TARGETING EPHB6 SYNTHETIC LETHALITY IN BREAST CANCER

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ABSTRACT

Traditionally, cancer treatments focus on proteins impacted by either an increase in expression or a gain-of-function mutation. Loss-of-function mutations or especially down-regulation usually cannot be targeted directly through stabilization or activation, so alternative approaches are needed to target these changes in cancer cells. One approach is based on the synthetic lethality concept, where lethality occurs only when inhibition of a gene partner to the loss-of-function alteration is inhibited. EphB6, a kinase-dead member of the Eph receptor tyrosine kinase family, has anti-malignant properties and is often down-regulated in breast cancer, making it a strong candidate for the synthetic lethality approach. To support the development of EphB6-based therapies, our lab previously completed a genome-wide shRNA screen in an effort to find novel EphB6 interactions, searching specifically for molecules synthetically lethal with EphB6 that also had FDA-approved inhibitors. One of the identified hits, the Src kinase, was successfully validated with and FDA-approved Src inhibitor, KX2-391, in triple-negative breast cancer models representing the most aggressive breast cancer subtype. Met was another promising target identified, but it was not properly validated. This thesis describes the further assessment of the EphB6-Src and EphB6-Met synthetic lethal interactions in various breast cancer subtypes. Also reported here is the screening of the library of over 1800 FDA-approved compounds that allowed us to identify a new drug (the INHIBITOR) synthetic lethal with EphB6 that selectively eliminates EphB6-deficient TNBC cells. The results laid out in this thesis strongly suggest that synthetic lethal interactions of EphB6 discovered in TNBC appear to be restricted to this breast subtype, and also indicate that using small-molecule Met inhibitors, especially ARQ-197, or the newly identified INHIBITOR could represent valuable options for treating TNBC patients with low EphB6 expression in tumor cells.

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LIST OF ABBREVIATIONS

CCK8	Cell Counting Kit 8
DMSO	Dimethyl sulfoxide
EPH receptor	Erythropoietin-producing hepatocyte receptor
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and drug administration
GFP	Green Fluorescent Protein
HER2	Epidermal growth factor receptor 2 (EGFR2)
ns	Non-silencing
PI3K	Phosphoinositide 3-kinases
RFP	Red Fluorescent Protein
shRNA	Short hairpin RNA
TNBC	Triple negative breast cancer

1.0 INTRODUCTION

1.1 Breast Cancer: Subtypes and Treatment

Of all malignancies affecting women, breast cancer leads in mortality. One in eight women end up with breast cancer and nearly 15% of tumor-related deaths in women are caused by breast malignancies (Makki, 2015; Ferlay *et al.*, 2015). For years, the scientific community has invested significant resources in finding effective therapies to combat the high rate of breast cancer and the associated mortality. New sequencing technologies and expression arrays have helped develop a molecular classification system for breast cancer tumors (Figure 1.1) based on the expression of three proteins: the estrogen receptor (ER), the progesterone receptor and the human epidermal growth factor receptor 2 (HER2) (Inic *et al.*, 2014). This has led to the identification of three main breast cancers subtypes: luminal (ER^+, $PR^{+/-}$, $HER2^{+/-}$), $HER2^+$, and triple-negative-breast cancer

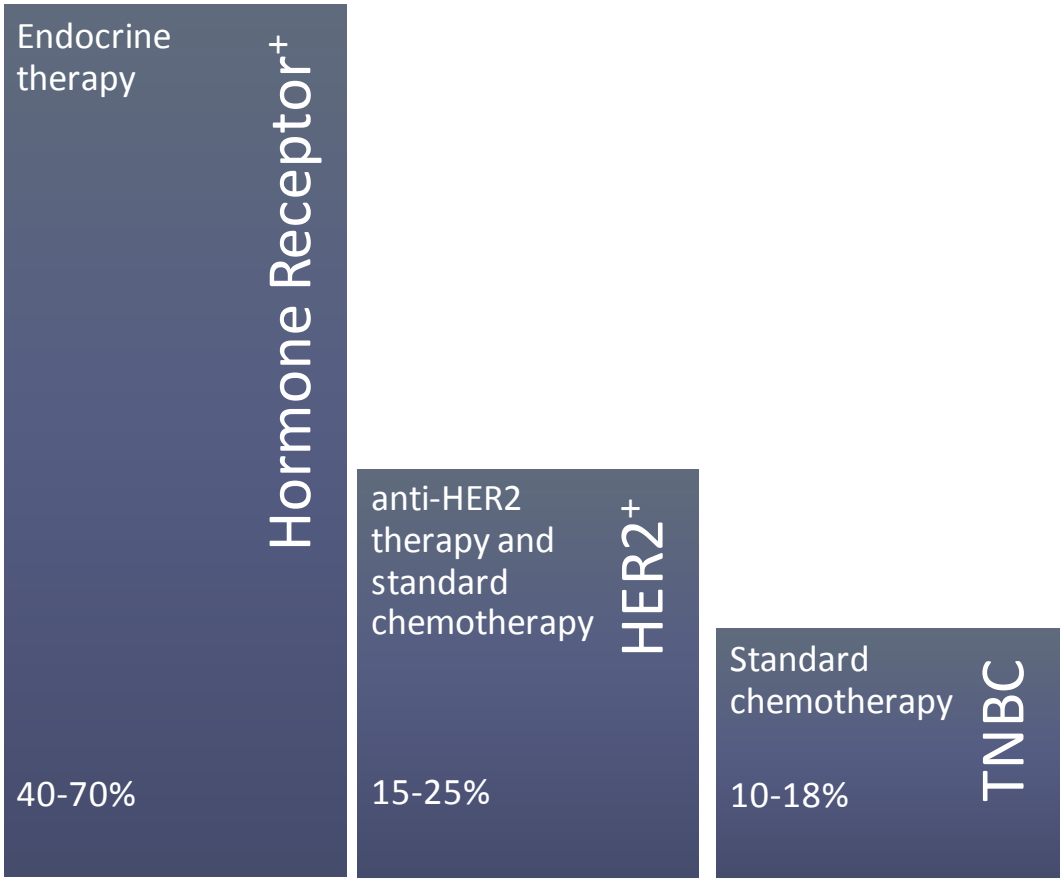


Figure 1.1. **Breast cancer subtypes.** Breast cancer subtypes determine the therapies used. Chemotherapy typically involves the use of a taxane in combination with an anthracycline. Percentages of respective subtypes are included on the bottom of the figure.

(TNBC), lacking all three proteins (Tang *et al*, 2016). While numbers vary significantly depending on ethnicity, tumors within the luminal subtype, hereon referred to as ER^+ breast cancer, account for 40-70% of breast cancer cases in North America, $HER2^+$ 15-25%, and TNBC 10-18% of cases (Makki, 2015). As cancer cells of the first two subtypes depend on hormone receptors or HER2 signaling, for their survival and propagation (Inic *et al.*, 2014), selection of treatments for a patient depends on the expression profiles of these proteins and matching targeted therapies are being used to eliminate these tumors. Unfortunately, resistance to these treatments still represents a significant challenge and there are no targeted therapies available for TNBC. New personalized therapies are urgently needed.

To understand the need for targeted therapies, a discussion of current chemical therapies and how they target their respective proteins is helpful. Treatments are typically used before surgery (neo-adjuvant) and then continued following recovery (adjuvant). ER^+ breast cancer depends on the estrogen receptor for growth and proliferation of the cancer cells. The estrogen receptor-alpha binds to estrogen with high affinity, leading to dimerization of the receptor and binding to both estrogen receptor elements within the chromatin structure and transcriptional regulators like activators and repressors. ER^+ breast cancer is treated mainly by endocrine therapy, either blocking estrogen from binding to the receptor (Selective Estrogen Receptor Modulators such as Tamoxifen) or reducing the amount of hormone available (aromatase inhibitors such as letrozole) (Brufski and Dickler, 2018; Tremont *et al.*, 2017). These treatments continue until the onset of resistance. Resistance in ER^+ breast cancer has several origins including crosstalk between ER and tyrosine kinases, ER mutations, and drug transport out of the cell (Brufski and Dickler, 2018). $HER2^+$ breast tumors depend on HER2 or EGFR2 for growth and proliferation. While the receptor does not have any ligands, it is the preferred binding partner of the other EGFR receptors and leads to activation of downstream pathways after dimerization (Murphy and Modi, 2009). $HER2^+$ breast tumors are treated either with antibodies targeting HER2 or small-molecule inhibitors of HER2, though these often target other proteins, such as the epidermal growth factor receptor (EGFR) as well (Murphy and Modi, 2009). Both treatment courses are typically combined with chemotherapy, or generally cytotoxic compounds that target either DNA or microtubules. Since HER2-targeting drugs were developed approximately 15 years ago, median survival has increased dramatically, from 20 months to 5 years (Tang *et al.* 2016), showing the power of these targeted therapies. Resistance in $HER2^+$ breast cancer results from increased

dependence on other pathways by other tyrosine kinases, hormone receptors, or the PI3K/Akt pathway and reduced ability of the antibody to bind to HER2 due to new mutations (Murphy and Modi, 2009). Unfortunately, effective targets have not been discovered until now for treating TNBC, meaning chemotherapy with DNA-damaging compounds or microtubule-targeting drugs is the primary method of both neoadjuvant and adjuvant therapies in this malignancy (Tang *et al.*, 2016; Szekely *et al.* 2017). While TNBC initially responds better than other subtypes to chemotherapy, it still has a higher mortality. Typically, TNBC patients receive alternating taxane and anthracycline regimens, which result in pathological complete response (pCR) values of only 30-40%, but a recent success story, platinum-containing drugs, have raised those numbers in recent clinical trials to approximately 50% (Sikov *et al.*, 2015; Rugo *et al.*, 2016; von Minckwitz *et al.*, 2014). However, patients with resistant tumors have such mortality that TNBC remains the most lethal among breast cancer subtypes with median survival in TNBC of only 1 year after recurrence and only 40% of patients not surviving 5 years when initially diagnosed in high grade tumors (Goncalves *et al.*, 2018). Overall, it remains vital to find a targeted therapy to combat cancers that are resistant to current regimens, especially as most of current cancer drugs are effective only in early stage tumors.

1.2 Eph Receptors

One class of proteins emerging as very promising therapeutic targets are proteins known as Eph receptors, notable as they are tyrosine kinases often deregulated in various malignancies, including breast cancer (Truitt and Freywald, 2011; Lisabeth *et al.*, 2013; Kou and Kandpal, 2018). Overall, 16 Eph receptors have been discovered, making them the largest group of receptor tyrosine kinases; 14 of these receptors are expressed in human cells. They are categorized into two classes based on their ephrin ligands: EphA receptors and EphB receptors. EphA receptors primarily bind to ephrinA ligands, which are attached to their host cells through a glycosylphosphatidylinositol anchor, while EphB receptors bind to ephrin B ligands that are transmembrane proteins that also have an intracellular portion. Overall, nine EphA receptors (EphA1-EphA8, EphA10) are expressed in humans, along with five EphB receptors (EphB1-EphB4, EphB6).

Both classes of Eph receptors have a conserved structure (Figure 1.2). Their extracellular portion includes an N-terminal ephrin-binding domain, a cysteine-rich region, and two fibronectin type III repeats. Following the transmembrane portion, a regulatory juxtamembrane region leads into the tyrosine kinase domain, and two domains involved in protein-protein interactions, the sterile alpha motif domain and the C-terminus PDZ-binding motif. Binding to ephrins leads to the dimerization and oligomerization of both ephrin-bounds receptors (Himanen *et al.*, 2007; Himanen *et al.*, 2010), as well as recruitment and clustering of ligand-free Eph molecules. Receptor clustering causes phosphorylation of two key residues in the juxtamembrane domain (Binns *et al.*, 2000; Wybenga-Groot *et al.*, 2001), relieving inhibitory interactions and resulting in the activation of the kinase domain. This culminates in *trans*-phosphorylation of receptors (Pasquale, 2005; Himanen *et al.*, 2007) and promiscuous activation of the aggregated Ephs, even across classes. The cysteine-rich region of Eph receptors, as well as their fibronectin type III repeats, are thought to be primarily involved in receptor dimerization. Sterile alpha motif domains are thought to stabilize receptor clustering (Smalla *et al.*, 1999). In other protein classes sterile alpha motifs can polymerize but only weak interactions have been shown in Eph receptors (Qiao and Bowie, 2005). Removal of the sterile alpha motif domain in EphA4 did not change signalling (Qiao and Bowie, 2005) though this has not been carried out in more members of the Eph receptor family. The PDZ-binding motifs are involved in signalling through PDZ-domain-containing proteins, such as AF-6 (Hock *et al.*, 1998; Torres *et al.*, 1998; Buchert *et al.*,2002; Richter *et al.*, 2007). AF-6 interacts with Eph receptors as it has a PDZ domain and is an adaptor shown to interact with members of Ras family of GTPases and potentially can interact with actin itself (Hock *et al.*, 1998). It must be noted that while PDZ-mediated interactions with other proteins do occur, the kinase domain is responsible for most of signalling. A major part of Eph receptor signalling relies on the recruitment of Rho family GTPases, such as RhoA, Rac1, and Cdc42 (Pasquale, 2008,2010), which are vital the regulation of actin cytoskeleton. Ras GTPases are also affected through Eph receptor signalling, though the effects can be divergent, either activating or inactivating the Ras-Erk pathway (Pasquale 2008, 2010; Xiao *et al.*, 2012). Phosphorylation at the juxtamembrane inhibitory sites as well as various residues in in the kinase domain allows recruitment of proteins with Src Homology type 2 or SH2 domains, which bind to phosphorylated tyrosines. This includes members of Src and Abl families of non-receptor-tyrosine kinases and adaptor proteins, including Crk (Jorgensen *et al.*, 2009).

Eph receptor signaling causes changes in the actin cytoskeleton and affects major signal transduction pathways such as the Ras-Erk and PI3K-Akt pathways, resulting in the changes in cell shape, adhesion, and movement, while also affecting cell survival and proliferation. In adults, normal biological functions controlled by Eph receptor signalling include remodeling of synapses (Kayser *et al.*, 2008; Filosa *et al.*, 2009), maintaining bone homeostasis (Zhao *et al.*, 2006), regulation of immune responses (Freywald *et al.*,2003,2008), tissue repair (reviewed in Adams and Eichmann, 2010), and epithelial differentiation (Vaught *et al.*, 2009). Eph receptors are also vital in the context of embryo development (reviewed in Pasquale 2008, 2010), especially in the brain, where they play a central role in initiating synaptic connections between neurons, spatial differentiation of different cell populations, and vascularisation. Finally, Eph receptors are implicated in almost every type of tumor, including breast cancer (reviewed in Truitt and Freywald, 2011).

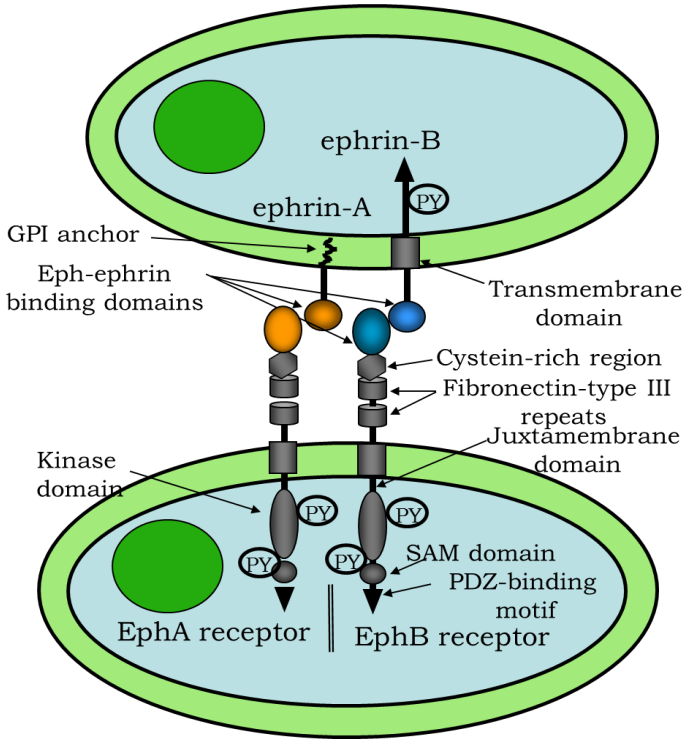


Figure 1.2. Eph-Ephrin Interaction. Interaction between ephrin-expressing cell and Eph receptor-expressing cells. This interaction leads to dimerization or oligomerization of the receptor and eventually, receptor phosphorylation. The scheme shows the conserved domains of Eph receptors.

Ephrin-B-mediated reverse signalling is not well characterized. It is thought to be primarily mediated by the Src kinases which creates binding sites for adaptors such as the adaptor protein Grb4 (Palmer *et al.*, 2002). PDZ-domain containing proteins binding to the PDZ-binding motif at the C-terminus of ephrin-B's (Qiu *et al.*, 2010). Biological functions mediated by ephrin-B reverse signalling include brain development, angiogenesis and neuronal migration (Lisabeth *et al.*, 2013). Ephrin-B activity in cancer is not well understood either. STAT3 has been shown to bind to ephrin-B1 which could impact cancer progression (Bong *et al.*, 2007).

1.3 EphB6 and Its Role in Malignancy

Unique to Eph receptors are the divergent effects that result from ligand-induced stimulation: activated Eph receptors do not *only* activate certain cellular and signaling responses. Rather, both activation and suppression of signaling can result from Eph-ephrin interactions. At least some part of this unusual activity is likely to be related to the two kinase-dead members of the Eph receptor family, EphB6 and EphA10. The existence of these receptors, one in each class, could indicate their importance in divergent signalling. EphB6, though it does not have an active kinase domain to phosphorylate proteins, can be phosphorylated when in complex with EphB1 (Freywald *et al.*, 2002) or EphB4 receptors (Truitt *et al*, 2010) and thus, initiate its signaling. EphB6 binds two ligands, ephrin-B1, and EphrinB2, and has been shown to interact with c-Cbl and Fyn, the former is a cytoplasmic ligase involved in protein ubiquitination and the latter is a Src kinase family member that appears to act in a tumor-suppressive manner (Freywald *et al.* 2002; Truitt *et al.*, 2010; Matsuoka *et al., 2005*). On the functional level, EphB6 regulates the actin cytoskeleton, expression of matrix metalloproteases, cell-matrix adhesion and cell migration (Freywald *et al*, 2002, Fox and Kandpal, 2009, Truitt *et al*, 2010). Interestingly, EphB6 seems to be able to modulate the effects of its partner proteins and is associated with tumor suppression rather than aggression (Fox and Kandpal, 2009), acting as an anti-malignant molecular switch in cancer cells. Indeed, EphB6 is activated by other Eph receptors (Freywald *et al.*, 2002; Truitt *et al.*, 2010) and can reverse the oncogenic activity of the EphB4 receptor (Noren *et al.*, 2009; Truitt *et al.*, 2010). When dimerized with EphB4, EphB6 facilitates the action of c-Cbl, leading to the activation of the Abl kinase resulting in increased adhesion of breast cancer cells. While acting without EphB6, EphB4 actively enhances cancer invasiveness. Thus, these two Eph receptors seem to oppose one another's functions, suggesting a model where EphB6 interacts with pro-

metastatic catalytically-active EphB receptors to actively interfere with their action and suppress metastasis (Truitt and Freywald, 2011). EphB6 also has been shown to act in a tumor-suppressive manner through increasing production of a matrix metalloprotease inhibitor, TIMP2 and a decrease in matrix Matrix metalloproteases 7 and 19 in several breast cancer cell lines (Fox and Kandpal, 2009).

As such, EphB6 is an intriguing target for use in cancer therapies. Unfortunately, while Eph receptors are often overexpressed in malignancy, EphB6 is down-regulated multiple tumor types (Paul *et al.*, 2016; Table 1), including breast cancer and plays a complex role in several cancers (Table 2). EphB6 expression is silenced through methylation in significant amount of cancers (Table 1), including non-small cell lung tumors (Yu *et al.*, 2010) and invasive breast cancer (Fox and Kandpal, 2006). Loss of EphB6 is associated with a poor prognosis in colorectal cancer, largely due to increased metastasis (Mateo-Lozano *et al.*, 2017). Similarly, loss of EphB6 is correlated with a poor prognosis in metastatic melanoma and neuroblastoma (Hafner *et al*, 2003). Other observations reveal that EphB6 is strongly expressed in normal breast epithelial cell and non-invasive breast cancer cells, but not in invasive breast cancer cells, further supporting the central role for EphB6 in suppressing breast cancer invasion (Fox and Kandpal 2004, 2009). Unfortunately, due to the fact that its expression is frequently lost in malignant tissue, EphB6 cannot be directly stimulated to take advantage of its tumor-suppressing function and therefore, other methodologies must be used to target EphB6 in cancer.

1.4 Approaches to Eliminating EphB6-Deficient Breast Cancer Cells and Tumors: Synthetic Lethality

Traditionally, cancer therapeutics target a mutated or over-activated pathway or gene that cancer cells are dependent on, including oncogenic driver genes. This strategy has resulted in treatments that have been quite successful; an easy example described earlier is the targeting of the ER and HER2 receptors in breast cancer. That said, this severely limits the genes that can be targeted and resistance to this type of therapy is a major obstacle in breast cancer treatment (Paul *et al.*, 2014).

Table 1.1. TCGA analysis of EphB6 expression and methylation in different cancers compared to matching normal tissue controls

Cancer Type	EphB6 expression reduced ($p < 0.05$)	EphB6 methylation increased ($p < 0.05$)
Bladder urothelial carcinoma		
Breast Invasive carcinoma	✓	✓
Cervical squamous cell carcinoma		✓
Colon adenocarcinoma	✓	✓
Esophageal carcinoma	✓	
Glioblastoma multiforme	✓	N/A
Head and neck squamous cell carcinoma	✓	N/A
Kidney chromophobe	✓	N/A
Kidney renal clear cell carcinoma		✓
Kidney renal papillary cell carcinoma		N/A
Liver hepatocellular carcinoma	✓	
Lung adenocarcinoma	✓	✓
Lung squamous cell carcinoma		✓
Pancreatic adenocarcinoma		✓
Pheochromocytoma and paraganglioma	✓	N/A
Prostate adenocarcinoma	✓	✓
Rectum adenocarcinoma		✓
Sarcoma		
Skin cutaneous melanoma		
Stomach adenocarcinoma	✓	
Thyroid carcinoma	✓	
Thymoma		N/A
Uterine corpus endometrial carcinoma	✓	

Data compiled using Paul *et al,* 2016 and Toosi *et al.,* 2018

Table 1.2. EphB6 and Cancer

Type of Cancer	Impact of EphB6	Reference
TNBC	-tumor suppressive when interacting with EphB4 -loss of EphB6 increases invasive activity -synthetically lethal with Src -modulates miRNAs, including those involved with PI3K, Akt pathway -reduced expression in breast cancer	(Truitt et al., 2010) (78) (Bhushan et al., 2014) (6) (Paul et al., 2016) (61) (Bhushan and Kandpal, 2011) (7)
Invasive melanoma	-reduced expression	(Hafner et al., 2003) (27)
Lung	-reduced expression in metastatic cancer -silenced through methylation in non-small cell lung cancer -blocks metastatic activity	(Muller-Tidow et al., 2005) (51) (Yu et al., 2010) (85) (Bulk *et al*., 2012) (13)
Colorectal	-loss of EphB6 associated with poor prognosis/metastasis	(Matea-Lozano et al., 2017) (47)
Prostate	-reduced expression	(Mohamed et al., 2015) (49)
Gastric	-reduced expression	(Liersch-Lohn et al., 2015) (41)
Ovarian	-reduced expression	(Gu et al., 2015) (26)
Neuroblastoma	-loss of EphB6 associated with poor prognosis	(Tang et al., 1999) (71)

Synthetic lethality is a concept that widens the scope of genes available to target—the idea that targeting a protein is insufficient to cause lethality, unless another protein is also either missing or inhibited (Paul *et al.*, 2014) (Figure 1.3). For example, in the case of EphB6, a tumor suppressing gene (EphB6) is missing, the cancer cell may have an increased dependence on a compensating pathway so our goal is to find a synthetic lethal partner, where it's targeting causes lethality selectively in EphB6-deficient cells.

1.4.1 Finding EphB6 Synthetic Lethal Partners with Genome-Wide shRNA Screen

To exploit downregulated EphB6 expression in breast cancer cells through the targeting of a synthetic lethal gene partner, finding a gene that displays a lethal phenotype selectively in the

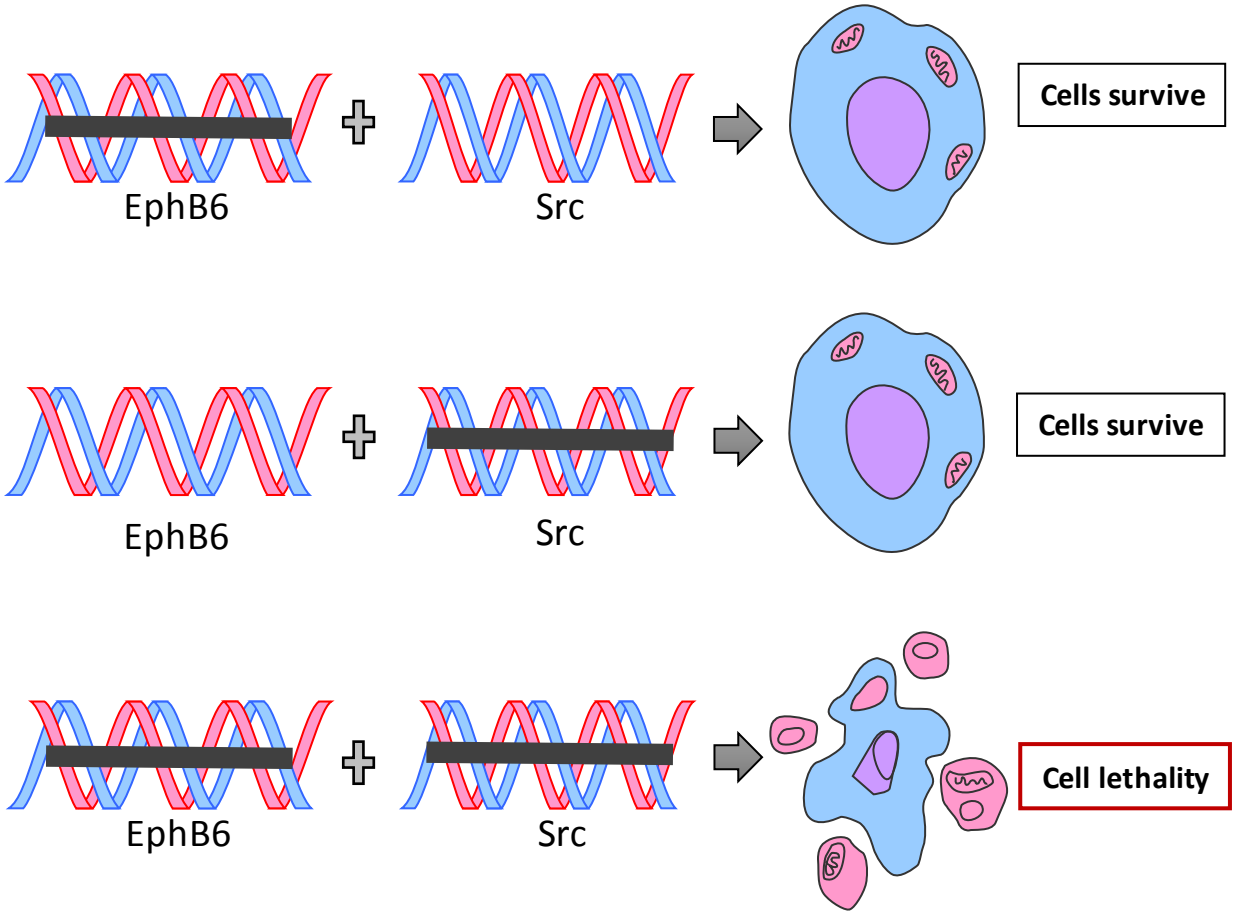


Figure 1.3. **Synthetic lethality with Src as example.** Downregulation or targeting of either EphB6 or Src is not effective in killing TNBC cells; Src inhibitors are more lethal when EphB6 is down-regulated. Adapted from Paul *et al.*, 2014.

context of EphB6 deficiency is necessary. One method to identify synthetic lethal partners of EphB6 in breast cancer is a pooled shRNA screen. Pooled screening is an approach that allows one to search for negative gene interactions on a broad scale, not simply focusing on oncogenic driver mutations but searching genome-wide for interactions. shRNAs mimic well the action of small-molecule inhibitors that are used in medicine, as gene knockdowns are typically incomplete. This approach allows multiple shRNAs to be used for targeting each gene to increase the confidence in the obtained data. While off-target effects might present challenges in such screens, the use of multiple shRNAs for each gene lessens the chance of a false-positive observations as the shRNAs target different sequences of mRNA: even if a lethal effect is due to off-target effects, the likelihood of additional shRNAs having identical off-target effects is quite low, allowing more confidence in the reliability of the lethal effects of targeting a specific gene.

1.4.2 Previous shRNA-based Screening for Synthetic Lethal Interactions done by our team

Using a library of 90,000 shRNAs targeting 18,000 genes, our collaborative team has compared survival of three TNBC cell lines isogenic with the exception of EphB6, two EphB6-positive (one with Myc-tagged EphB6 and one wild-type EphB6) and one EphB6-negative (with a control expression vector) (Paul *et al.*, 2016). After lentiviral transduction of the cell lines, lethality of individual shRNAs was analyzed by amplifying the surviving shRNAs and comparing to the sequencing of shRNAs at the beginning of the experiment. The reliability of these results was confirmed by checking a set of reference genes essential to all cells, and the screen yielded strong scores for reliability—essential genes properly dropped out. In these screens, survival of MDA-B6 cells (MDA-MB-231 TNBC cells expressing wild-type EphB6) was compared to MDA-pc3 (MDA-MB-231 cells with the control pcDNA3 vector), as was survival of MDA-B6myc cells (MDA-MB-231 cells expressing Myc-tagged EphB6). Scores from the two comparisons were then overlapped. Overall, 113 statistically significant overlapping hits were identified. The synthetic lethal data was coupled with transcript expression levels of EphB6 using TCGA data, searching for hits whose expression was inversely correlated to EphB6 expression. The non-receptor tyrosine kinase Src was identified as a potential target for TNBC tumours with low or no EphB6; this hypothesis was confirmed in a variety of cellular assays and a xenograft mouse model of human TNBC. The initial observations in TNBC have been published (Paul *et al.*, 2016) along

with this screen, but no analysis was completed for EphB6-Src synthetic lethality in $HER2^+$ or ER^+ breast cancers. Another promising hit that was identified in the initial screen was the Met receptor, though no further investigation was done in regards to the relevance of the EphB6-Met synthetic lethal interaction.

1.4.2.1 Selection of Synthetic Lethal Targets for Validation: Src

The cytoplasmic Src tyrosine kinase is one of the first proto-oncogenes discovered. Viral Src had been discovered as a gene that resulted in cancer in chickens (Stehelin *et al.*,1977). Following this, a gene similar to viral Src was discovered, a momentous discovery as it clued scientists to the idea that cancer could be caused by aberrant signalling or expression of a protein normally expressed by the cells (Oppermann *et al.*, 1979). Src is quite well characterized with regards to cancer. It is either activated or overexpressed in a majority of breast cancer cells (Dehm and Bonham 2004, Elsberger 2014). Src has been reported to be involved in breast cancer metastasis, especially metastasis of cancer to the bone, which accounts for approximately 30% of all breast cancer metastasis (Zhang *et al.*, 2009). Brain-targeted breast cancer metastases have been also linked to Src (Zhang *et al.*, 2013). Several studies have shown a relationship between HER2 and Src expression (Belsches-Jablonski *et al.*, 2001, Madrid-Paredes *et al.*, 2015). In addition, Src has been shown to support tumor drug resistance, tumor growth, proliferation and survival of breast cancer cells (Wheeler *et al.*, 2009). One of the reasons the Src kinase was selected for a more detailed investigation, when found amongst other synthetic lethal screen hits, was the fact that Src already had several FDA-approved inhibitors, allowing an easier translation towards a potential therapy. As well, Src contains an SH2 domain that could bind with phosphorylated tyrosines in EphB6 giving a logical potential interaction that could explain a synthetic lethal relationship. While this inhibitor has not been very successful in initial breast cancer clinical trials (Elias and Ditzel, 2015), personalisation of its application might improve its efficiency. All the initial assessments of EphB6-Src synthetic lethality were done by our team exclusively in TNBC models and no research was done in other breast cancer subtypes after the synthetic lethal relationship between EphB6 and Src was confirmed in TNBC (Paul *et al.*, 2016). Determining whether this relationship is viable across all breast subtypes would be valuable for developing new therapies.

1.4.2.2 Selection of Synthetic Lethal Targets for Validation: Met

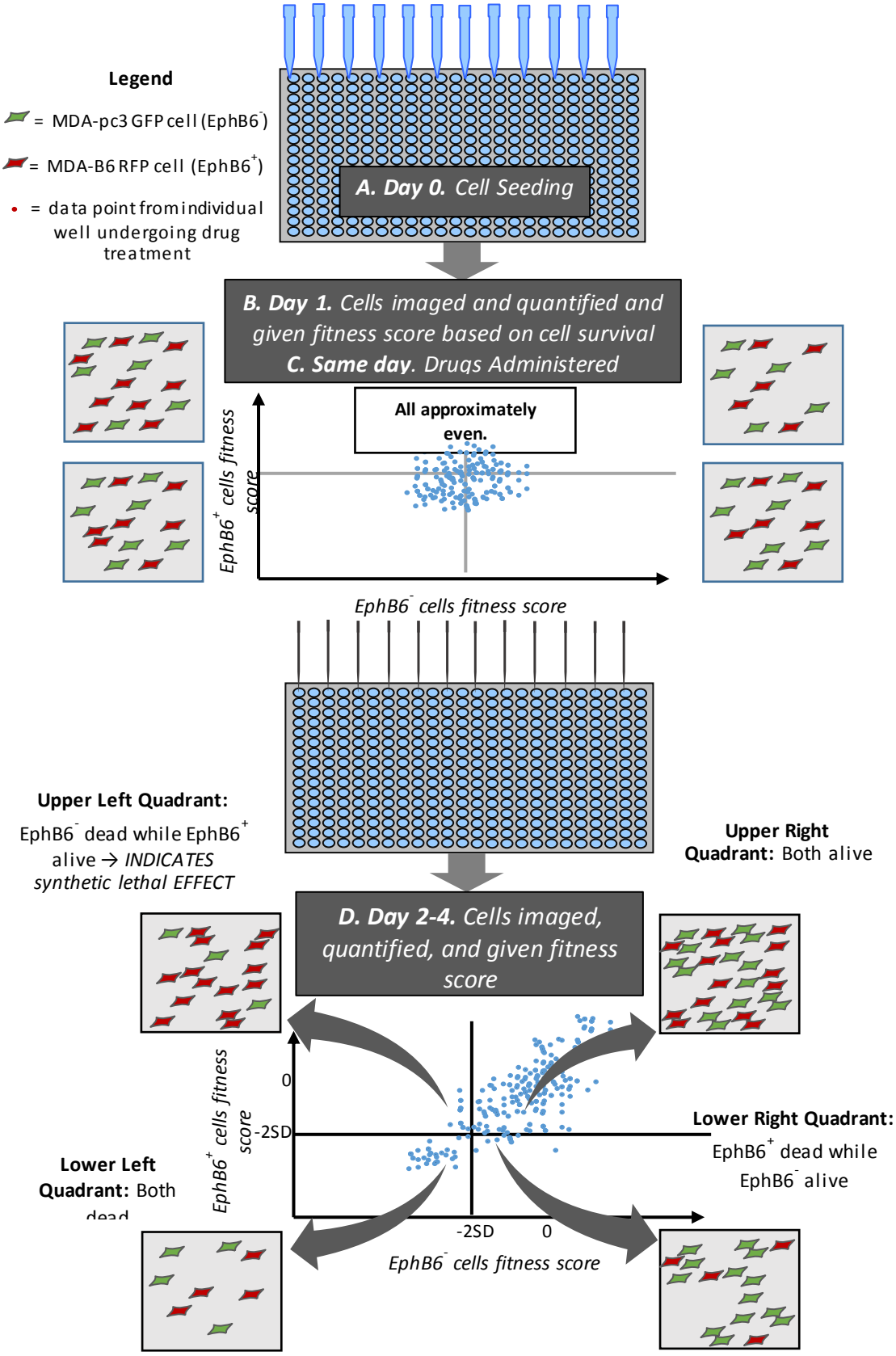
Met is a receptor tyrosine kinase with a single ligand, hepatocyte growth factor (HGF) that is overexpressed in breast cancer, especially in TNBC (Ho-Yen *et al.*, 2014). Met is strongly phosphorylated in HER2+ breast cancer cells resistant to EGFR inhibition and this response is thought to be mediated by the Src kinase (Ho-Yen *et al.*, 2015). In addition, there is also compensatory cross-talk between Met and HER2 receptors in breast cancer cells (Ho-Yen *et al.*, 2015). Crosstalk between Met and HER2 or HER3 receptors supports resistance to EGFR inhibitors in lung cancer and this may be the case in breast cancer: where HER2 is inhibited, Met is overexpressed (Karamouzis *et al.*, 2009). Interestingly, Met and HGF also seem to be actively involved in cancer invasiveness, and metastasis. Recent research indicates that HGF induces invasion through the activation of the PI3K/Akt and MAPK pathways, and by increasing activities of matrix metalloprotease in breast cancer (Kuang *et al.*, 2017). HGF treatment induces loss of cell contacts between cancer cells via the endocytosis of E-cadherin and induces several other pro-invasive events, including phosphorylation of the focal adhesion kinase (FAK) and upregulation of several matrix metalloproteases. This same publication also revealed that Met and HGF seem to play an important role in hypoxia-triggered invasion. Interestingly, a recently published paper shows the co-inhibition of Met and Src to be a promising therapeutic strategy for treating colon cancer (Song *et al.*, 2017), which also often displays reduced EphB6 expression (Table 2). Similar to Src, Met has FDA-approved inhibitors already available, making it an exciting potential target, especially in HER2+ and TNBC subtypes. This is less relevant to ER+ breast cancer, since analysis of ER^+ breast cancer tumours showed a surprisingly low expression of Met — only 5% of ER+ tumours expressed the Met receptor (Zagouri *et al.*, 2014). While Met inhibitors have not been very effective in clinical setting so far (Tolaney *et al.*, 2015), personalising these therapies may improve their efficiency.

1.4.2 Finding Novel EphB6 Synthetic Lethal Interactions with High-Throughput Imaging Drug Screen with Library of FDA-Approved Compounds

While shRNA screens represent an excellent method of identifying genetic interactions and provide a guidance for choosing optimal small-molecule inhibitors, effects of drugs are often more

complex than that of silencing an individual gene, since inhibitory compounds usually affect more than one target. High-throughput screening using libraries of actual FDA-approved small-molecule compounds is an alternative approach conducive to overcoming this limitation (Broach and Thorner, 1996) (Figure 1.4). While this may be a stretch of the term, finding drugs that are synthetically lethal with EphB6 would represent another way to target EphB6-deficient cells, potentially providing a clinically relevant solution. The development of both robotic drug screen platforms and extensive drug libraries allows high-throughput screening to be carried out with a high number of drugs quickly and efficiently. One type of library available to our team contains only current FDA-approved compounds providing an approach to repurposing current drugs towards a personalized cancer therapy. Similar to the shRNA screen, drug screens carried out with both EphB6 expressing and deficient isogenic cell lines could facilitate the discovery of drugs that have potential for use in treating breast cancer dependent on the absence EphB6 expression.

Figure 1.4. Protocol for Drug Screen (NEXT PAGE) A. DAY 0. MDA-B6-RFP (red) cells and MDA-pc3-GFP (green) cells are mixed and seeded into 384 well plates. **B.** DAY 1: Cells are imaged and quantified and score on cell survival with a fitness score (known as B-score) the next day. **C.** DAY 1: The same day, drugs are added to the plate immediately after imaging. **D.** DAY 2-4: Cells are imaged and quantified. Later, fitness scores (B-scores) for each drug/well were quantified. Potential synthetic lethal hits are in the upper left quadrant, where EphB6-positive cells survive better than EphB6-deficient cells.


Legend
= MDA-pc3 GFP cell (EphB6-)
= MDA-B6 RFP cell (EphB6+)
• = data point from individual well undergoing drug treatment
A. Day 0. Cell Seeding
B. Day 1. Cells imaged and quantified and given fitness score based on cell survival
C. Same day. Drugs Administered
All approximately even.
EphB6+ cells fitness score
EphB6- cells fitness score
Upper Left Quadrant: EphB6- dead while EphB6+ alive → INDICATES synthetic lethal EFFECT
Upper Right Quadrant: Both alive
D. Day 2-4. Cells imaged, quantified, and given fitness score
0
-2SD
Lower Right Quadrant: EphB6+ dead while EphB6- alive
Lower Left Quadrant: Both dead

2.0 HYPOTHESIS AND RESEARCH AIMS

2.1 Hypothesis

The hypothesis was that breast cancer cells of various subtypes can be eliminated through EphB6's synthetic lethal relationships.

2.2 Research Aims

To test my hypothesis and explore synthetic lethal relations of EphB6, this project had three specific aims:

1. To examine if EphB6 has synthetic lethal relations with Met and Src across the ER^+, $HER2^+$, and TNBC breast cancer subtypes.
2. Identify EphB6 chemical genetic synthetic lethal interactions by screening a library of FDA-approved compounds and validating potential hit.
3. Investigate molecular mechanisms underlying synthetic lethal interactions of EphB6.

3.0 MATERIALS AND METHODS

3.1 Uncommon Reagents

Reagent	Supplier
Anti-Akt	Cell Signalling Technology (Danvers, MA, USA)
Anti-C-Met	New England Biolabs (Canada)
Anti-C-Src	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-β-tubulin	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-GAPDH	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-EphB6	Santa Cruz Biotechnology (Dallas, TX, USA)
Anti-EphB6	Sigma-Aldrich (St. Louis, MO, USA)
ARQ-197	APEX Biotechnology (Houston, TX, USA)
Bovine serum albumin (BSA)	BioShop Canada Inc. (Burlington, ON, Canada)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, MO, USA)
INHIBITOR	Selleckchem (Houston, TX, USA)
KX2-391	Selleckchem (Houston, TX, USA)
Polybrene	Sigma-Aldrich (St. Louis, MO, USA)
Puromycin	ThermoFisher Scientific (Burlinton, ON, Canada)
Resazurin	R&D Systems (Minneapolis, MN, USA)
XL184	APEX Biotechnology (Houston, TX, USA)

3.2 Cell Lines

3.2.1 Cell Lines and Culture Conditions

TNBC cell lines MDA-MB-231 (ATCC HTB-26), hcc70 (ATCC Crl-2315), and BT20 (ATCC HTB-19) were purchased from the American Type Culture Collection, as were $HER2^+$ cell lines hcc1954 (ATCC Crl 2338) and SKBR3 (ATCC HTB-30), and ER^+ cell lines T47D (ATCC HTB-133) and MCF7 (ATCC HTB-22). MDA-MB-231, BT20, SKBR3, and MCF7 were cultured at 37ºC and 5% CO_2 in DMEM medium with 10% FBS (Gibco, Life Technologies), 1 mM sodium pyruvate (HyClone, GE Life Sciences), and 1% penicillin/streptomycin (Gibco, Life Technologies). Hcc70, hcc1954, and T47D were cultured in RPMI-1640 media with 10% FBS

and 1% penicillin/streptomycin. Cells lines were analyzed for mycoplasma contamination using the MycoAlert kit (Lonza) at thawing and were not passaged longer than three months at a time.

3.2.2 Generation of Lentiviral Particles for altering EphB6 expression

Lentiviral particles were produced in HEK293T using a third-generation lentiviral system containing three plasmids, one containing Gag and Pol (pMDLg/pRRE was a gift from Didier Trono; Addgene plasmid # 12251), one encoding Rev (pRSV-Rev was a gift from Didier Trono; Addgene plasmid # 12253), and an envelope plasmid (pMD2.G was a gift from Didier Trono; Addgene plasmid # 12259). The transfer plasmid used was the pLenti CMV Puro DEST plasmid (gift from Eric Campeau & Paul Kaufman; Addgene plasmid # 17452) with EphB6 wildtype cDNA cloned into the plasmid, while again, EPHB6 MISSION shRNA Lentiviral Transduction Particles with pLKO.1 vector (Sigma-Aldrich, shRNA TRCN0000010677). Particles were generated by co-transfecting ~80% confluent HEK293T cells on 10cm plates with aforementioned plasmids in DMEM containing 2% FBS along with 30 µL METAFECTENE PRO (Biontex Laboratories, Munchen, Germany) in the late afternoon. The next morning, the media was replaced with media containing 10% FBS. Viral particles were collected twice, at 48 hrs and 72 hrs. Media was filtered through 0.44 µm filter. Virus was used fresh if possible, or was stored at -80ºC.

3.2.3 Generation of Stable Cell Lines

EphB6 was previously re-introduced into MDA-MB-231 cells using a mammalian expression vector, pcDNA3 (provided to Addgene by Invitrogen) (Truitt *et al.* 2010). Wild-type EphB6 was re-introduced into SKBR3 using lentiviral transductions. In cells with high EphB6 levels its expression was silenced using shRNA lentiviral constructs (non-silencing shRNa's used as control (ns)). Polyclonal cells lines were used rather than monoclonal to avoid artefactual results. Introduction of green fluorescent protein (GFP) or red fluorescent protein (RFP) had been previously carried out using lentiviral transduction (Paul *et al.* 2016).

3.2.3.1 shRNA-based Gene Silencing

Stable knockdowns were generated using an shRNA that targets EphB6 encoded in EPHB6 MISSION shRNA Lentiviral Transduction Particles (Sigma-Aldrich, shRNA TRCN0000010677) prior to selection with 10 µg/mL puromycin (Sigma-Aldrich) for 5 days. Parental cell lines at ~80-90% confluency were washed with PBS, detached using 0.25% Trypsin EDTA and pelleted. Cells were resuspended in complete media containing 10 µg/mL polybrene (Sigma-Aldrich) and seeded in six well plates. Lentiviral particles were added dropwise to the cells and cells were incubated overnight at 37° C and 5% CO_2. The next morning, media was changed to DMEM containing 10% FBS. 48 hrs after transduction, selection media was added—DMEM with 10% FBS and 10 µg/mL puromycin. Protein expression was monitored using Western blotting. Lentiviral particles were produced using protocol described in Section 3.4.

3.3 Immunoblotting and Western Blotting

Cells at ~80% confluency were rinsed with ice-cold PBS and then lysed in lysis buffer after cell collection by incubating on ice for 30 minutes. Lysis buffer used contained 0.2% NP-40, 5 mM EDTA, 20 mM Tris, 150 mM NaCl, 6 mM PMSF, and 3 mM sodium ortho-vanadate. Cell debris was removed by high speed centrifugation at 9615xg for 15 minutes at 4ºC. Cell lysates were resolved using SDS-PAGE gels (8% SDS, running buffer contained 0.1% SDS, 0.3% Tris, and 1.4% glycine) and then transferred to a nitrocellulose membrane (Amersham, GE Healthcare Life Sciences, transfer buffer contained 20% methanol, 0.3% Tris, and 1.4% glycine), followed by blocking in a 7% non-fat milk solution in PBS/Tween20 or, in the case phospho-antibodies were to be used, 7% BSA in TBS/Tween20. Loading controls used were either GAPDH or tubulin, both structural proteins assumed to be consistent across samples. Membranes were then washed in PBS/Tween20 or TBS/Tween20 three times and incubated at 4ºC overnight with indicated primary antibodies (see Section 3.1 for list of primary antibodies, typically diluted 1:1000 in PBS/Tween20 with 0.05% sodium azide). Membranes were then washed in PBS/Tween20 or TBS/Tween20 three times once more before being incubated at room temperature with secondary, fluorescent antibodies (LI-COR Biotechnology, Guelph, ON, Canada) in a 5% non-fat milk or BSA solution, and protein images were acquired using the LI-COR Odyssey imaging system (LI-COR Biotechnology).

Figures were exported from Odyssey and cropped using Powerpoint. Representative images are shown and each experiment was carried out three times unless otherwise indicated.

3.4 Cell Survival/Cell Death Assays

3.4.1 Resazurin Assay

Cells were seeded in 96 well plates at varying cell concentrations (indicated in results sections, ~1000-3000 cells/well), depending on the cell line in complete media. 18-24 hrs later, drugs were added in 100 µL media with the indicated serum concentrations. Src inhibitor KX2-391, Met inhibitors XL184 and ARQ-197, and the INHIBITOR discovered in the drug screen were used in resazurin experiments. After drug addition, cells were incubated at 37º C and 5% CO_2 for 24-96 hrs. Optimal drug ranges were chosen using literature containing the respective inhibitors and further optimized using initial experiments. At experiment endpoint, 10 µL resazurin stain was added to each individual well and fluorescence was measured using SpectraMax M5 plate reader at 544/590 nm. Five replicates per concentration of drug were used (n=5). Representative experiments are shown. DMSO concentrations matching highest inhibitor concentration points were used as solvent controls. Graphs were produced using Microsoft Excel.

3.4.2 Cell Counting Kit 8 (CCK8) Assay

Cells were seeded in 96 well plates at varying cell concentrations depending on the cell line in complete media and 18-24 hrs later, drugs were added, in 100 µL media with the indicated serum concentrations. Several inhibitors were used, including Src inhibitor, KX2-391, Met inhibitors, XL184 and ARQ-197, and the INHIBITOR discovered in the drug screen. After drug addition, cells were incubated at 37º C and 5% CO_2 for 24-96 hrs, depending on the experiment. At experiment endpoints, 10 µL CCK8 stain was added to each individual well and absorbance was measured using SpectraMax M5 plate reader at 450 nm. Representative experiments are shown. DMSO was used as a solvent control as all drugs were dissolved in DMSO. Graphs were produced using Microsoft Excel.

3.5 Caspase 3 Activation Assay

Analysis of Caspase 3 activity was carried out using the Enchek Caspase 3 Activation Kit #1 (Z-DEVD-AMC substrate) (Thermofisher), according to the provided protocol. Cells were seeded in 10 cm plates in four groups: a solvent control and a drug group for each EphB6-positive, and EphB6 deficient cells, and drugs were added 18-24 hrs after seeding. Optimal concentration was chosen using the resazurin assays previously completed. At indicated time points, cells were collected with EDTA and Caspase 3 activity was analyzed in cell lysate. Three technical replicates were carried out per each experiment.

3.6 Real-time Cell Imaging with Incucyte

Similar to resazurin and CCK8, EphB6-expressing and EphB6-deficient cells were seeded in 96-well plates at indicated amounts. In 18-24 hrs, drugs were added at indicated concentrations with DMSO control matching highest inhibitor concentration. Plates were immediately placed in Incucyte and imaged over time at indicated intervals until indicated endpoints. Analysis was optimized for each cell line and graphs were created by comparing INHIBITOR treated cells to DMSO control in Microsoft Excel.

3.7 High Throughput Drug Screen with a Library of 1800 FDA-Approved Compounds

MDA-B6 (expressing wild type EphB6) and MDA-pc3 (with control vector) were used as models of TNBC with and without EphB6. The drug screen is an imaging-based assay—MDA-B6-RFP and MDA-pc3-GFP previously produced in our lab (Paul *et al.* 2016) were co-seeded in 384 well plates and imaged prior to the addition of the drugs, and then every 24 hrs for 72 hrs. 250 MDA-B6 GFP and 250 MDA-pc3 RFP cells were co-seeded using the Beckman Coulter Biomek NX(P) in DMEM complete media (described in Section 3.3.1), but media was changed after initial imaging (approximately 24 hrs after seeding) to a low serum media (DMEM, 1% FBS) to reduce risk of any potential interaction between proteins in FBS and the small-molecules and to highlight drugs that work through induction of apoptosis. Drugs were added using the Biomek Laboratory Automation Workstation robot giving a final concentration of either 100 nM or 500 nM. These low concentrations were used to try find a therapeutic window at a low concentration to reduce

off-target effects and side effects of the drugs. Six 384-well plates were seeded per each repetition of the experiment. Plates were imaged at 24, 48, and 72 hrs using a high-throughput confocal microscope. Four images were taken per well. The experiment was repeated four times, two times at the two different concentrations of the drugs—100 nM and 500 nM.

3.8 Computational Analysis of Screen Results

After imaging each day, images were analyzed using MetaXPress, the software that also was used to image the plates, and cells of each colour and thus EphB6 genotype were counted. Plate effects were normalized by using the B-score algorithm, with a script written in Python by Bjorn Haave and Frederick Vizeacoumar. The B-score handles variability by removing positional effects through a median polish. A residual is calculated after subtracting the median from each row and each column, and then this is divided by the median absolute deviation. The result is a normalized measurement value. DMSO control wells were present on the outermost layer of the plate and were omitted for the B score analyses as random organization is required for proper analysis. Data analysis was carried out using Anaconda. B-score data was organized by day and plotted. Graphs were created using Microsoft Excel. Drugs with a normal MDA-B6 B score but a negative MDA-pc3 B-score (two standard deviations less than the mean) were monitored by day and trends of B-score were studied for potential hits to confirm synthetic lethal effect and cell death over time. Images were checked to ensure the synthetic lethal effect was real.

3.9 Statistical Practices and Methods

Experiments were repeated three times unless otherwise indicated. Statistical significance was calculated with student t-test, comparing each individual set of data points to its control, typically the DMSO control. “n” values were calculated as the number of technical replicates per experiment conducted and experiments were repeated three times unless otherwise indicated. Data in graphs are presented as mean +/- standard deviation.

4.0 RESULTS

4.1 Generation of EphB6-Positive and EphB6-Deficient Isogenic Cell Lines

In order to validate the hits that arose in the earlier shRNA synthetic lethality screen (Paul *et al.*, 2016), cell line pairs different *only* in EphB6 expression were needed to test the effect of EphB6 on cell survival after treatment with small-molecule inhibitors intended to approximate the synthetic lethal effect that shRNA molecules proved to have in the screen. Expression of EphB6 was assessed in parental breast cancer cell lines by Western. Following either silencing of EphB6 or introduction of EphB6 (depending on the cell line's original expression), cell lysates were made and Westerns were used to assess EphB6 expression (Figure 4.1). To ensure stable EphB6 silencing/expression, EphB6 levels were also monitored by Westerns, while cells were being used in my experiments. On some occasions, suppression of EphB6 by shRNA decreased and parental cells were "re-silenced" (Figure 4.1).

Src and Met expression also had to be confirmed at the protein level (Figure 6). Src expression in TNBC cell lines was not examined, as this information had been previously published (Paul *et al.*, 2016). Src expression, while slightly variable, was observed across subtypes in all tested cell lines (Figure 4.2). Unfortunately, ER^+ cell lines, T47D and MCF7, do not express the Met receptor, matching previous research concerning Met expression (Kim *et al.*, 2014; Zagouri *et al.*, 2014) so Met-related experiments were not carried out in ER^+ cell lines. Interestingly, some TNBC and $HER2^+$ breast cancer cell lines had varying expression of the Met receptor, negatively correlating with EphB6 levels, further supporting a synthetic lethal relationship between EphB6 and Met. Cells that had lower EphB6 expression appeared to increase their Met levels, notably all three tested TNBC cell lines and one $HER2^+$ breast cancer cell line (Figure 4.2). Nevertheless, all TNBC and $HER2^+$ breast cancer cell lines, with the exception of $HER2^+$ SKBR3, expressed the Met receptor at some level. SKBR3 cells proved to be MET deficient (not shown) and therefore were not included in our analysis of EphB6-Met synthetic lethal interaction.

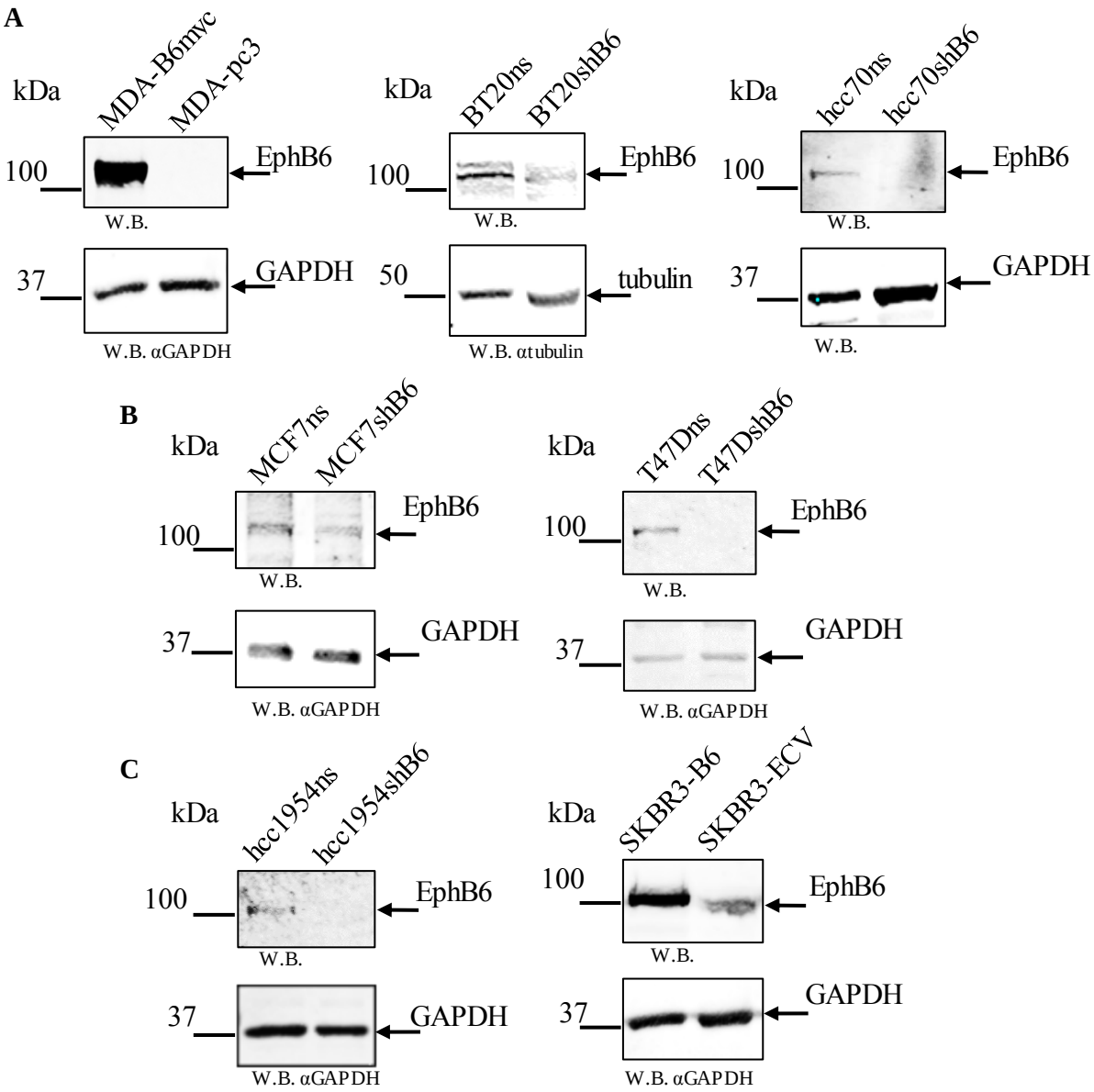


Figure 4.1. Creation of cell line pairs isogenic with the exception of EphB6. A. TNBC cell lines used. **B.** ER^+ breast cancer cell lines used. **C.** $HER2^+$ breast cancer cell lines used. Myc-tagged EphB6 was previously re-introduced into TNBC MDA-MB-231 cells using pcDNA3 expression vector (MDA-B6myc). Empty pcDNA3 vector was used as a control (MDA-pc3). Wild-type EphB6 was re-introduced into $HER2^+$ SKBR3 cells using a lentiviral transduction with pCMV3 vector (SKB3-B6) with empty vector used as control (SKBR3-ECV). Cells that expressed high levels of EphB6 were silenced with shRNA lentiviral constructs (shB6) with non-silencing shRNAs used as control (ns). Stable transfections and transductions were performed using selection with 10 ug/mL puromycin for 5 days. Lentiviral particles were produced using protocol described in Section 3.4.

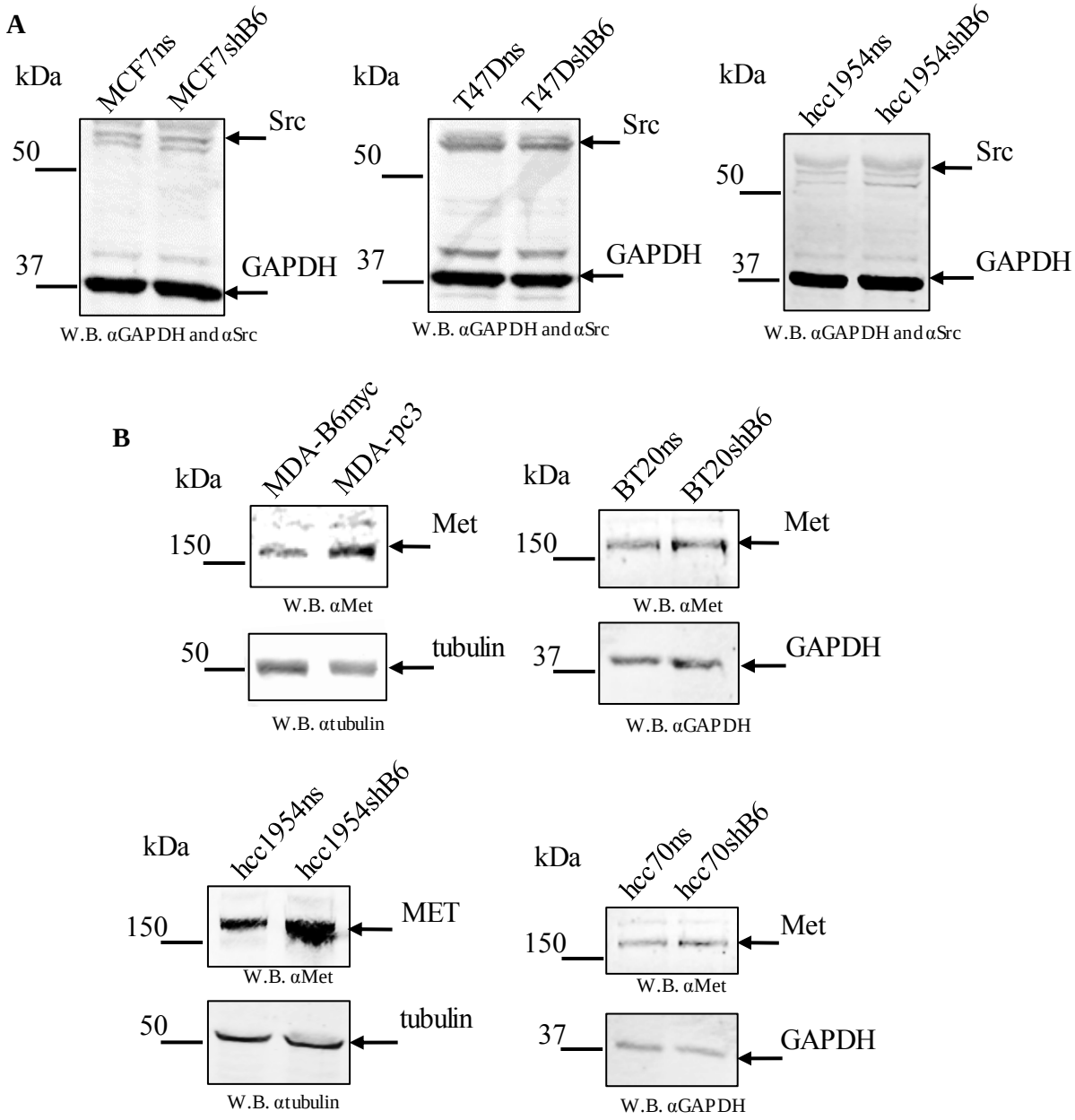


Figure 4.2. Expression of Src and Met in various cell lines used in experiments. **A**. Src expression in ER^+ and $HER2^+$ breast cancer cell lines MCF7 (ER^+), T47D (ER^+), and hcc1954 ($HER2^+$) cells. **B**. Met expression in TNBC cell lines BT20, hcc70 and MDA-MB-231 and in $HER2^+$ breast cancer cell line hcc1954. Met expression appears to increase inversely to EphB6 expression.

4.2 Validation of EphB6-Met and EphB6-Src Synthetic Lethal Interactions across Major Breast Cancer Subtypes

4.2.1 Validation of Synthetic Lethal Relationships using Resazurin Assay

Interestingly, an FDA-approved Src inhibitor that binds the substrate binding site of Src (Antonarakis *et al.*, 2013), KX2-391 showed no synthetic lethal effects in ER^+ cell lines (Figure 4.3), when tested in the resazurin-staining assay. The effect in $HER2^+$ cell line hcc1954 was not consistent across different concentrations, nor were the same concentrations effective in different experiments (Figure 4.3) and no effect was seen in another HER2+ cell line, SKBR3 (Figure 4.3). Thus, it seems that the synthetic lethal relationship between EphB6 and Src is mostly restricted to TNBC. This is not very surprising, since this synthetic lethal interaction was initially identified and characterized in TNBC cells (Paul *et al.*, 2016), and TNBC biology differs dramatically from the biology of $HER2^+$ and ER^+ tumors (Inic *et al*. 2014).

Treatment with an FDA-approved Met inhibitor, ARQ-197, which binds to the ATP-binding site of Met and also has some tubulin-binding properties (Basilico *et al.*, 2013), resulted in preferential killing of EphB6-deficient TNBC cells, MDA-pc3, compared to MDA-B6myc (Figure 4.4), consistent with the synthetic lethal effect of the Met-targeting shRNAs in the original screen. Another FDA-approved Met-inhibitor that acts by stabilizing Met in it's inactive conformation (De Falco *et al.*, 2017), XL184, was also tested with MDA-MB-231 cells (Figure 4.4). Similar to ARQ-197, it showed a stronger effect in MDA-pc3 compared to MDA-B6-myc, exciting as the synthetic lethal effect proved not to be limited to a specific inhibitor. Further, the effect was not limited to a specific cell line as BT20, another TNBC cell line, also showed a synthetic lethal effect with ARQ-197 (Figure 4.4). Using a second cell line of the same subtype gave more confidence in the results being broadly applicable. As MDA-MB-231 needed expression restored and BT20 EphB6 expression was silenced, it appears to be reliant on the expression of EphB6 rather than any method of changing EphB6 expression.

Met inhibitors were not tested in ER^+ breast cancer cells due to the lack of Met expression in ER^+ breast cancer cell lines, which was consistent with previously published observations (Zagouri *et al.*, 2014). Resazurin assays with $HER2^+$ breast cancer cell line, hcc1954, did not show consistent effects that that would indicate a synthetic lethal relation of Met inhibitors with EphB6 (Figure 4.4). SKBR3 does not express the Met receptor either, but the lack of a response in

hcc1954 combined with lower expression in Met in HER2$^+$ breast cancer in general convinced us to move on to other parts of the project.

4.2.2 Analysis of Signalling Events Involved in EphB6-Met Synthetic Lethality

4.2.2.1 Caspase 3 Activation Increases More in EphB6-Deficient Cells after Treatment with Met Inhibitor

Drugs that are used for cancer treatment often trigger cell death through an increase in apoptotic signalling. Therefore, after preferential killing of EphB6-deficient cells with FDA-approved Met inhibitors was observed, activation of a central pro-apoptotic molecule, Caspase 3 was studied to try elucidate the mechanism underlying the synthetic lethal action. Caspase 3 is an effector in apoptotic signalling, an indicator that cells are currently undergoing apoptosis. Caspase 3 activation was tested in MDA-MB-231 cells with or without EphB6 expression in response to treatment with ARQ-197, with the concentration used in experiments determined using resazurin assay data. Cells that lacked EphB6 showed a stronger increase in Caspase 3 activity after treatment with ARQ-197, compared to cells that expressed EphB6 (Figure 4.5).

4.2.2.2 EphB6 enhances Akt activation in Met inhibitor-treated cells

After studying Caspase 3 activation and noting a difference in apoptotic signalling depending on EphB6 presence, pro-survival/anti-apoptotic signalling further upstream was examined, predicting that pro-survival signal activation is the reason EphB6-expressing cells exhibit a reduced apoptotic response. Apoptosis can be negatively controlled by the Akt kinase or Protein Kinase B (Manning and Toker, 2017) and confirming our hypothesis, further experiments showed that activating Akt phosphorylation on S473 residue, one of the residues where phosphorylation is required for Akt kinase activation, was far higher in ARQ-197-treated cells that expressed EphB6 (Figure 4.6). Since Akt is known to block the Caspase 3 activation through phosphorylation and inhibition of the Bcl-2 family protein Bad (Manning and Toker, 2017), this effect is likely to represent the mechanism of EphB6 action in supporting TNBC cell survival following Met inhibition.

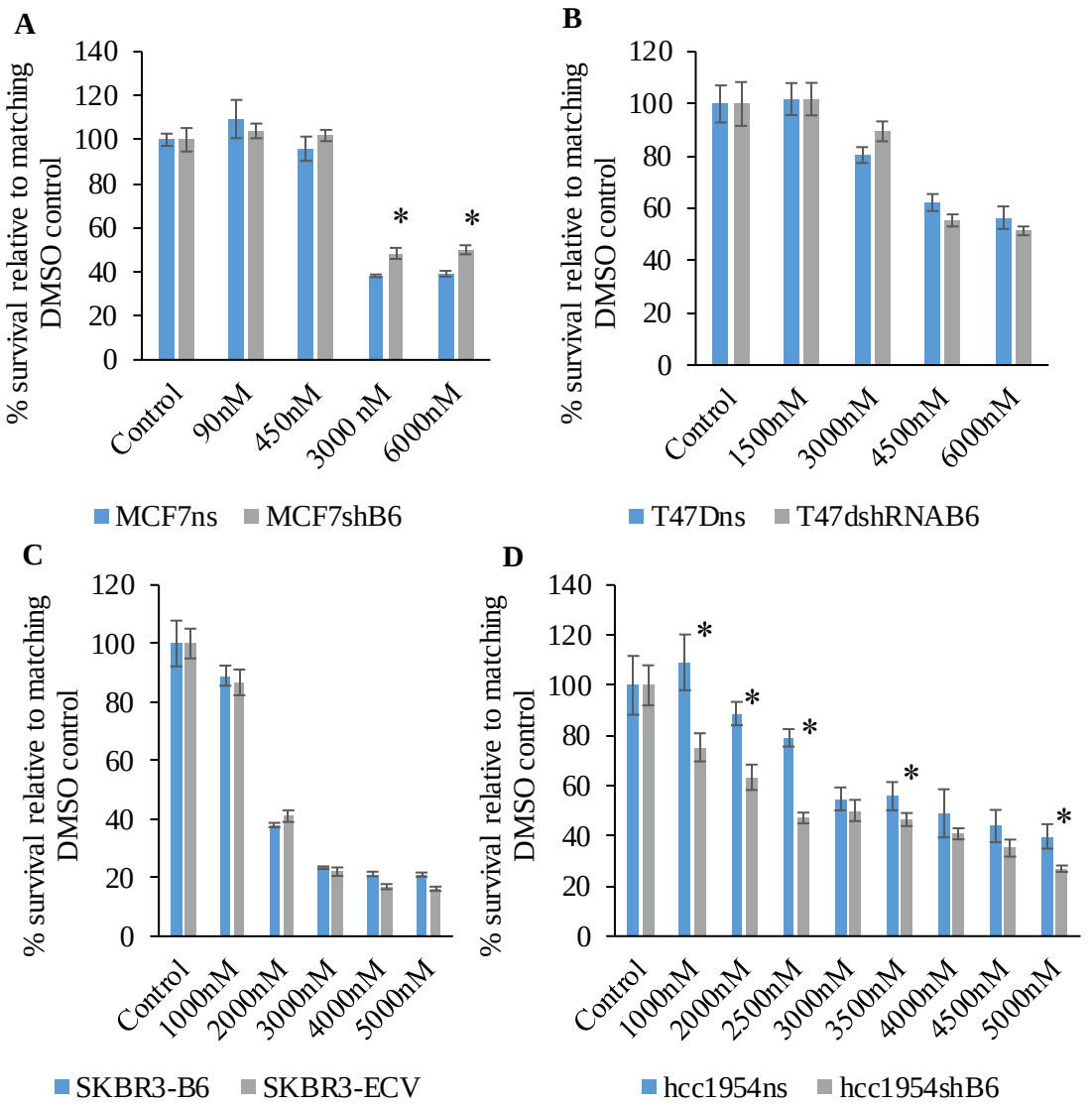


Figure 4.3. EphB6-Src synthetic lethality restricted to TNBC. Cells were seeded in 96 well plates and cultured with indicated concentrations of KX2-391 or DMSO control matching highest inhibitor concentration at normal serum (10% FBS) for 72 hrs. Resazurin was added and absorbance was measured using SpectraMax M5. Five wells were analyzed per condition. The graph shows percentage of cell survival relative to DMSO control. **A.** MCF7 with KX2-391 seeded at 5 x 10^3 cells/well (two replicates). **B.** T47D with KX2-391 seeded at 5 x 10^3 cells/well (two replicates) **C.** SKBR3 with KX2-391 at 5 x 10^3 cells/well (two replicates). **D.** hcc1954 with KX2-391 seeded at 3 x 10^3 cells/well. *P < 0.05, *versus* EphB6 expressing cell line

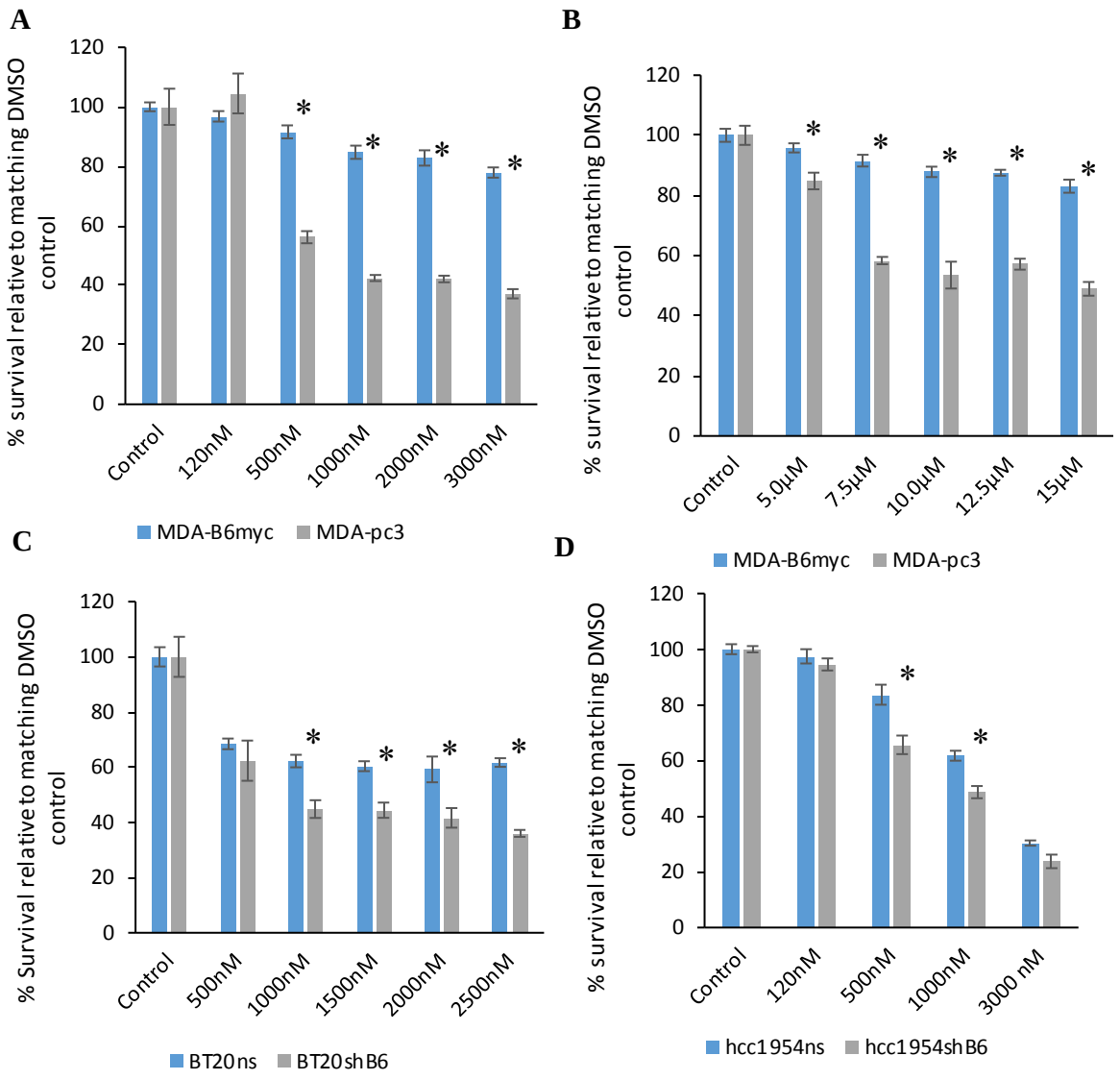


Figure 4.4. EphB6-Met synthetic lethality restricted to TNBC. Cells were seeded in 96 well plates and cultured with indicated concentrations of Met inhibitors or DMSO control matching highest concentration at normal serum (10% FBS) for 72 hrs. Resazurin was added and absorbance was measured using SpectraMax M5. Five wells were analyzed per condition. The graph shows percentage of cell survival relative to DMSO control. **A.** ARQ-197 treated MDA-MB-231 seeded at 1 x 10^3 cells/well and **B.** XL184 treated MDA-MB-231 seeded at 1 x 10^3 cells/well **C.** BT20 with ARQ-197 seeded at 1 x 10^3 cells/well **D.** hcc1954 with ARQ-197 seeded at 3 x 10^3 cells/well. *P < 0.05, *versus* EphB6 expressing cell line significant

4.3 Identifying EphB6 Synthetic Lethal Interactions using Drug Screen with a Library of FDA-approved Compounds

To find alternative clinically-relevant synthetic lethal interactions of EphB6, a drug screen using high-throughput imaging technology was performed, trying to find a drug that selectively kills EphB6-deficient breast cancer cells, as again, EphB6 is downregulated in breast cancer. For EphB6-expressing cells, MDA-MB-231 cells stably expressing wild-type EphB6 (MDA-B6) (Truitt *et al.*, 2010) were used and MDA-MB-231 cells with empty vector (MDA-pc3) were used as cells that did not express EphB6. MDA-B6 cells were engineered to express RFP, while MDA-pc3 cells were designed to express GFP (Paul *et al.*, 2016). One potential caveat to a drug screen, like any other screen, is the issue of potential artefactual observations due to systemic errors, including "plate effects" caused by the uneven medium evaporation in different areas of tissue culture plates, issues related to drug interactions with some parts of the experimental system, such as the media or serum, or cell-line specific responses. To avoid cell-line specific responses, any hits would need to be validated using additional cell lines. To reduce the likelihood of drug-protein

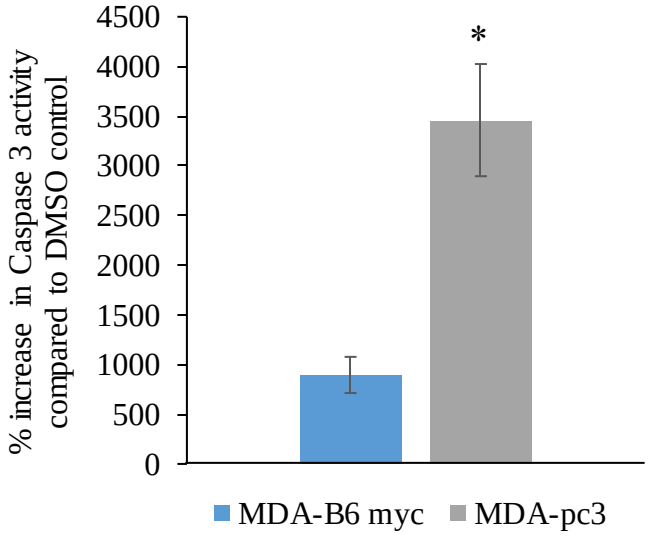


Figure 4.5. **Increase in pro-apoptotic Caspase 3 activity after treatment with ARQ-197 enhanced in EphB6-deficient cells**. Cells were seeded in 10 cm plates and 18-24 hrs later, 1 μM ARQ-197 or matching volume DMSO control (3 replicates per condition) was added. Cells were treated for 65 hrs at 10% FBS. Caspase 3 activity was determined using the Enzchek Caspase 3 Kit #1, with a SpectraMax M5 plate reader used to measure activity. Two replicates.

*$P < 0.05$. *versus* EphB6 expressing cell line

interactions, lower serum media can be used; this also helps to better display potential apoptotic effects. Plate effects are normalized using what is known as the B-score algorithm described in Brideau *et al.* (2003). The B-score handles variability by removing positional effects through a median polish. A residual is calculated after subtracting the median from each row and each column, and then this is divided by the median absolute deviation (MAD). The result is a normalized measurement value. MDA-pc3 and MDA-B6 cells were treated with the library of FDA approved drugs at 100 nM and 500 nM in the presence of 0.5% of FBS. The screen was carried out twice at each condition. The data from the 100 nM screens are shown in Figure 4.7 and the 500 nM in Figure 4.8. The bolded lines represent two standard deviations less than normal for each B-score. Only one of the drugs (further on referred to as "INHIBITOR"; name of the INHIBITOR not disclosed in thesis for patenting purposes) produced significant synthetic lethal responses in all four screens.

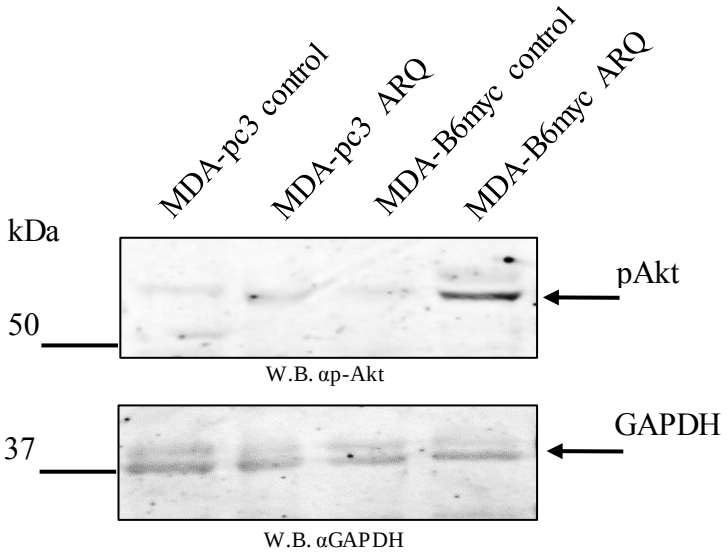


Figure 4.6. Pro-survival signaling after treatment with MET Inhibitor increased more in EphB6-expressing cells. Cells were seeded in 6 well plates and 18-24 hrs later, 1 μM ARQ-197 or matching volume DMSO control was added. Cells were treated for 48 hrs at 10% FBS before lysates were prepared. Akt phosphorylation was analysed by Western blotting with anti-phospho-Akt (S473). Western blotting with anti-GAPDH was used as a loading control. Experiment was repeated twice.

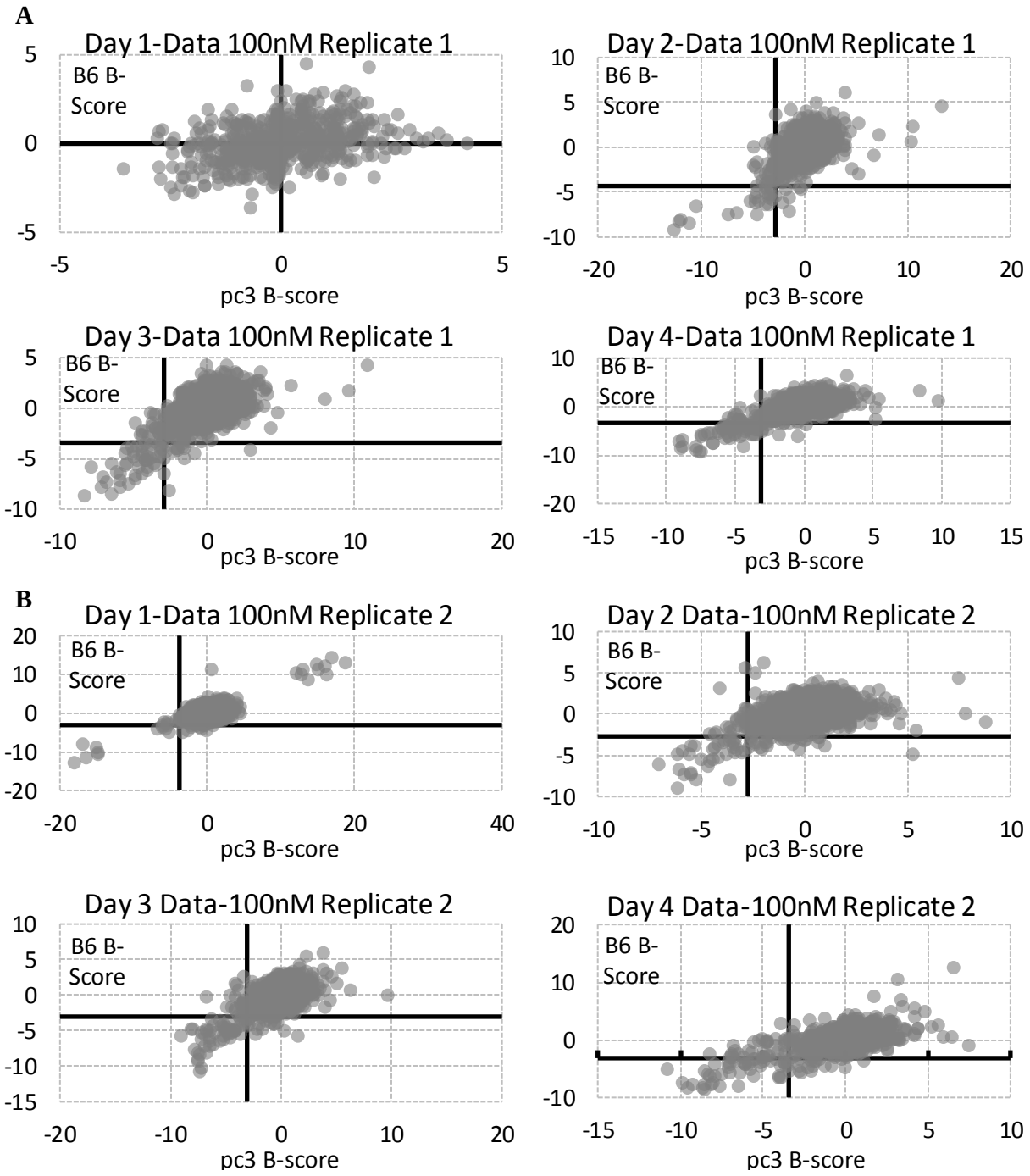


Figure 4.7. **B-scores from both 100 nM screens.** MDA-B6-RFP and MDA-pc3-GFP were seeded in 384 well plates at 250 cells/cell line/well and cultured with 100 nM of drugs in FDA-approved library or DMSO control matching volume of drugs at low serum (0.5% FBS). Day 1 was after cells adhered but prior to addition of drugs; Day 2-4 were 24, 48, and 72 hours after addition of drugs. Cells quantified and B-score calculated. MDA-B6 B-score is on the Y axis and MDA-pc3 B-score is on the X-axis. Note that darkened lines indicate level two standard deviations below normal. Drugs with synthetic lethal potential are those in the upper left quadrant. Graphs from the 100 nM first replicate (**A**) and second replicate (**B**).

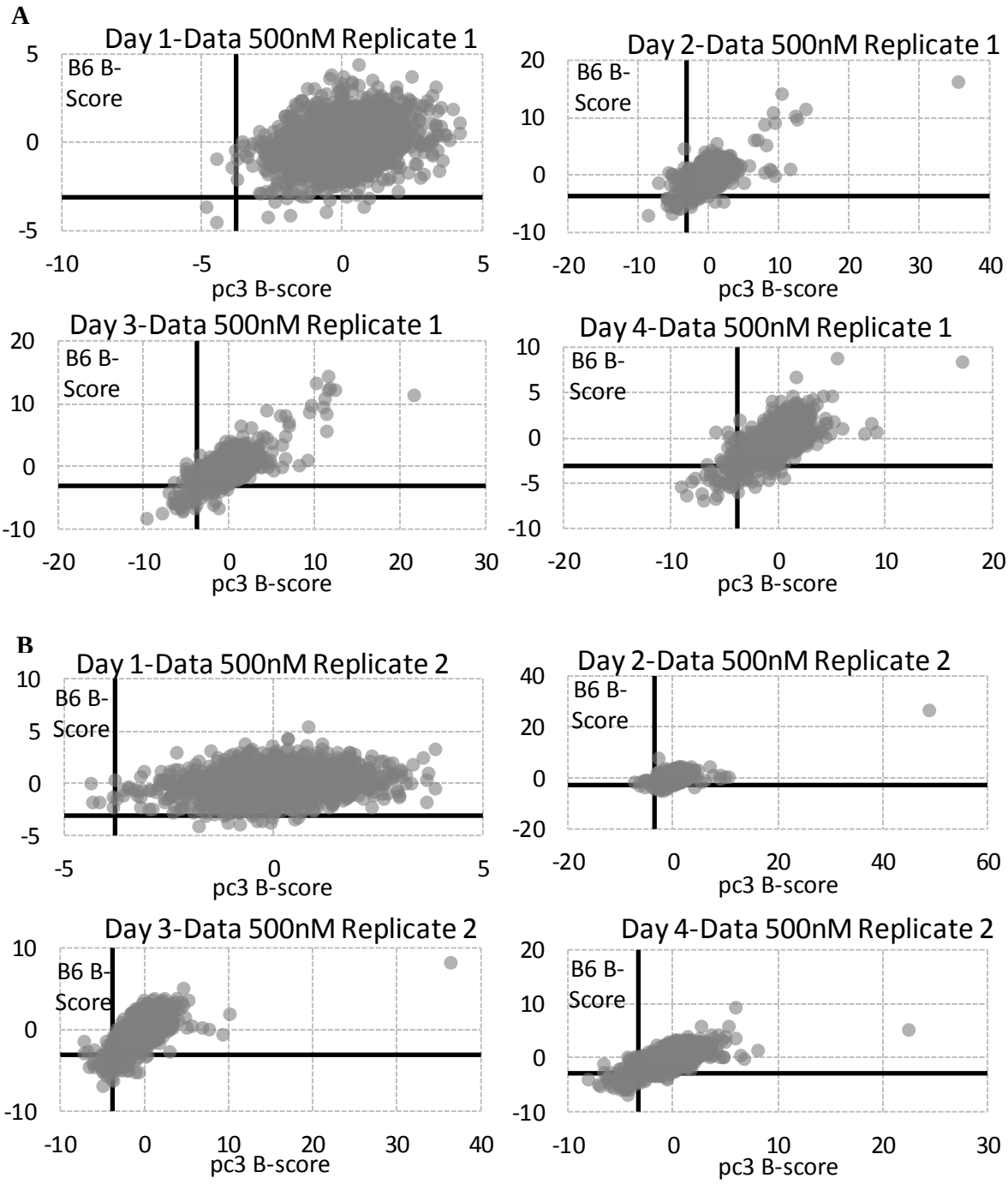


Figure 4.8. **B-scores from both 500 nM screens.** MDA-B6-RFP and MDA-pc3-GFP were seeded in 384 well plates at 250 cells/cell line/well and cultured with 500 nM of drugs in FDA-approved library or DMSO control matching volume of drugs at low serum (0.5% FBS). Day 1 was after cells adhered but prior to addition of drugs; Day 2-4 were 24, 48, and 72 hours after addition of drugs. Cells quantified and B-score calculated. MDA-B6 B-score is on the Y axis and MDA-pc3 B-score is on the X-axis. Darkened lines indicate two standard deviations below normal. Drugs with synthetic lethal potential are those in the upper left quadrant. Graphs from the 500 nM first replicate (**A**) and second replicate (**B**).

As the plates were imaged for 72 hrs, it was vital to watch the trend of the B-score for those hits that showed up in the different screens. Again, a drug with a low MDA-pc3 B-score and a normal MDA-B6 B-score was needed for a potential hit.

4.4 Validation of EphB6 Synthetic Lethal Interaction with INHIBITOR in Different Breast Cancer Subtypes

4.4.1 Cell Survival Assays to Study EphB6-INHIBITOR Synthetic Lethality

4.4.1.1 Resazurin Assays Confirm EphB6-INHIBITOR Synthetic Lethality in TNBC

Initially, resazurin assays were carried out with INHIBITOR in two TNBC cell lines, MDA-MB-231 and hcc70 at varying experimental conditions, both 72 hours and 96 hours and with both lower serum (1%) and normal serum (10%), and in both cell lines INHIBITOR showed a synthetic lethal effect with EphB6 (Figure 4.9). Similar to the work with Met inhibitors, MDA-MB-231 represents a cell line where EphB6 expression needed restoration while hcc70 is a TNBC cell line that still expresses EphB6 so the result does not depend on the method of EphB6 expression manipulation. Again, the synthetic lethal effect proved to be not reproducible in ER^+ breast cancer cell lines, and was not observed in $HER2^+$ breast cancer cells, suggesting significant differences in EphB6's role across different breast cancer subtypes (Figure 4.10).

4.4.1.2 CCK8 Assays Confirm EphB6-INHIBITOR Synthetic Lethality in TNBC

A second method based on cell's metabolism, CCK8 assays, also showed a similar synthetic lethal effect of the INHIBITOR, since the application of INHIBITOR selectively suppressed EphB6-deficient TNBC cells at all tested INHIBITOR concentrations (Figure 4.11).

4.4.2 Incucyte Analysis Confirm EphB6-INHIBITOR Synthetic Lethality in TNBC

Incucyte allows to monitor cell propagation/survival based on the optical analysis of cell density. This experimental approach also showed stronger effects of INHIBITOR in EphB6-

deficient TNBC cells (Figure 4.12), once again, confirming the synthetic lethal relation between INHIBITOR and EphB6 in TNBC.

4.4.3 Comparing Cell Death-Related Signalling After Treatment with INHIBITOR in EphB6-Positive and EphB6-Deficient Cells

4.4.3.1 Caspase 3 Activation Increases more in EphB6-deficient TNBC Cells after Treatment with INHIBITOR

As expected, the activation of Caspase 3 was much greater in EphB6-deficient MDA-pc3 cells than in EphB6-expressing MDA-B6 cells following INHIBITOR application (Figure 4.13). A similar synthetic lethal effect was observed in hcc70, where differences in responses to INHIBITOR between EphB6-positive and EphB6-negative cells were also statistically significant (Figure 17B).

4.4.3.2 EphB6 enhances Akt activation in INHIBITOR Treated TNBC Cells

Pro-survival Akt signalling was tested by Western blotting with anti-phospho-Akt antibody that specifically recognizes Akt phosphorylation on the activating residue, S473, and showed the Akt activation was consistently higher following INHIBITOR treatment in TNBC cells that expressed EphB6. This suggests that EphB6 uses Akt activation to protect TNBC cells form the pro-apoptotic effects of INHIBITOR (Figure 4.14).

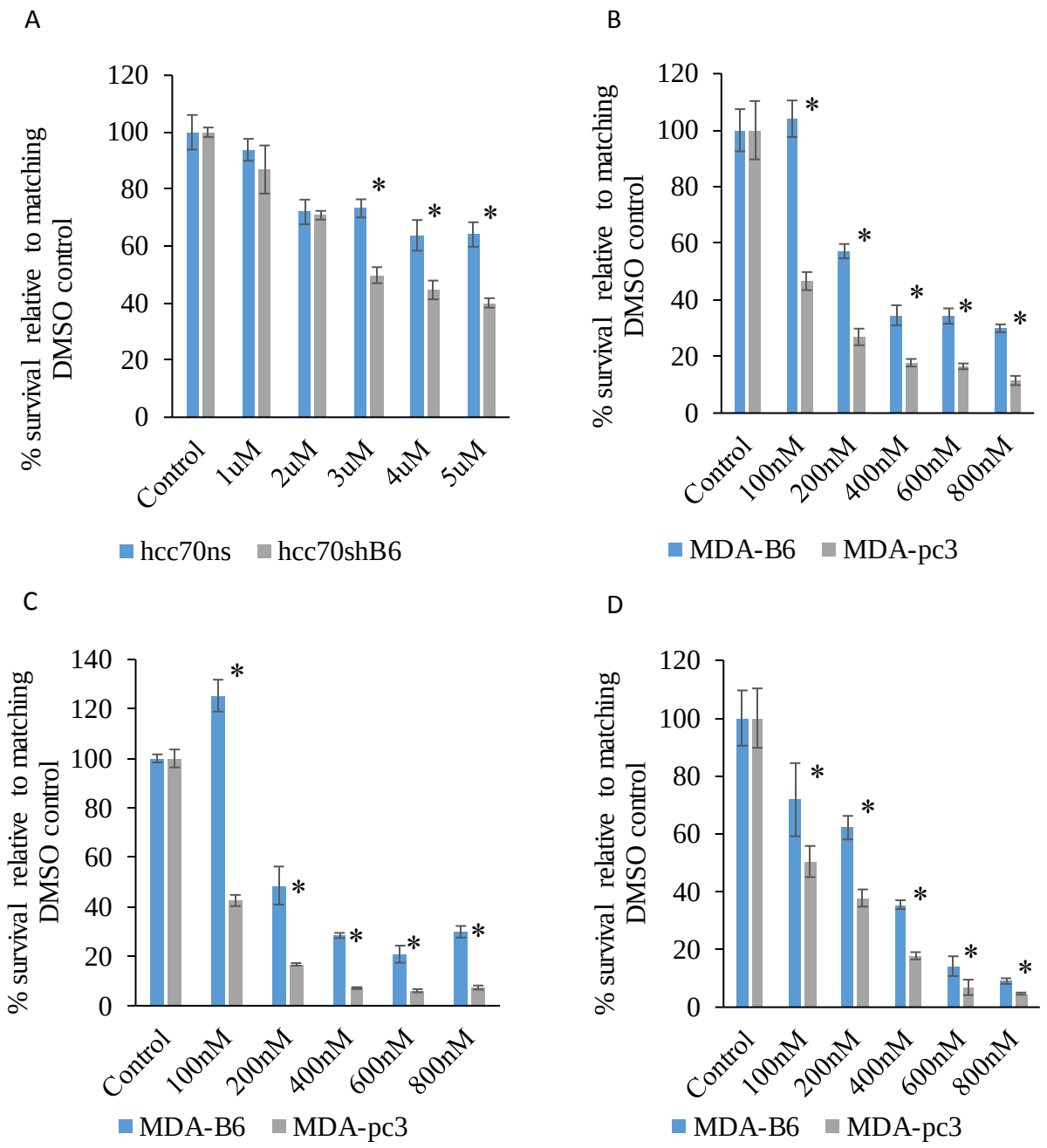


Figure 4.9. EphB6-INHIBITOR synthetic lethal interaction validated in TNBC. Cells were seeded in 96 well plates at 1 x 10^3 cells/well for MDA-MB-231 and 3 x 10^3 cells/well for hcc70 and cultured with indicated concentrations of INHIBITOR or DMSO control matching highest INHIBITOR concentration. Resazurin was added and absorbance was measured using SpectraMax M5. Five wells were analyzed per condition. The graph shows percentage of cell survival relative to DMSO control. **A**. hcc70 treated for 96 hours with low serum media (1% FBS). **B.** MDA-B6 and MDA-pc3 treated for 72 hours with low serum media (1% FBS). **C.** MDA-B6 and MDA-pc3 treated for 96 hours with low serum media (1% FBS). **D.** MDA-B6 and MDA-pc3 treated for 96 hours with normal serum (10% FBS). *P < 0.05, *versus* EphB6 expressing cell line

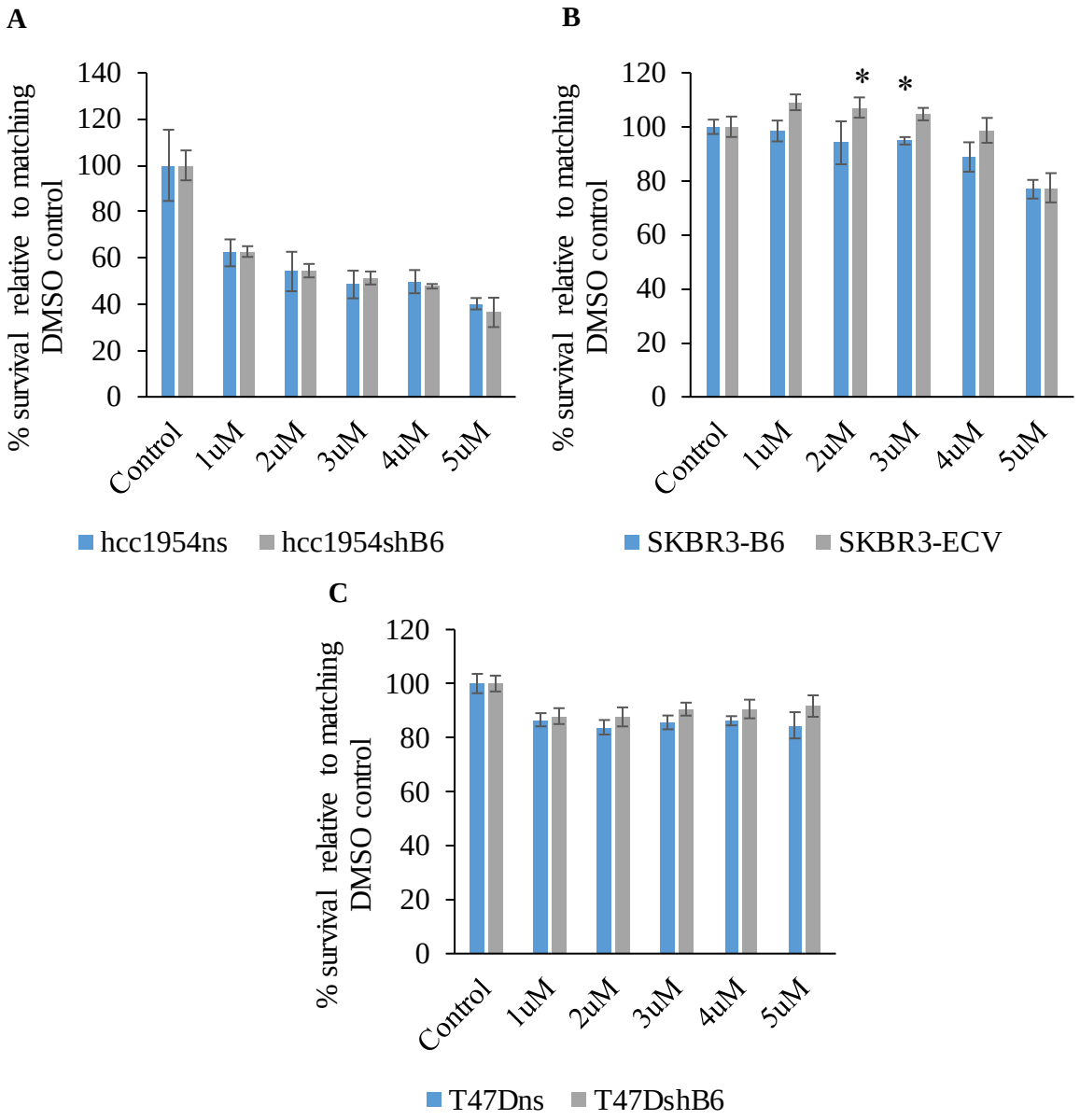


Figure 4.10. EphB6-INHIBITOR synthetic lethal interaction not present in ER$^+$ and HER2$^+$ breast cancer cell lines. Cells were seeded in 96 well plates and cultured with indicated concentrations of INHIBITOR or DMSO control matching highest INHIBITOR concentration at 1% FBS for 96 hours (**A** and **B**) or 72 hours (**C**). Resazurin was added and absorbance was measured using SpectraMax M5. Five wells were analyzed per condition. The graph shows percentage of cell survival relative to DMSO control. **A**. hcc1954 seeded at 3 x 10^3 cells/well (two replicates) **B** SKBR3 seeded at 5 x 10^3 cells/well (two replicates). **C**. T47D seeded at 5 x 10^3 cells/well (two replicates).
*P < 0.05, *versus* EphB6 expressing cell line

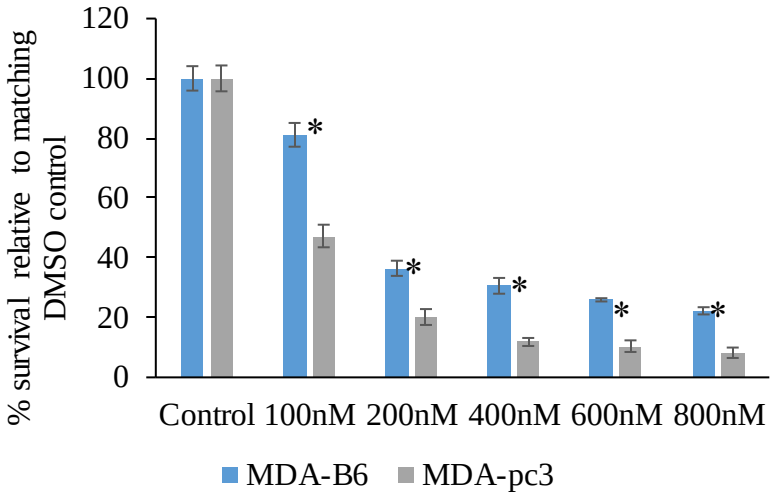


Figure 4.11. Validation of EphB6-INHIBITOR synthetic lethal interaction using CCK8 in TNBC. MDA-B6 and MDA-pc3 were seeded in 96 well plates at 1 x 10^3 cells/well and cultured with indicated concentrations of INHIBITOR or DMSO control matching highest INHIBITOR concentration at low serum (1% FBS) for 72 hours. CCK8 was added and absorbance was measured using SpectraMax M5. Five wells were analyzed per condition. The graph shows percentage of cell survival relative to DMSO control. *P < 0.05. *versus* EphB6 expressing cell line

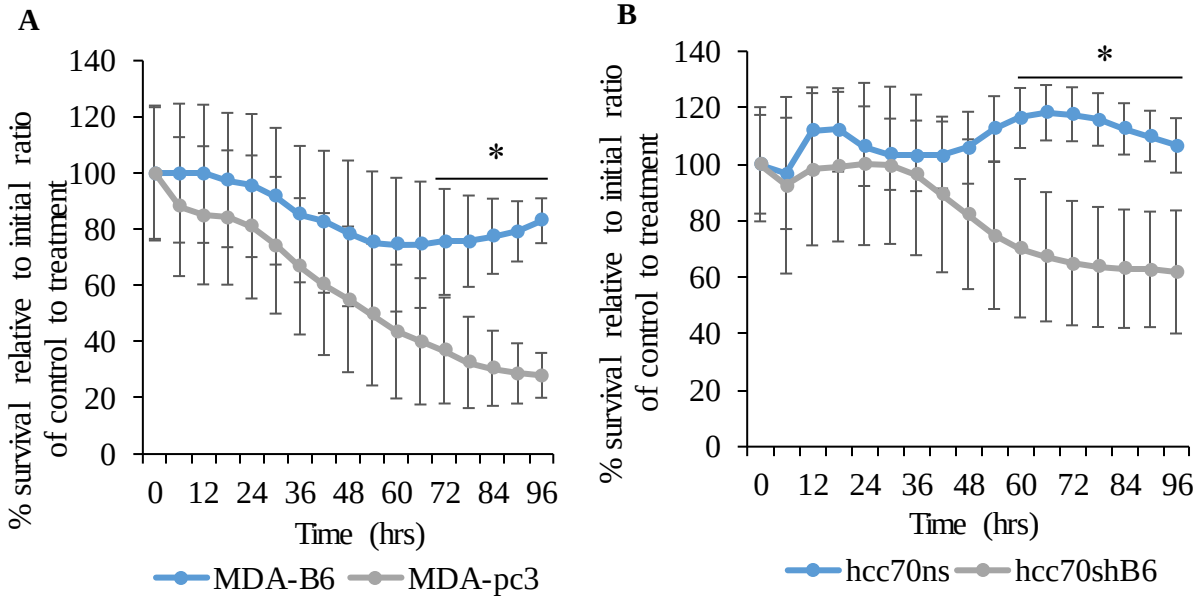


Figure 4.12. Validation of EphB6-INHIBITOR synthetic lethal interaction over time in TNBC. Cells were seeded in 96 well plates at indicated cell concentrations and cultured with indicated concentrations of INHIBITOR or DMSO control matching highest INHIBITOR concentration at indicated serum for 96 hours. **A.** MDA-MB-231 seeded at 1 x 10^3 cells/well and 4% FBS **B.** hcc70, seeded at 3 x 10^3 cells/well at 1% FBS. Cell survival was tracked over time using Incucyte. Five wells were analyzed per condition with four images taken at each time point per well. *p < 0.05 *versus* EphB6 expressing cell line

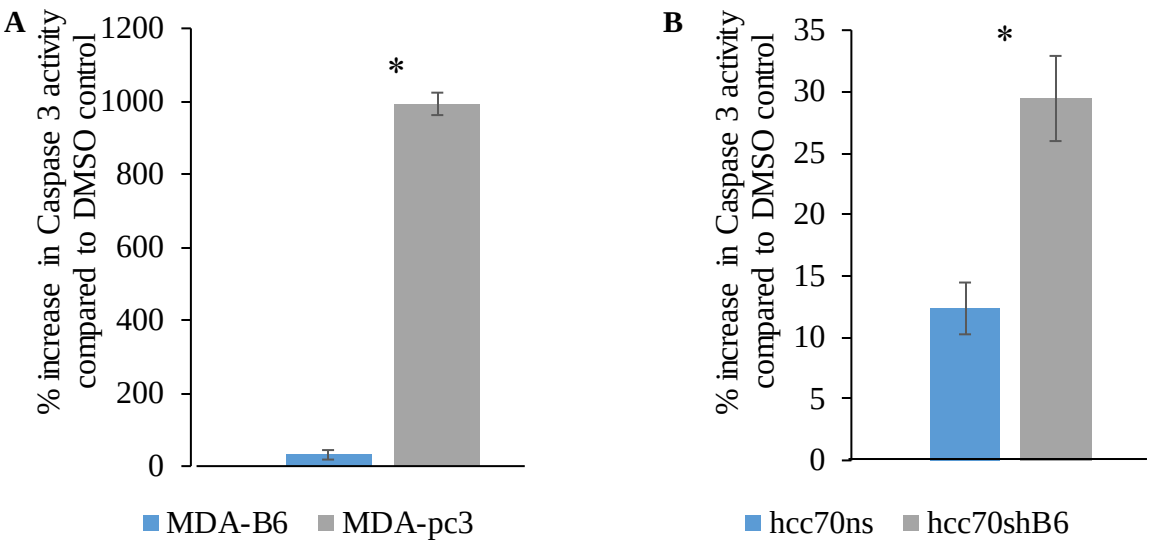


Figure 4.13. Capase 3 activation increases more in EphB6-deficient cells after treatment with INHIBITOR. Cells were seeded in 10 cm plates and 18-24 hours later, indicated concentration or matching volume DMSO control (3 replicates per condition) was added. Cells were treated for indicated time at indicated serum level. Caspase 3 activity was determined using the Enzchek Caspase 3 Kit #1, with a SpectraMax M5 plate reader used to measure activity. *P < 0.05, Student's t-test. **A.** MDA-MB-231 cells treated with 200 nM INHIBITOR at 10% FBS for 72 hours **B.** hcc70 cells treated with 4 µM INHIBITOR at 1% FBS for 96 hours. *p < 0.05. *versus* EphB6 expressing cell line

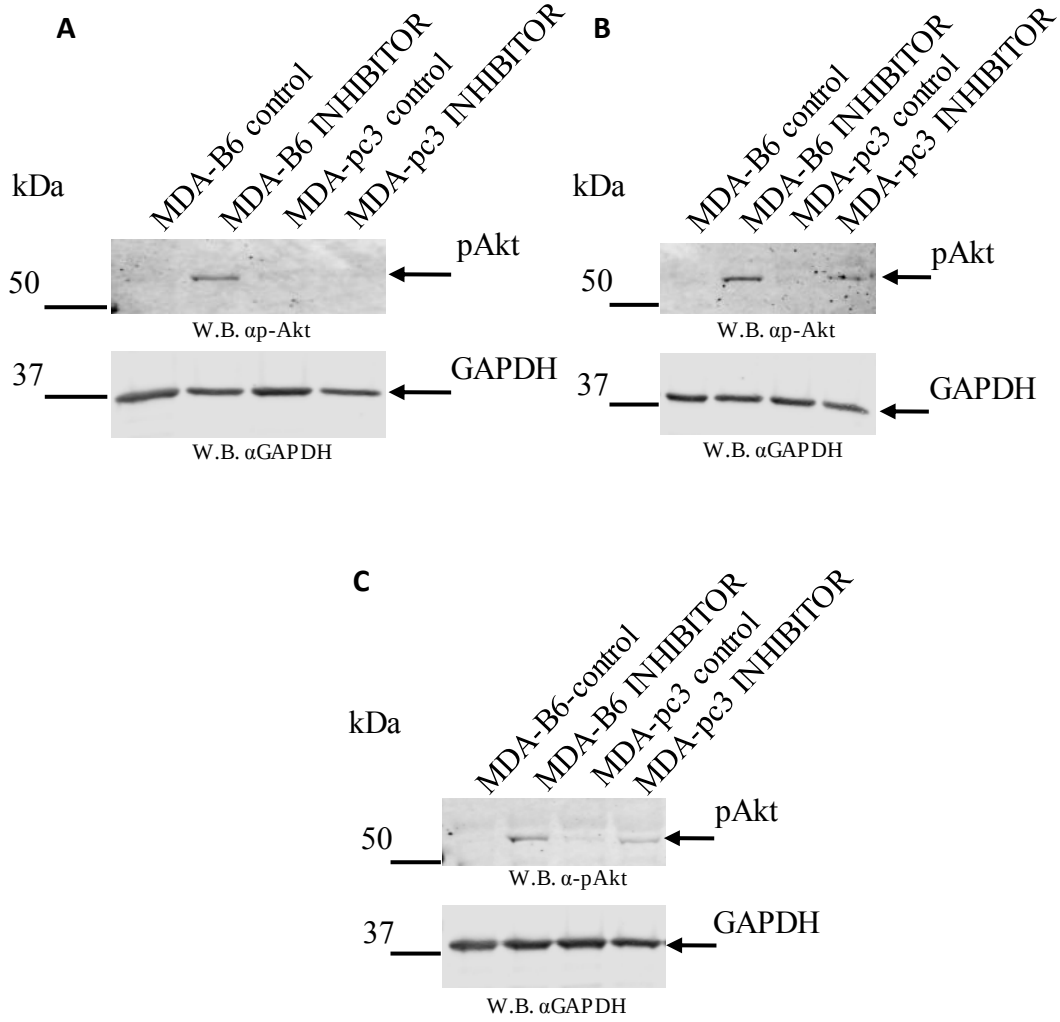


Figure 4.14. Cell survival signalling is enhanced in EphB6-expressing cells after treatment with INHIBITOR. MDA-MB-231 cells were seeded in 6 well plates and 18-24 hours later, 0.2 μM INHIBITOR or matching volume DMSO control was added. Cells were treated for various time periods before lysates were prepared. Akt phosphorylation was analyzed by Western blotting with anti-phospho-Akt (S473). Western blotting with anti-GAPDH was using as a loading control. **A**. 72hrs, 1%FBS. **B**. 72hrs, 10% FBS. **C**. 48hrs, 1% FBS (only two replicates for C).

5.0 DISCUSSION

5.1 Analyzing Significant Synthetic Lethal Hits

My analyses did not reveal a synthetic lethal relationship between EphB6 and Src in ER^+ breast cancer cells. Experiments with KX2-391 did not yield a consistent selective elimination of breast cancer cells deficient in EphB6 in $HER2^+$ cells lines either. Thus, at this point, it seems that the synthetically lethal relationship between EphB6 and Src is restricted to TNBC. The most likely reason for this would be that the screen was originally done in TNBC. As discussed in the background, treatment choice for breast cancer patients is based on tumor subtypes and they respond to various therapies, even general cytotoxic compounds, very differently. Therefore, it is not entirely surprising that the synthetic lethal interaction between EphB6 and Src, initially observed in TNBC, cannot be expanded to other breast cancer subtypes.

While, similar to the pattern observed with Src inhibition, there was not a consistent synthetic lethal effect involving EphB6 and Met receptors in ER^+ breast cancer or $HER2^+$ breast cancer, targeting Met in EphB6-deficient TNBC proved quite promising. This EphB6/Met synthetic lethal was first observed in the shRNA screen carried out in TNBC (Paul *et al.* 2016) and small molecular Met inhibitors produced the synthetic lethal effect as well in my experiments. According to my observations, EphB6 expression is inversely correlated to Met levels in TNBC cell lines (Figure 4.2), which indicates that cells that lose EphB6 might become more dependent on Met, making them vulnerable to Met inhibition. Experiments in xenograft models of human TNBC should be carried out to confirm that selective targeting of TNBC tumors based on their EphB6 deficiency could lead to better efficiency of Met-inhibiting therapies.

Pro-survival Akt signaling is much more increased in EphB6-positive than EphB6-deficient TNBC cells treated with an FDA-approved Met inhibitor, suggesting EphB6 facilitates Akt activation to protect TNBC cells from the consequences of Met inhibition. This activation likely is through the PI3K which contains a SH2 domain that could interact with EphB6's phosphorylated domains. While this treatment still causes some level of death response in EphB6-expressing cells, treatments are far more lethal when EphB6 is not present. Since the Met receptor also activates the Akt kinase in its downstream signaling pathways (Kulmann *et al.*, 2005; Tsao, 2011), the absence of EphB6 makes cells more sensitive to Met inhibition. This model matches

the definition of a synthetic lethal interaction and suggests that there is a strong potential for the use of Met inhibitors in treating patients with EphB6-deficient TNBC tumors.

5.2 Novel EPHB6 synthetic lethal Interaction with the INHIBITOR and its Therapeutic Potential

High-throughput compound screening is a method becoming quite popular in academic settings (Macarron *et al.*, 2011). While not the traditional method of finding synthetic lethal interactions, finding an FDA-approved drug rather than a target gene was an exciting possibility due to the EphB6's behaviour as a down-regulated molecule with tumor-suppressive characteristics (Truitt and Freywald, 2011). The high-throughput imaging drug screen that was carried out and described in this thesis revealed an FDA-approved drug (INHIBITOR) that, similar to Met and Src inhibitors, exploits a potentially complex synthetic lethal relationship because, again, drug's effects are not as specific as shRNA molecules. Overall, this alternative approach allowed us to find an FDA-approved small-molecular inhibitor selectively targeting EphB6-deficient cells.

The experimental data obtained with the identified INHIBITOR have been quite promising. While the INHIBITOR did not exhibit synthetic lethal properties with EphB6 in ER^+ and $HER2^+$ breast cancer cells, the results in TNBC, with synthetic lethal effects being shown in both MDA-MB-231 and hcc70, suggest a strong potential for this drug in TNBC treatment. The fact that very similar effects of the INHIBITOR have been observed in two independent cell lines of the TNBC origin provides a greater confidence in the biological relevance of my findings. As with the promising results with Met inhibitors, pre-clinical trials in matching xenograft mouse models have to be carried out prior to future publication and patenting.

The INHIBITOR is currently in clinical trials for TNBC as a part of a combination therapy, so it may be wise to also consider including combination therapies in the further studies of the observed synthetic lethal effects. That said, new effective treatments are urgently needed for TNBC patients, so validation of drugs in preclinical in vivo experiments has to be completed in a timely manner.

Similar to the work with Met inhibitors, the fact that Akt signalling is increasing in cells after treatment with these inhibitors gives a clue as to the signalling partners of EphB6. Akt is primarily activated through the binding of the PH domain of Akt to tri-phosphorylated

phosphatidylinositol, the product of the PI3K kinase, which contains an SH2 domain that could easily be binding to EphB6's phosphorylated tyrosine's or that of its signalling partners. This interaction between EphB6 and the PI3K would be a novel finding and so could be further studied (discussed further in section 6.2).

6.0 CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

FDA-approved Src and Met inhibitors have been undergoing clinical trials for treating a variety of malignancies, including breast cancer (Elias and Ditzel, 2015; Tolaney *et al.*, 2015), but have not shown great efficacy in patients so far compared to current treatments. Better personalising the use of these inhibitors for treating specifically EphB6-deficient tumors could be a positive development and would hopefully help solve some of the issues with fighting TNBC, including the lack of targeted therapies and resistance to currently used drugs. The INHIBITOR discovered in the drug screen could be potentially re-purposed into a new treatment approach, which might significantly advance this pursuit. In this approach, EphB6 could be used as a biomarker providing an indication for the INHIBITOR application. Not only would EphB6-deficient tumors be targeted with the synthetic lethal therapy, but tumors that have high EphB6 expression would also be excluded from therapies with the INHIBITOR, leading to a more efficient use of societal resources, while also reducing patient suffering under ineffective treatment and improving patient survival.

Our findings could lead to several new research avenues as well. Looking through many of the current clinical trials in TNBC, combination therapies including generally cytotoxic chemotherapy compounds are quite common (ClinicalTrials.Gov IDs: NCT02657889, NCT02730130, NCT02648477, NCT02489448, NCT01042379, NCT02282345, NCT02393794) and so further investigation that involves administration of cytotoxic compounds in combination with suppressing newly identified synthetic lethal targets in preclinical mouse models could be quite productive. Inhibiting EphB6 synthetic lethal partners simultaneously with cytotoxic treatments, such as doxorubicin or docetaxel, may be a good strategy to developing a therapeutic approach closer to what is likely would be used in the clinic for treating TNBC patients.

The results presented here indicate that EphB6-based responses are breast cancer subtype specific. To try find a new therapy for breast cancer that is not so subtype-specific, one could do a screen in $HER2^+$ or ER^+ breast cancer cells, either a drug screen or an shRNA screen and compare the results with the results from TNBC screening. While there is no guarantee that it would lead to a therapy as EphB6 may play different roles in different subtypes of breast cancer, there would

be potential to benefit a much greater patient population if one could find a common synthetic lethal target in these screens.

6.1 Future Work

Another potential route to finding new treatments would be to carry out a CRISPR/Cas9 synthetic lethal screen that should be expected to identify a different subset of synthetic lethal targets (Morgens *et al.*, 2016). Several hits from the initial shRNA screen in TNBC cells still have not been studied either, so future projects could be designed to target breast cancer cells with small molecule inhibitors that target the uncharacterised hits from that screen. Of the 113 statistically significant synthetic lethal hits identified by our team, only two have now been studied comprehensively. Further analysis of the drug screen may lead to other promising therapies as well. While the drug selected had a strong synthetic lethal effect at both 100 nM and 500 nM, other drugs were effective at either of these concentrations and so could potentially prove to be promising avenues of treatment for EphB6-deficient tumors as well.

Further research is needed to establish a consistent way to measure EphB6 expression levels based in patients' tumor samples. This would allow to further validate EphB6 as a reliable biomarker and help to select a proper treatment protocol for each individual patient. One way to determine EphB6 expression would be to have a antibody for immunohistochemistry testing in tumor samples. Research into an appropriate antibody for this research must be carried out at this time.

A great opportunity for further research would be further studying and clarifying downstream signalling that leads to greater efficacy of both Met inhibitors and INHIBITOR in EphB6-deficient cells. While Akt activation is a strong indicator of pro-survival signalling upstream of Caspase 3, checking signalling of FOX01, Tsc2, or even Bad phosphorylation would be an excellent progression to check whether all Akt signalling is increased. Akt is primarily activated through the binding of the PH domain of Akt to tri-phosphorylated phosphatidylinositol, the product of the PI3K kinase so to determine the upstream signalling between EphB6 and Akt, co-immunoprecipitation between EphB6 and the PI3K might be worthwhile to study. Westerns could be used for this further signalling. Comparing the kinome after treatment with inhibitors may also give clues into what other signalling is changing. Finally, using flow cytometry to analyze

differences in Annexin V or 7-AAD at different time points might better clarify the timing of the process of apoptotic induction and might be beneficial.

One another avenue of future research would be studying whether these synthetic lethal interactions are consistent in a more complex *in vivo* mouse model. Treatment groups would include DMSO control, inhibitor treatment, current chemotherapy, and a combined therapy group, with all of these groups studied in both EphB6-expressing and EphB6-deficient human xenograft cell lines, rather than established cell lines used in this project. This would add more confidence in the efficacy of this treatment.

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