitz, 2013; Van Lacum *et al.*, 2014). A study by Krontiris-Litowitz (2013) demonstrated that students enrolled in an introductory biology course that focused on development of scientific literacy skills had difficulties connecting the goal of investigation to the previous studies and understanding the study's hypotheses and the purpose of using a certain method to test the stated hypothesis. Data interpretation was another challenge (Krontiris-Litowitz, 2013), and using the data to justify the authors' conclusions presented a difficulty to both lower-division and upper-division biology undergraduates (Gehring and Eastman, 2008; Krontiris-Litowitz, 2013). Van Lacum and colleagues reported that 44–88% of students enrolled in a freshmen-level life sciences course could not correctly identify such elements of scientific articles as the motivation for a study, its main conclusion, and its implications (Van Lacum *et al.*, 2014). Gehring and Eastman reported that upper-division biology students had difficulties integrating an article's data with other published results (Gehring and Eastman, 2008). Among other recognized barriers to the primary literature are the unique characteristics of scientific texts: their high informational content, discipline-specific jargon, and the formal writing style that differs from textbooks or informal texts that students often find online (Gehring and Eastman, 2008; Snow, 2010). Together, these studies have revealed what instructors find their students struggling with as they begin to read the primary literature. However, students' perspectives on the challenges they face when reading scientific articles have not been systematically studied. Understanding these challenges is essential for developing effective teaching methods that help students to engage with the primary literature.

METHODS

Participants

This study focused on students enrolled in the contiguous BS/MS program of the Division of Biological Sciences in a large public university classified as RU/VH (very high research activity) in Southern California. Students enter this program during their senior year as undergraduates, complete at least six quarters of research (typically, three quarters as undergraduates, followed by at least three quarters of graduate research in the same lab), and defend a research-based thesis.

Data on students' demographics, majors, and prior experiences with primary literature were collected over four quarters from 69 students (representing 86% of students enrolled in this course during these quarters) via anonymous pre- and post-course surveys (Figure 1 and Supplemental Figure S1, B and C). The majority of students participating in this study were in their first three quarters of the master's program (88% of the students; Supplemental Figure S1A).

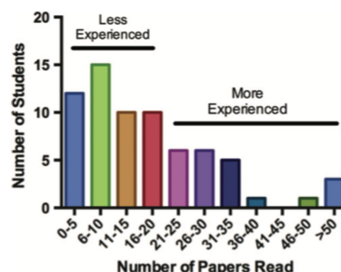

FIGURE 1. Self-reported previous exposure to primary literature, based on an anonymous survey completed before instruction. Students were asked "Approximately how many research papers have you carefully read and analyzed during your undergraduate and graduate studies?" and the response choices are shown on the X axis. $n = 69$ students. An arbitrary cutoff of 20 papers read was used to subdivide the students into "less experienced" and "more experienced" groups.

Additionally, we were interested in our students' previous experience with scientific papers and the types of instruction they had received on how to read research papers before taking this course. Only 26% of students had been explicitly taught how to read scientific articles. The distribution of self-reported number of papers read before taking this class is shown in Figure 1 ($n = 69$ students). Previous exposure to scientific literature varied among the students. More than two-thirds of the students ($n = 47$) had read 20 or fewer papers before taking this course, while the rest ($n = 22$) reported having read more than 20 articles. These two groups will be referred to as "less experienced" and "more experienced" in reading primary literature, respectively (Figure 1).

Demographic differences between the less experienced and the more experienced students were examined using Fisher's exact test (Supplemental Table S1). The two groups did not differ in terms of how long they had been in the master's program, major, ethnicity, grade point average (GPA), or gender (the data for GPA and gender were only available for the Fall 2013 and Winter 2014 quarters; Supplemental Table S1). We detected a positive but not significant relationship between belonging to the more experienced group and reporting having been taught a strategy for reading scientific papers and being more likely to participate in a journal club (Supplemental Table S1 and Supplemental Figure S1C).

Course Structure

A detailed description of this course is provided elsewhere (Abdullah *et al.*, 2015). In brief, the course was designed for master's students with the goal of providing training in critical analysis of primary literature. Rather than being focused on a specific topic, the papers discussed in this course were taken from different fields in biology, with the aim of providing students with universal skills that can be implemented in reading a paper on any topic in biology. Four articles were used: a paper that contained flaws in experimental design and data interpretation, an exemplary paper, and a pair of conflicting papers that

examined the same biological phenomenon but reached opposing conclusions. The papers are listed in the Supplemental Material, Appendix B. In selecting a flawed paper (paper 1), the course instructor (E.T.) was looking for a study in which detection of the flaws in experimental design and data interpretation did not require expertise in the topic of the paper or in the experimental procedures used in the paper. Similarly, we think that in selecting the appropriate pair of conflicting papers, it is best to select a pair in which determination of which paper presents a more convincing argument should not require specialized technical knowledge. In this sense, one of the pairs (papers 3 and 4 used in Winter 2014) was preferable to the other pair (used in Fall 2013), which required a more specialized knowledge to determine which was more convincing. Finding suitable papers for this course was serendipitous for some of the papers (papers 1 and 2 used in Fall 2013; papers 3 and 4 used in Winter 2014). The rest of the papers were identified by University of California, San Diego (UCSD), faculty after the instructor (E.T.) emailed them a brief description of the course and a request for flawed, exemplary, or conflicting papers.

Examination of each paper included five meetings consisting of the following activities performed by the students: 1) identification of unfamiliar methodology, terminology, and background; 2) presentation of unfamiliar methodology, terminology, and background; 3) critical interpretation of experimental data; 4) discussion of authors' conclusions and identification of unanswered research questions; and 5) design and presentation of a follow-up experiment and critique of experiments proposed by others. Students were graded on all of the activities described.

Surveys

Anonymous surveys were administered online via SurveyMonkey (www.surveymonkey.com), during the first and last weeks of a 10-week quarter. In addition to the question about the most challenging aspects of the primary literature, the survey included questions about students' self-efficacy in a number of skills related to the primary literature (Abdullah *et al.*, 2015; Supplemental Material, Appendix C). The precourse (but not the postcourse) survey also included questions about students' demographics, majors, and previous exposure to primary literature (Supplemental Material, Appendix C). To allow matching between the pre and post surveys while preserving the anonymity of responses, we asked students to provide the same five-digit number in both surveys. The students received a small number of course points for completing the surveys. Overall, 69 students (86% of students enrolled in this course) completed both pre and post surveys in the Fall 2012, Winter 2013, Fall 2013, and Winter 2014 quarters.

Qualitative Analysis of Students' Surveys

Using the anonymous surveys, three raters examined 69 pairs of pre- and postcourse free responses to the question "What aspects of understanding and analyzing scientific papers do you find most challenging?" The raters (E.T., a faculty member, and R.L. and C.A., both biology graduate students) were blind to both the students' identity and the pre- or postcourse status of the responses. Our analysis was aligned with grounded theory: we examined students' responses, seeking to describe all challenges identified by the students, as opposed to approaching the data with preconceived hypotheses about the nature of these challenges (Corbin and Strauss, 2008; Andrews *et al.*, 2012). Finally, we wanted to determine whether the nature or the frequency of challenges identified by the students changed after instruction and, if so, what hypotheses could be produced to explain this phenomenon. The three raters discussed each response until a consensus on the challenges described in the response was reached. Table 1 presents examples of students' responses and the challenges identified in these responses. Many students chose to write about more than one challenge in reading and analyzing scientific papers (Table 1). The average number of challenges per response was 1.6, with a range of zero to four challenges per response. Of the responses from pre- and postcourse surveys, 19 and 17%, respectively, contained statements that could not be interpreted unambiguously,

TABLE 1. Examples of the analysis of students' responses to the question: "What aspects of understanding and analyzing scientific papers do you find most challenging?"

Student's responses[a]	Difficulty 1 (assigned Bloom's level)[b]	Difficulty 2 (assigned Bloom's level)[b]	Difficulty 3 (assigned Bloom's level)[b]
Being able to follow up an experiment[1]	Designing follow-up experiment (synthesis)		
Terminology, terms describing techniques,[1] most-widely known and used methods in the field but not known to others outside of the field.[2] Figures and images that scientists of other fields would not know immediately.[3]	Unfamiliar terminology (knowledge)	Techniques/methods (knowledge)	Data presentation (knowledge)
Interpreting figures[1] and understanding the techniques used.[2]	Experimental data (analysis)	Techniques/methods (comprehension)	
Understanding the language.[1] It is hard to grasp a paper when you are unfamiliar with the techniques used[2] and what their results mean.[3]	Scientific language/ Writing style (comprehension)	Techniques/methods (knowledge)	Experimental data (comprehension)
Figuring out what new techniques are[1] or what specific terminology mean.[2]	Techniques/methods (comprehension)	Unfamiliar terminology (knowledge)	
Papers on subjects I don't find fulfilling.[1]	Lack of interest/motivation (no Bloom's level assigned)		

[a]Representative responses and individual challenges (superscript) identified in each response. Parts of the responses that were critical in identifying each challenge are underlined.

[b]Challenges identified in each of the responses and their assigned Bloom's level.

and such statements were coded as "vague." The determination of categories of challenges was carried out after all challenges were identified. Differences between pre- and postinstruction survey results were determined using the McNemar's chi-square test, followed by the Benjamini-Hochberg false discovery rate correction for multiple comparisons. The numbers of challenges observed in each of the quarters included in this study are shown in Supplemental Figure S2.

The implied Bloom's level of the challenges identified by the students was determined by the same three raters. The raters were blind to the pre/post identity of the responses. Identification of the Bloom's cognitive levels of the challenges was based on the published descriptions of the cognitive activities associated with each of the Bloom's levels (Bloom *et al.*, 1956; Anderson *et al.*, 2001; Crowe *et al.*, 2008). A detailed description of the rating process can be found in Appendix A in the Supplemental Material. Briefly, the verbs students used to describe difficulties were very important in determining the Bloom's level of the difficulties. Table 2 presents the verbs frequently used in students' responses and their assigned Bloom's levels. However, the context in which the verb was used in students' responses was important as well, as demonstrated in the students' responses rated at application level in Table 2. In identifying the Bloom's levels of challenges related to the experimental data category, we differentiated between student responses that described difficulties with *understanding* experimental data (coded as comprehension) and *interpreting* or *analyzing* experimental data (coded as analysis). Our rationale for making this distinction is provided in Appendix A in the Supplemental Material.

The raters used students' responses from the quarters not included in this study as a training data set. During the rating of the data set included in this study, each rater separately determined the Bloom's levels of the challenges, and the interrater reliability was calculated using Fleiss's kappa statistic. Initial interrater reliability for determining Bloom's levels of the challenges was 0.65 (substantial agreement), while the interrater reliability for lower-order cognitive skills (LOCS) versus higher-order cognitive skills (HOCS) rating of challenges was 0.74 (substantial agreement as defined in Landis and Koch, 1977). The three raters then discussed the disagreements between the Bloom's level assignments. The aim of these discussions was not to reach a consensus on the rating of each response but rather to articulate the reasoning of each rater for selecting a particular Bloom's level. This at times resulted in raters changing their initial Bloom's level assignment. After the discussion, the interrater reliability value rose to 0.89 (substantial agreement as defined in Landis and Koch, 1977). A Bloom's level was assigned to a challenge if at least two of the three raters rated the challenge at that level. Some challenges were determined to have no associated Bloom's level (e.g., lack of motivation or interest in the topic). The number of challenges assigned to each of the Bloom's levels in each of the quarters included in this study is shown in Supplemental Figure S3. Statistical analysis of the pre- and postcourse changes in the Bloom's levels of the challenges was performed using a paired *t* test, followed by the Benjamini-Hochberg false discovery rate correction for multiple comparisons.

TABLE 2. Typical verbs or expressions from students' responses used for identifying the Bloom's level of the challenges with the primary literature

Bloom's level	Typical verbs or expressions used by the students
Knowledge	Unfamiliar (being unfamiliar with)
Comprehension	Understand
	Being confused by
Application	"Understanding how small changes in methods might affect outcomes"[a]
Analysis	Analyze
	Draw conclusions
	Interpret
Synthesis	Design (think of, synthesize) a follow-up experiment
Evaluation	Evaluate
	Assess validity
	Critique
	Determine importance

[a]Note that although the student is using the verb "understanding" in this response, the context of the response indicates that the student is trying to *predict an outcome*, which is an application-level activity.

Institutional Review Board

Protocols used in this study were approved by the UCSD Human Research Protections Program (project 111351SX).

RESULTS

What Do the Students Perceive as the Most Difficult Aspects of Primary Literature?

To guide us in the future design of instructional approaches aimed to teach students how to critically read and analyze scientific papers, we wished to understand what barriers our students face while reading primary literature. From our analysis of students' responses to the question "What aspects of understanding and analyzing scientific papers do you find most challenging?," we derived a comprehensive list of challenges (Table 3). We also determined the frequency of each of the challenges before and after instruction (i.e., the percentage of students who identified a particular challenge; Table 3). We then identified overarching categories that combined related challenges (indicated in bold in Table 3). Our students perceived six major categories of difficulties in the primary literature:

1. Background and terminology: challenges related to unfamiliar biological background, concepts, and terms
2. Techniques: challenges related to experimental methods and techniques
3. Experimental data: challenges related to understanding and analyzing a paper's data
4. Conclusions: challenges related to understanding conclusions, drawing independent conclusions, and evaluating authors' conclusions
5. Paper as a whole: challenges related to the overall structure of the study; for example, connection between the hypothesis and experiments, connection between individual experiments
6. Generic attributes of a scientific paper: difficulties that did not relate to the specific content of a scientific paper but to generic aspects of primary literature, such as scientific language and high density of unfamiliar information

TABLE 3. Most difficult aspects of reading and analyzing scientific papers

Categories of challenges[a] Challenges comprising each category	Representative quotes from students' responses[b] *"Most difficult aspect of understanding and analyzing scientific papers is …"*	Percentage of responses before instruction[c] ($n = 69$)	Percentage of responses after instruction[c] ($n = 69$)
Background and terminology			
Unfamiliar background	"Reading papers outside my field of expertise (don't have the necessary background)" "Background information if I am unfamiliar with the field"	15	13
Unfamiliar terminology	"Understanding terminology" "The mass of technical jargon"	16	6
Techniques			
Techniques/methods	"Understanding the experimental methods used to produce figures (I feel that they are often not well defined in the text of the paper)." "Not fully understanding methods can make interpreting figures difficult as well." (Note that this response was also coded as including "experimental data" challenge)	35	23
Limited by lack of technical expertise	"I think trying to understand the specific[s] of different methods and trying to figure out what kinds of errors can occur and what effects they might have on experimental data is difficult for me but extremely important."	3	4
Experimental data			
Experimental data	"I have difficulties in understanding the figures." "Analyzing all of the data and experiments" "Thinking critically about figures" "Probably the data interpretation"	26	13
Data presentation	"Sometimes the analysis of the graphs or data is shown in a different way than I am used to, and may take extra time to decipher the way that the paper presents the data."	7	1
Statistics	"The statistics"	3	1
Evaluating quality of data	"Determining the validity/reliability of data"	1	4
Results (not clear whether the reference is to the data or to the *Results* section)	"Results"	0	1
Conclusions			
Drawing your own conclusions	"Drawing my own conclusions based on the data presented" "Coming to my own conclusions instead of blindly agreeing with the authors"	1	15
Evaluating author's conclusions	"Determining how credible an author's conclusion is from the data they present" "Probably still assessing whether the conclusions are valid"	3	13
Understanding conclusions	"Understanding how they [the authors] were able to draw a conclusion from the … experiment"	1	1
Discussion	"The discussion"	1	1
Paper as a whole			
Broad experimental design of the paper	"Connections with the hypothesis are vague." "Usually understanding how all experiments fit into the paper as a whole"	7	6
Design of a follow-up experiment	"Thinking up additional/follow-up experiments"	0	6
Relationship between experiments in the paper	"Since many scientific papers will touch on several questions/conclusions, sometimes I have a hard time understanding … how they all come together."	4	1
Relating the paper's findings to the broader field	"Determining if the relatively logical conclusions reached in the paper are congruous with what is already known and what other scientists are also discovering concurrently"	1	3
Main ideas of the paper	"I… lose sight of the main idea of the paper."	3	1
Impact of the paper	"You don't particularly know enough to know … whether the results will impact the field in any way."	3	3
Assumptions	"The most challenging for me is trying think outside the paper about … potential problems with assumptions the paper makes. Basically, I tend to accept what the paper proposes without asking too many questions."	3	0

(*Continued*)

TABLE 3. Continued

Categories of challenges[a] Challenges comprising each category	Representative quotes from students' responses[b] *"Most difficult aspect of understanding and analyzing scientific papers is …"*	Percentage of responses before instruction[c] ($n = 69$)	Percentage of responses after instruction[c] ($n = 69$)
Generic attributes of a scientific paper			
Scientific writing style	"Deciphering the scientific language to get to the main points." "The vocabulary and sentence structure"	13	0
Excessive time required	"Timing, I struggle with reading the papers quickly and efficiently."	6	1
Getting lost in the details	"I get lost in the details."	4	0
Lack of motivation or interest in the topic	"The greatest challenge is finding the motivation to spend time and energy to read, and interpret the meaning of the paper."	1	4
The first attempt at reading	"I still have some trouble dealing with difficult concepts on a first read."	0	3
Amount of unfamiliar information	"I find it most difficult when there are large portions of text that only contain information that I am unfamiliar with. In these cases, I get overwhelmed and have trouble grasping onto any information at all."	1	0
Organization (format) of a scientific paper	"I am easily distracted when I have to flip between different pages with corresponding text, images, and supplementary endnotes."	1	0
No difficulties		0	3
Vague		19	14

[a]Challenges identified by the students, grouped into broad categories (bold).
[b]Students' responses representative of each type of challenge (in the interest of brevity, some of the quotes include only the most relevant parts of students' response).
[c]Percentage of responses that included each challenge, before and after instruction, respectively. Each response could contain more than one challenge; therefore percentages add up to more than 100%.

We also wanted to examine whether students' prior experiences with reading primary literature impacted the types and frequencies of challenges they perceived. Since, to our knowledge, no studies have been done on the relationship between the number of scientific papers read and development of expertise in reading primary literature, we decided to use 20 papers as an arbitrary threshold, with students who reported reading 20 or fewer papers before taking this course ($n = 47$ students) considered "less experienced" and students who reported reading more than 20 papers ($n = 22$ students) considered "more experienced."

Before instruction, "techniques" was one of the two most frequent categories of challenges, present in 38% of students' responses (Figure 2A). Students' responses that included this challenge often described lack of familiarity with the techniques used in the paper or difficulties understanding the techniques (Table 3). More than one-third of these students (13%) linked their lack of familiarity with techniques to their difficulty in understanding or interpreting the data (such responses were also coded as including an "experimental data" challenge):

> "New techniques you have not yet encountered so it is difficult to interpret the data."

> "Not fully understanding methods can make interpreting figures difficult as well."

Techniques presented a major challenge to the less experienced and to the more experienced students alike (Figure 2, B and C). After instruction, we detected no statistically significant decrease in the frequency of this category, with more than a quarter of the students still identifying techniques as one of the most challenging aspects of reading primary literature (Figure 2, A–C).

"Experimental data" was the other most frequently identified category of challenge (Figure 2A). Responses in this category described difficulties ranging from understanding the data to data analysis and evaluation (Table 3). After instruction, the frequency of student responses that identified "experimental data" as the most challenging aspect of scientific papers decreased by 42%, but this decrease was not statistically significant. Interestingly, the only type of challenge that increased in frequency in the "experimental data" category was "evaluating quality of data" (Table 3).

The third most frequent category of challenges before instruction was "background and terminology" (Figure 2A and Table 3). After instruction, the number of students who identified challenges from this category did not decrease significantly. Among more experienced students, the frequency of the challenges in "background and terminology" category exhibited a threefold decrease after instruction (however, this decrease was not significant, possibly due to the small sample size), while remaining a persistent challenge for less experienced students (Figure 2, B and C).

"Conclusions" was the only category of challenges that exhibited a significant increase in frequency after instruction ($p = 0.02$, Cramer's V = 0.38; Figure 2A). Before instruction, only 7% of the students identified any aspect of conclusions as a major challenge, while after instruction, almost one-third (30%) of the students did so, making this category the most frequent in the postinstruction responses (Figure 2A). The largest postinstructional increase within the category of "conclusions" was seen in "drawing your own conclusions from the data," followed by "evaluating author's conclusions" (Table 3). This effect was especially pronounced among the less

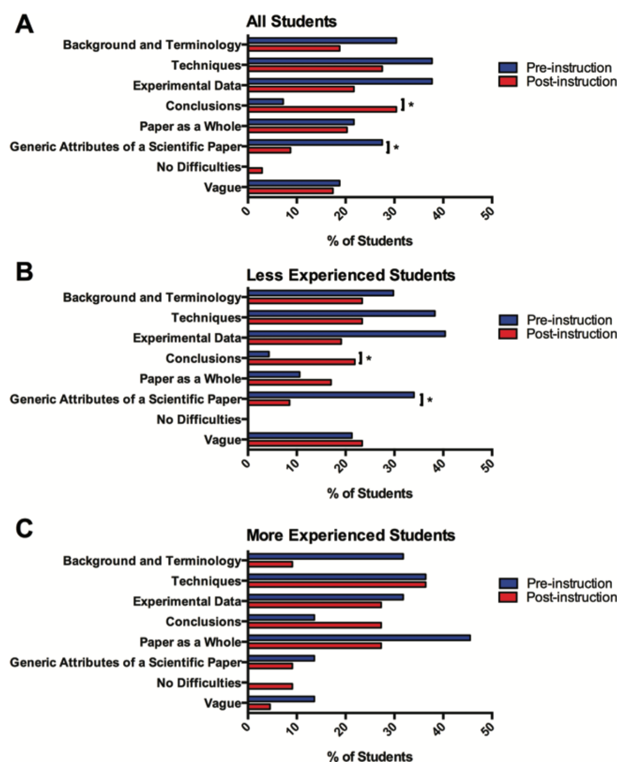

FIGURE 2. Perceived categories of challenges in the primary literature before and after instruction. (A) Frequency of each category of challenges in the pre- and postcourse responses of all students ($n = 69$ pairs of students' responses). (B) Frequency of the categories of challenges reported by less experienced readers ($n = 47$ paired responses). (C) Frequency of the categories of challenges reported by more experienced readers ($n = 22$ paired responses). *, $p < 0.05$.

experienced group, where the frequency of this challenge increased more than sevenfold after instruction ($p = 0.02$, Cramer's V = 0.37; Figure 2B).

The category "paper as a whole" included challenges related to the connection between a paper's hypothesis and its experiments, the relationship between the experiments, and designing a follow-up experiment (Table 3). Before instruction, the more experienced group identified this challenge four times more frequently than the less experienced group (Figure 2, B and C), but this difference was not statistically significant. Overall, the number of responses that included this category did not change significantly between the pre- and postquarter surveys (Figure 2A); however, the two groups of students exhibited different trends: a sharp decline among the more experienced students and a small increase among the less experienced students (Figure 2, B and C). Again, the small size of the more experienced group might have accounted for the lack of statistical significance in the change in this category of difficulty. A type of challenge that did exhibit an increase in the postsurvey was "designing a follow-up experiment" (from 1 to 6%; Table 3). This is not surprising, since students designed and presented follow-up experiments to the papers discussed in this course, which probably brought this aspect and its difficulty into students' awareness.

Surprisingly, more than a quarter of the students identified generic, surface features of scientific articles (e.g. "scientific writing style," "getting lost in details") or motivational aspects (lack of motivation or perceived excessive amount of time it takes to read an article) as a major difficulty in the primary literature (Table 3). Before instruction, these challenges were especially common among the less experienced students, who identified them 2.5 times more frequently than the more experienced readers (Figure 2, B and C), but this difference was not statistically significant. After instruction, the share of these difficulties decreased significantly among all students ($p = 0.032$, Cramer's V = 0.33; Figure 2A), with a fourfold drop among less experienced students ($p = 0.032$, Cramer's V = 0.33), bringing its frequency to the level observed in the more experienced group (Figure 2, B and C). The most frequent challenge in this category, "scientific writing style," decreased from 13 to 0% after instruction (Table 3).

Changes in Bloom's Cognitive Levels of Perceived Challenges Posed by Primary Literature

We also noticed a distinction in the level of cognitive skills implied in some of the challenges in students' responses. This prompted us to re-examine our data and, where appropriate, to identify the level of Bloom's taxonomy that corresponded to each challenge (Bloom *et al.*, 1956; Anderson *et al.*, 2001). Before instruction, the challenges corresponding to LOCS dominated the responses, comprising 71% of all challenges (Figure 3A). Comprehension-level challenges appeared more common among the less experienced students, while HOCS-level challenges appeared more frequently among the more experienced group (Figure 3, C and E); however, these differences were not statistically significant.

After instruction, we observed a significant overall decrease in challenges aligned with LOCS ($p = 0.001$, Cohen's $d = 0.63$; Figure 3A). This decrease was significant among the less experienced students ($p = 0.005$, Cohen's $d = 0.70$; Figure 3C), but not in the more experienced group. The overall decrease in

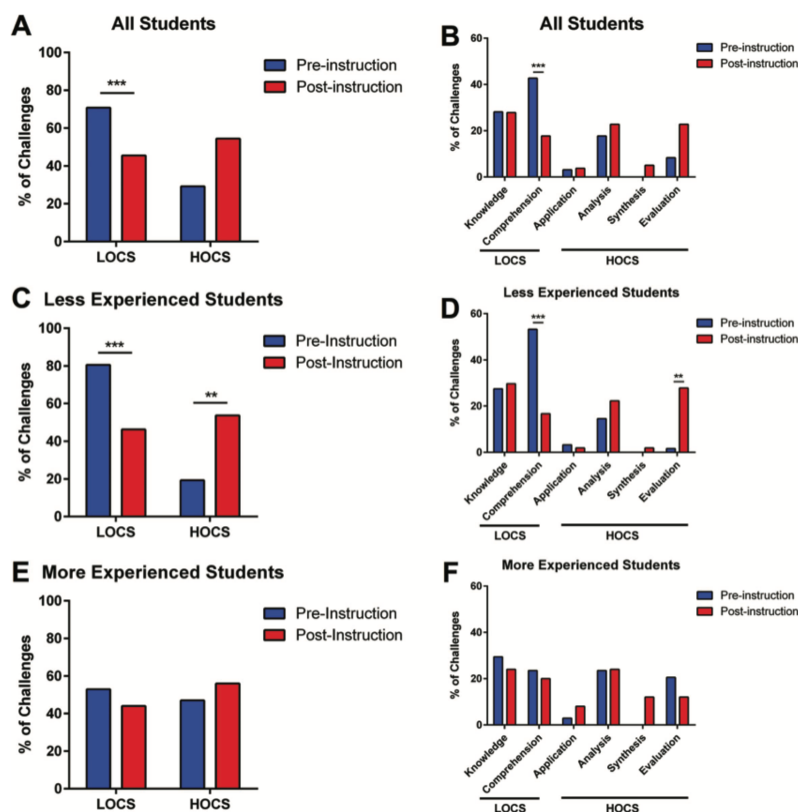

FIGURE 3. Bloom's levels of challenges students perceived in the primary literature. (A, C, and E) Frequency of challenges that correspond-ed to LOCS and HOCS, as reported by (A) all students, (C) less experienced readers, and (E) more experienced readers before and after instruction. (B, D, and F) Frequency of challenges corresponding to each Bloom's level, as identified by (B) all students, (D) less experienced readers, and (F) more experienced readers before and after instruction. *p* Values are reported as follows: **, $p < 0.01$; ***, $p < 0.001$. The overall change in the frequency of HOCS-level challenges (A) was not significant ($p = 0.12$, Cohen's $d = 0.33$).

LOCS-level challenges was largely driven by the drop in the frequency of challenges associated with comprehension, ($p = 0.001$, Cohen's $d = 0.67$; Figure 3B). This decrease was significant in the less experienced group ($p = 0.001$, Cohen's $d = 0.83$; Figure 3D), while no significant change was detected in the more experienced group (Figure 3F).

After instruction, the frequency of challenges that correspond to HOCS significantly increased among the less experienced students ($p = 0.002$, Cohen's $d = 0.7$; Figure 3C) but not among the more experienced group (Figure 3E). This increase in HOCS-level challenges in the less experienced group was driven primarily by the rise of evaluation-level challenges ($p = 0.005$, Cohen's $d = 0.73$; Figure 3D). Taken together, these data suggest that instruction provided in this course was associated with a significant decrease in the perceived LOCS-level challenges in the primary literature and an increased identification of HOCS-level challenges among students with less experience in reading primary literature.

DISCUSSION

Understanding the difficulties students perceive when they engage in critical reading and analysis of scientific papers is important for designing proper instructional approaches to help students in this task. To our knowledge, this study provides the first analysis of what very recent postgraduate

students perceive to be the most challenging aspects of primary literature.

Previous studies have presented the instructors' perspectives on students' difficulties with the primary literature (Gehring and Eastman, 2008; Krontiris-Litowitz, 2013; Van Lacum *et al.*, 2014). Interpretation of data presented in papers was one such difficulty (Krontiris-Litowitz, 2013), and our study shows that students also perceive understanding and interpreting the data and evaluating their quality as major challenges. Using data to draw conclusions or to justify authors' conclusions was another difficulty detected by the instructors (Gehring and Eastman, 2008; Krontiris-Litowitz, 2013). Very few of our students identified "conclusions" as a difficulty before instruction; however, it became the most frequently identified challenge after instruction.

Instructors have also found that students struggle connecting a paper's hypothesis with its experiments or placing a paper's findings in the broad context of its field (Gehring and Eastman, 2008; Krontiris-Litowitz, 2013). Our students also identified these challenges (category "paper as a whole"). More experienced students were more likely to identify this category as a challenge, implying that many less experienced students might not be even trying to make these connections and thus are unaware of these difficulties. Another previously recognized barrier is scientific language, due to its high informational density and its formal writing style (Samuels, 1983; Snow, 2010). Difficulties with scientific language were also identified by our students and were especially frequent among less experienced students, suggesting that experience helps students to overcome this barrier. Students' difficulty with terminology, or scientific jargon, has been long recognized by instructors (McDonnell *et al.*, 2016, and the references within), and our students also perceived it as a substantial challenge before instruction.

Some of the difficulties with primary literature we identify here have not been described previously. Experimental techniques were among the most frequent challenges, despite the fact that our students were enrolled in a research-based master's program. We predict that techniques are likely to present an even bigger challenge to undergraduate students with less laboratory research experience. The link that some of our students made between understanding methods and being able to interpret data indicates that, if we want our students to seriously engage with data in papers, students' difficulty with techniques needs to be addressed instructionally. Unfamiliar background was another challenge not previously documented in the literature.

Another novel finding of this study is that, before instruction, the majority of the challenges students perceived in the primary literature were LOCS-level challenges: difficulties that related to lack of knowledge or understanding. Although the instructor in this course (E.T.) wanted her students to focus on activities that involve HOCS (analyzing data, evaluating conclusions, thinking of follow-up experiments), the students were deterred by, and therefore focused on, LOCS-level challenges.

Effect of the Instruction on the Perceived Challenges

Structured instruction on how to read primary literature that was provided in this course was associated with significant changes in the frequency of two categories of challenges. The frequency of "generic attributes of scientific papers" significantly decreased, while challenges associated with "conclusions" significantly increased. This postinstructional increase in "conclusions" suggests that instruction helped our students become aware of the need for and the difficulty of critically evaluating authors' conclusions ("instead of blindly agreeing with the authors," as one student put it). We also detected changes in the cognitive level of students' difficulties. The frequency of the challenges corresponding to LOCS significantly decreased, while the frequency of challenges aligned with HOCS significantly increased among less experienced students. Together, these changes suggest a shift in less experienced students' perceptions of the primary literature toward a deeper, more critical thinking–oriented approach. The frequency of the remaining categories of challenges did not change significantly, indicating that these aspects of scientific articles require additional instructional support. Among such categories were "techniques," "experimental data," "unfamiliar background and terminology," and "paper as a whole."

Gradual Acquisition of Competency in Reading Primary Literature

The model of domain learning (Alexander *et al.*, 1997; Alexander and Jetton, 2000; Alexander, 2003) provides a useful perspective on the changes in students' perceptions of the primary literature. This model describes how text-based learning changes with acquisition of expertise and identifies three progressive stages in this process (Figure 4):

Stage 1. Acclimated learner: At this stage, the reader lacks the knowledge about the subject area of the text (such as scientific paper), has difficulty discerning between what is important and unimportant, and has mostly situational interest in the subject (such as a course requirement). When faced with a difficulty in the text, this reader employs primarily superficial strategies, such as rereading, focusing mainly on reading comprehension.

Stage 2. Competent learner: A more experienced reader, who has acquired some subject matter knowledge, has strategies to efficiently find information about unfamiliar topics and has more intrinsic interest in the subject. Less deterred by surface-level barriers (such as high informational content or the formal writing style of scientific language), a competent reader is concerned not only with comprehension, but also with analysis and evaluation of the information provided in the text.

Stage 3. Proficient learner: An expert reader, who is highly knowledgeable about the subject matter of the text and deeply interested in the subject. This proficient reader habitually employs deep strategic processing of the text: analyzing and evaluating new information, coming up with new models that integrate this information, and identifying new avenues of investigation (Figure 4).

We suggest that the process of acquisition of primary literature reading skills can be viewed as a continuum, with instruction and experience enabling readers to progress from acclimation to proficiency. In the context of our class, we do not expect our students to achieve proficiency in reading articles in just one quarter, but our data indicate that, at least in their perceptions of the challenges posed by scientific articles, our students

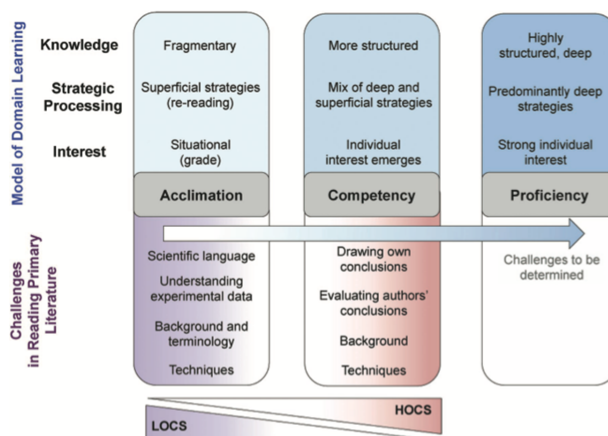

FIGURE 4. Students' difficulties with primary literature map to the acclimation and competency stages in the model of domain learning. Top, the model of domain learning, which describes how text-based learning changes as readers become more knowledgeable and skillful (Alexander *et al.*, 1997; Alexander, 2003). Three stages are identified in this process: acclimation, competency, and proficiency, with each stage characterized by the state of the reader's subject knowledge, strategic processing of the text by the reader, and the source of his or her interest and motivation (blue rectangles). Bottom, the challenges students identify in primary literature (purple-pink rectangles) are consistent with these students being in the acclimation or competency stages. The challenges perceived by the experts (proficiency stage) and the Bloom's levels of these challenges remain to be investigated. Previous exposure to scientific papers and instruction in reading primary literature help students progress along the acclimation–competency continuum (blue arrow).

are progressing toward competency. We argue that students who identified "generic attributes of a scientific paper" as a major challenge are likely to be in the acclimation stage with respect to reading primary literature (Figure 4). These readers are likely to be deterred by such surface-level barriers to reading comprehension as scientific language. Discrimination between important and unimportant concepts also presents a difficulty to readers in the acclimation stage (Alexander and Jetton, 2000), and challenges such as "getting lost in details" and "amount of unfamiliar information" reflect this difficulty. Conversely, more experienced readers were less deterred by the surface features of scientific articles. They also identified more HOCS-level challenges, although this difference was not statistically significant. These findings suggest that experience and previous instruction enabled these students to progress along the acclimation–competency continuum.

Postinstructional decrease in LOCS and increase in HOCS-level challenges, decrease in the difficulties with "generic attributes of a scientific paper," and increase in the frequency of difficulties with "conclusions" suggest that instruction in this course helped students to progress along the continuum toward competency in their perception of scientific articles (Figure 4). This effect was especially noticeable among less experienced students.

Limitations and Future Directions

One limitation of this study is that we describe what students *perceived* as the major difficulties in reading and analyzing primary literature. Our students likely had other difficulties with the primary literature, which they might not have been aware of or did not perceive as the *most* challenging. Furthermore, an increase in the awareness of HOCS-level challenges does not necessarily mean that the students are now performing better in these HOCS-level tasks. Previous studies have reported that undergraduate courses that offered structured analysis of primary literature were associated with significant increases in students' performance in questions assessing Bloom's HOCS (Hoskins *et al.*, 2007; Gottesman and Hoskins, 2013; Segura-Totten and Dalman, 2013). In a study conducted in the course we described here, we detected a significant increase in students' ability to design an experiment but not in students' ability to interpret data, draw conclusions, or evaluate a hypothesis based on the data, although our conclusions were limited by high preinstructional performance of our students in this test (Abdullah *et al.*, 2015). A primary literature–based assessment that systematically evaluates students' skills in the various aspects of reading primary literature (i.e., identifying hypothesis, understanding experimental setup, interpreting data, placing the paper in its broader context) would be very valuable in providing an objective evaluation of students' skills and difficulties in critically reading scientific articles.

Some of the changes in students' perceptions of the challenges in primary literature could be due to factors other than the instruction provided in this course. Among such factors are students' own research, research-related literature they read, other courses they took, journal clubs, and lab meetings in which they participated during that quarter. Additionally, activities students completed in this class were likely to have an impact on what students perceived as most difficult in scientific literature: for example, while attempting to evaluate evidence from two conflicting papers or design a follow-up experiment for the first time, students might have realized that these activities are challenging.

Another limitation of this study is that, in our analysis of students' difficulties and their alignment with the Bloom's cognitive skills, we were limited by what students chose to include in their written responses. For example, it is possible that, for a master's student, "data interpretation" may mean a level of engagement with the data different from that for a more advanced graduate student, postdoc, or a faculty member. Interviews with students could provide additional insights into

students' perceptions about primary literature and the strategies they apply when reading it.

In this study, we used 20 papers as an arbitrary threshold to differentiate between the less experienced and the more experienced students. We acknowledge that such dichotomization might have resulted in a loss of information about the relationship between the number of papers read and the challenges perceived by our students. We also acknowledge that there is no reason to assume that a student who has read 21 papers has a substantially different experience from a student who has read 20 papers, and other factors (e.g., instruction they received on how to read papers, superficial vs. in-depth reading by the student) will also play a role in a student's experience. We suggest that future studies in this topic should treat the number of papers read as a continuous variable and use regression analysis to examine the relationship between students' experience with scientific papers and the challenges they perceive. Also, rather than selecting a range of numbers, as we did in this study, it would be preferable to ask the students to report the actual number of papers they have read. As in any study that assesses past behavior, a possibility of inaccurate recall of this number will remain a limitation, and students will be likely to recall more reliably the number of papers they read in the more recent past.

In this study, we assessed a population of students who are postgraduates, have demonstrated good academic achievement (undergraduate GPA of 3.0 or higher), and are engaged in active research. It will be of great interest to investigate the difficulties that undergraduate students face when approaching primary literature and how these perceptions change in the course of their studies. A sample that also includes PhD students, postdocs, and faculty can provide a better understanding of the progression from an acclimated to a competent and then to a proficient reader.

Implications for Instruction

Only 26% of the students who participated in this study reported receiving an explicit instruction on how to read scientific articles at any point of their undergraduate or graduate career. We think that such training is essential if we want to empower our students to critically engage with the primary literature. Our findings that students perceived unfamiliar techniques, background, terminology, and even scientific language as substantial challenges before instruction suggest that these difficulties will need to be explicitly addressed by instructors, both at the level of skills, by teaching students how to find information about unfamiliar concepts and techniques, and at the metacognitive level, by helping students to be aware of these difficulties, to realize that these difficulties are common and can be overcome. New online resources that can help address these challenges have recently become available. The *Journal of Visualized Experiments* (www.jove.com) provides videos of a wide variety of techniques in biology performed by experts in the field. Instructors could refer students to use these videos as a source outside class or incorporate the videos into the classroom instruction. Another tool is Science in the Classroom, a free collection of annotated articles from different fields of science, developed by a team of *Science* journal editors, which provides rich embedded descriptions of terminology, prior research background, and techniques (AAAS, 2014; http://scienceintheclassroom.org/?tid=22). These papers could be used by instructors as a basis for introducing students to primary literature.

In addition to providing students with strategies to address the LOCS challenges, we suggest that instructors should also guide students toward engagement with the literature at a higher cognitive level: critical analysis and evaluation of a paper's data, experimental design, and conclusions and thinking about questions yet to be addressed. We suggest inclusion of papers with flaws that students can identify without relying on extensive knowledge or technical expertise. Such articles provide an opportunity for students to critically assess and evaluate the authors' claims and assertions. In addition, including papers from new, developing fields, in which many questions are still unanswered, can offer an opportunity for students to appreciate the dynamic, sometimes uncertain nature of scientific process and exercise their skills of synthesis by developing new or alternative hypotheses and proposing original follow-up experiments (Hoskins *et al.*, 2007; Abdullah *et al.*, 2015). Moving forward, it is important to develop effective teaching strategies that address the barriers that students encounter in their journey toward competence in engaging with primary literature.

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