# CHARACTERIZING METFORMIN RESPONSE AND SENSITIVITY BIOMARKERS IN BREAST CANCER

by

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Dedications

To David, Momma, and Paige, without whom my soul would have crumbled.

(It has been concluded that the survival prognosis of Lindor is < 48hr post-purchase.)

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**Abstract**

The anti-diabetic drug metformin has been shown to exhibit broad *in vitro* and *in vivo* anti-neoplastic activity in various cancer types, including breast cancer. Despite numerous clinical trials ongoing, no targeted biomarkers currently exist to predict or monitor patient response to metformin treatment. Our lab previously conducted quantitative proteomics on MDA-MB-231 triple-negative breast cancer cells conditioned to physiologically-relevant doses of metformin. In this study, I have aimed to evaluate the genes encoding the top 12 most up-regulated proteins for their potential use as metformin biomarkers. I have found that several genes may modulate metformin sensitivity in MDA-MB-231 cells in a glucose-dependent manner. Most notably, I have identified aldo-keto reductase family 1 member C3 (AKR1C3) to be a novel metformin response marker and a modifier of metformin sensitivity in breast cancer via both up- and down-regulation. AKR1C3 protein expression may also have some predictive value to estimate metformin response *in vitro*.

**List of Abbreviations Used**

2-DG – 2-deoxyglucose

3-D – 3 dimensional

4E-BP-1 - eukaryotic translation initiation factor 4E-binding protein 1

AKR1C3 – aldo-keto reductase family 1 member C3

Akt – protein kinase B

AMP – adenosine monophosphate

AMPK – AMP-activated kinase

APC - adenomatous polyposis coli

ARE – antioxidant response element

ATCC – American Type Culture Collection

ATP – adenosine triphosphate

AUP1 – ancient ubiquitous protein 1

BL1 – basal-like 1

BL2 – basal-like 2

BLAST - Basic Local Alignment Search Tool

BRCA1 – breast cancer 1

BRCA2 – breast cancer 2

BSA – bovine serum albumin

CI – confidence interval (95% unless otherwise specified)

CCDC14 - coiled-coil domain containing 14

CD24 – cluster of differentiation 24

CD44 – cluster of differentiation 44

CD8 – cluster of differentiation 8

cDNA – complementary DNA

CTGF – connective tissue growth factor

DAPI – 4',6-diamidino-2-phenylindole

dd$H_2O$ - double-distilled water

DMEM – Dulbecco's Modified Eagle's Medium

DNA – deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

EGFP - enhanced green fluorescent protein

EGFR – epidermal growth factor receptor

EMT – epithelial-to-mesenchymal transition

ENPP4 – ectonucleotide pyrophosphatase/phosphodiesterase 4

ER – estrogen receptor

FBS – fetal bovine serum

GLUT1 – glucose transporter 1

GFP – green fluorescent protein

HER2 – human epidermal growth factor receptor 2

HK 1 – hexokinase 1

HK 2 – hexokinase 2

HMGN5 – high mobility group nucleosome binding domain 5

HMOX1 – heme oxygenase 1

Hr – hour

HR - hazard ratio

HRP – horseradish peroxidase

ID – identification

IDC – invasive ductal carcinoma

IF – immunofluorescence

IGFBP7 – insulin-like growth factor binding protein 7

IGF-1(R) – insulin-like growth factor 1 (receptor)

IL-6 – interleukin 6

IM - immunomodulatory

IR – insulin receptor

KEAP1 - Kelch-like ECH-associated protein 1

L-15 – Leibovitz's Media

LAR – luminal androgen receptor

LKB1 – liver kinase B1

M – mesenchymal

MAPK – mitogen-activated protein kinase

MEM – minimum essential media

METABRIC – Molecular Taxonomy of Breast Cancer International Consortium

Min – minute

MIQE – Minimum Information for Publication of Quantitative Real-Time PCR Experiments

mM – millimolar

mRNA – messenger ribonucleic acid

MSL – mesenchymal stem-like

mTOR – mammalian target of rapamycin

MW - molecular weight in grams per mol (g/mol)

N - sample size, biological replicates

NCBI – National Centre for Biotechnology Information

NEAA – non-essential amino acids

NF-κB - nuclear factor kappa-light chain-enhancer of activated B cells

NIH – National Institutes of Health

NOD/scid – non-obese diabetic, severe combined immunodeficiency

Nrf2 – transcription factor encoded by nuclear factor erythroid-derived 2-like 2 (NFE2L2) gene

OCT – organic cation transporter

ORF – open reading frame

PAM50 – Prediction Analysis of Microarray 50

PBS – phosphate-buffered saline

PCOS – polycystic ovary syndrome

PCR – polymerase chain reaction

PDE3B - phosphodiesterase 3B

PDX – patient-derived xenograft

PFA – paraformaldehyde

EGAD – Enhanced Gene Analysis and Discovery Facility

PHGDH – phosphoglycerate dehydrogenase

PI3-K – phosphatidylinositol 3 kinase

POLRMT – RNA polymerase mitochondrial

PR – progesterone receptor

pS6 – phospho-S6 ribosomal protein

PTEN - phosphatase and tensin homolog

RIPA – radioimmunoprecipitation assay (buffer)

RNA – ribonucleic acid

RNAi – ribonucleic acid interference

ROS – reactive oxygen species

Rpm – revolutions per minute

RPMI-1640 – Roswell Park Memorial Institute medium 1640

RT-qPCR – reverse-transcriptase quantitative polymerase chain reaction

SD – standard deviation

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – standard error of the mean

SH3BGRL – SH3 domain binding glutamate rich protein like

shRNA – short hairpin ribonucleic acid

SPANXB1 – sperm protein associated with the nucleus, x chromosome, family member B1

SQSTM1 – sequestosome 1

SSR3 - signal sequence receptor subunit 3

STAT3 – signal transducer and activator of transcription 3

TAM – tumour-associated macrophage

TCGA – The Cancer Genome Atlas

TGF-ß1 - transforming growth factor beta 1

TNBC – triple-negative breast cancer

TP53 - tumour protein 53

TTN – titin

TWIST1 - twist family BHLH transcription factor 1

Unk – Unknown

UTR – untranslated region

VEGF – vascular endothelial growth factor

WHO – World Health Organization

WT – wild-type

ZEB1 – zinc finger E-box-binding homeobox 1

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# Chapter 1: Introduction

## 1.1 Preamble

The central focus of this thesis project is to characterize novel response and sensitivity biomarkers for the re-purposed anti-diabetic drug metformin to lay the foundational groundwork for more targeted therapies in breast cancer. I will begin by discussing the molecular characteristics and existing clinical treatments for breast cancer, with emphasis on the difficult-to-target subtype of triple-negative breast cancer (TNBC). Subsequently, I will discuss how metformin can be re-purposed to potentially treat and prevent cancer, and will outline existing knowledge about the mechanisms underlying metformin's anti-neoplastic activity. I will focus on the existing applications and limitations of metformin in various cancer models including triple-negative breast cancer, specifically focusing on biomarkers and their potential uses for optimizing treatment. Finally, I will outline the previous work our lab has done to help characterize metformin mechanism in triple-negative breast cancer, and how I have proceeded to investigate novel putative modifiers of metformin action to validate as biomarkers *in vitro*.

## 1.2 Breast cancer

### *1.2.1 Clinical presentation*

Breast cancer is the most common malignancy in women, accounting for 25.8% of all new cancer cases in Canadian females in 2016 (Canadian Cancer Society's Advisory Committee, 2016). Although individuals carrying certain hereditary gene variants (such as mutations in tumour-suppressor genes like breast cancer 1 [BRCA1] and breast cancer 2 [BRCA2]) have an increased risk of developing breast cancer during their lifetime (Ghoussaini et al., 2012; Hedenfalk et al., 2001), most cases have no clear familial

association and are highly multifactorial. These cancers develop spontaneously due to a variety of risk factors including age, ethnic status, and lifestyle (Mcpherson, Steel, & Dixon, 2000). The 5-year survival rate for breast cancer patients in Canada is 87% (Canadian Cancer Society's Advisory Committee, 2016), although exact prognosis varies significantly with histological and molecular subtype, stage, and grade of tumour at time of diagnosis (Malhotra et al. 2010).

### *1.2.2 Pathological origin and histological subtypes*

Breast cancer is generally epithelial in origin and deemed a carcinoma, with only rare instances of connective tissue sarcomas being reported (Adem et al., 2004; Breastcancer.org, 2017). Carcinomas are broadly classified into two categories: *in situ*, meaning the cancer is isolated to the location of origin, or invasive, indicating that the cancer has infiltrated the surrounding normal tissue. The most common type of invasive carcinoma of the breast originates from the epithelial lining of milk ducts, and is classified as invasive ductal carcinoma (IDC). A carcinoma may also form in the lobular tissue of the breast, thus characterizing it as invasive lobular carcinoma (ILC) (Fisher et al., 1975). Invasive ductal carcinomas account for about 80% of all invasive breast cancer cases, with invasive lobular carcinomas representing roughly 10% (Breastcancer.org, 2017; Makki, 2015). Each carcinoma type may be further sub-classified based on its histopathological profile of structural and cytomorphological features (such as papillary, medullary, or mucinous invasive ductal carcinomas), with each sub-type being associated with modest prognostic variability (Fisher et al., 1975; Makki, 2015; Malhotra et al., 2010).

### *1.2.3 Molecular subtypes*

Under current pathology guidelines, breast cancers are generally classified into 5 broad subtypes based on their intrinsic molecular characteristics, gene expression, and hormone receptor status: luminal A, luminal B, HER2-enriched, triple-negative phenotype (sometimes referred to as 'basal like'), and normal-like (Parker et al., 2009; Perou et al., 2000; Voduc et al., 2010). Numerous assays have been developed to further delineate these subtypes according to their gene expression profile to make predictions about clinical behaviour. These include the 21-gene panel (Paik et al., 2004) developed into the Oncotype DX® (Genomic Health) test, as well as the "PAM50" (Prediction Analysis of Microarray 50) gene signature (Parker et al., 2009) recently adapted for clinical use by Prosigna. The molecular details and prognostic implications of each of the five main breast cancer subtypes will be discussed further below.

#### *1.2.3.1 Luminal A*

Luminal A cancers express the estrogen receptor (ER+) and/ or the progesterone receptor (PR+), do not over-express the human epidermal growth factor 2 receptor (HER2-), and have low levels of the tumour proliferation marker Ki-67. These tumours are typically low-grade and present the best prognoses of all breast cancers, with a 5-year relative survival rate of 92-96% (Minicozzi et al., 2013).

#### *1.2.3.2 Luminal B*

Luminal B cancers may be ER+ and/or PR+ and have high levels of Ki-67. This category can be subdivided into luminal B-HER2-enriched where HER2 is over-

expressed, and luminal B-HER2- where it is not (Voduc et al., 2010). The 5-year relative survival rate is about 82-92%, slightly lower than luminal A due to a higher proliferative index (Minicozzi et al., 2013).

#### *1.2.3.3 HER2-enriched*

Breast cancers that are ER-/PR- but enriched for HER2 expression tend to have worse prognosis than luminal subtypes, with a 5-year survival rate around 68-81% (Minicozzi et al., 2013). Survival statistics for these cancers is expected to improve significantly over the coming years with the advent of HER2-targeted therapies (see *1.2.4. Existing treatments for breast cancer*).

#### *1.2.3.4 Triple-negative*

Triple-negative breast cancers (TNBC) are negative for all three major molecular markers (ER-/PR-/HER2-) and represent 12-17% of all breast cancer cases (Foulkes, Smith, & Reis-Filho, 2010). They are associated with BRCA1 germline mutations and occur more frequently in pre-menopausal women and populations of African descent than other subtypes (Carey et al., 2006). Triple-negative malignancies tend to present at a later stage and more advanced grade than other breast cancers, and carry the worst prognosis of all subtypes with a 5-year relative survival rate of 69-80% (Dent et al., 2007; Minicozzi et al., 2013). Triple-negative breast cancers are highly molecularly heterogeneous, making consensus on further sub-classification challenging. Intrinsic histological properties sometimes divide the triple-negative category into two subgroups, basal-like and non-basal, depending if they are positive for epidermal growth factor

receptor (EGFR+) and/or cytokeratin 5/6 (CK5/6+) (Voduc et al., 2010). More recently it has been shown that non-basal TNBCs make up the majority of claudin low breast cancers, a molecular subtype that is characterized by an absence of luminal differentiation markers and an enrichment in gene expression related to immune response, epithelial-to-mesenchymal transition (EMT), and cancer stem cell phenotype (Prat et al., 2010). Through detailed clustering analysis of TNBC gene expression profiles, Lehmann *et al*. (2011) identified 6 alternative molecular subgroups all with sizeable representation, which include: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal-like (M), mesenchymal stem-like (MSL), and luminal androgen-receptor (LAR). It was shown that all subtypes are represented in panels of commercially-available cell lines, thus confirming that *in vitro* models of triple-negative breast cancer are useful in capturing the molecular heterogeneity of TNBC biology (Lehmann et al., 2011).

*1.2.3.5 Normal-like*

Breast tumours defined as "normal-like" have similar gene expression patterns to those found in normal breast tissue; they mimic the gene signatures of basal epithelial or adipose cells rather than luminal epithelial cells (Perou et al., 2000). They are the rarest subtype of breast cancer and only account for 3.5-5% of breast malignancies. Hormone status (ER/PR/HER2) and prognosis are similar to that of luminal A cancers, although detailed gene expression assays have proven more effective for predicting their clinical behaviour than that of other subtypes (Liu, Zhang, & Zhang, 2014; Sweeney et al., 2014).

### *1.2.4 Existing treatments for breast cancer*

It is estimated that with advances in breast cancer screening and more effective therapies, over 32,000 breast cancer deaths have been prevented in Canada since 1988 (Canadian Cancer Society's Advisory Committee, 2016). Primary breast tumours are removed surgically, either through lumpectomy for localized cancer, or mastectomy in cases of further invasion. Following surgery, chemotherapy regimens are chosen depending on molecular subtype (Prat et al., 2015). Luminal cancers are frequently treated with taxanes or anthracyclines, with some successes also reported using nucleoside analogs (such as 5-fluorouracil) in luminal B cancers. HER2-enriched tumours generally do not respond well to endocrine therapies such as tamoxifen, but recent advances in HER2-targeted treatments (i.e. trastuzumab or lapatinib anti-HER2 therapy) have been shown to produce a complete response in a modest proportion of patients, and may even benefit luminal HER2+ patients when combined with other therapies such as paclitaxel. Triple-negative breast cancers cannot be effectively targeted by any single therapy and are comparatively difficult to treat (Dent et al., 2007; Foulkes et al., 2010; Prat et al., 2015). A treatment regimen would typically involve multiple drugs used in combination (polychemotherapy), sometimes incorporating platinum-based agents as well. Anti-androgen therapies have been proposed for triple-negative cancers with high rates of androgen signalling (LAR subtype, Lehmann et al., 2011) but the clinical benefit of this approach is not yet clear (Prat et al., 2015). Overall, better treatments are required for triple-negative breast cancer either as a singular therapy or as a polychemotherapy adjuvant.

### *1.2.5 Models for studying breast cancer*

Immortalized breast cancer cell lines are by far the most common model for investigating the signalling pathways underlying breast cancer behavior. Cell lines are generally robust, survive much longer than primary cultures, and are easy to adapt to a wide variety of experimental assays (Burdall et al., 2003). In contrast to malignancies like prostate cancer, for which there are only a handful of *in vitro* models, there are dozens of cell lines available to study breast cancer (American Type Culture Collection, 2017). This allows researchers to better capture the genetic heterogeneity of the disease. It is easy to control individual variables under *in vitro* conditions, making cell lines the model of choice for initial drug sensitivity screening and genetic manipulation (Burdall et al., 2003; Holliday & Speirs, 2011; Vargo-Gogola & Rosen, 2007). However, a major limitation of these cell models is that they are mostly cultured 2-dimensionally on plastic surfaces, which do not accurately represent the 3-dimensional (3-D) growing patterns of tumours *in vivo*. Such models cannot account for the impact of the structural and biological components of the tumour microenvironment, and have been shown to potentially affect a cancer's molecular signalling profile and treatment response (Kenny et al., 2007). Although 3-D culturing substrates such as Matrigel (Corning) may address some of these concerns (Lee et al., 2007), xenotransplantation of human cell lines into immunocompromised animals (for example, NOD/scid rodents or zebrafish embryos) is often the preferred model to address these *in vivo* considerations (Konantz et al., 2012; Vargo-Gogola & Rosen, 2007). If an immune component is suspected, one could employ either a chemically-induced or oncogene-induced spontaneous tumour rodent model, or a syngeneic mammary epithelial cancer cell model (such as mouse 4T1 cells) that can be

manipulated *in vitro* and injected into an immunocompetent animal (Fantozzi & Christofori, 2006). These approaches come with their own limitations - rodents and humans are sufficiently different organisms genetically such that cancer biology is usually not identically modeled (Vargo-Gogola & Rosen, 2007). Alternatively, patient-derived xenografts (PDX) have been shown to harness considerable predictive power for clinical drug response (DeRose et al., 2011; Whittle et al., 2015). However, aside from the difficulty in obtaining clinical samples, PDX models are strongly affected by the non-human host microenvironment, and tend to under-represent certain subtypes of breast cancer, such as luminal A/ ER+ cancers (Whittle et al., 2015). PDX models are also generally not well-suited for foundational studies of gene knock-down or over-expression to assess changes in cellular behaviour.

Overall, *in vitro* cultures of immortalized human breast cancer cells are likely to remain the preferred model for foundational work into drug response and molecular signalling pathways, with xenotransplantation and syngeneic rodent models playing important roles in pre-clinical evaluation and validation.

### 1.3 Cancer risk and energy metabolism

Chronic states of energy surplus and physical inactivity often result in obesity, metabolic syndrome, and type-2 diabetes mellitus, but are also strongly associated with increased cancer risk (Fair & Montgomery, 2009; Gallagher & LeRoith, 2015; Peairs et al., 2011). This may be due to a multitude of factors including the release of inflammatory cytokines, adipokines, hormones, and growth factors from adipose tissue (Diaz, Herzig, & Schafmeier, 2016), as well as high circulating concentrations of blood

glucose and insulin that may promote tumorigenesis (Gallagher & LeRoith, 2015; Wahdan-Alaswad et al., 2013). Impaired energy homeostasis has been associated with immunosuppression and changes in tissue oxygenation, which are known to promote immune-evading and aggressive cancer phenotypes, respectively (Conroy et al., 2016; Park et al., 2010; Young & Anderson, 2008). Meta-analyses have shown that type 2 diabetics have a 1.5-fold or higher increased risk of developing liver, pancreatic, kidney, and endometrial cancer, and a modest increase in risk (relative risk: 1.12-1.43) of colorectal, bladder, and breast cancer compared with non-diabetics (Vigneri et al., 2009). This phenomenon is thought to be due to dysfunctional energy homeostasis, increased insulin-like growth factor 1 (IGF-1) signalling, and the potential mitogenic effects of insulin-based or insulin-stimulating anti-diabetic therapies like insulin analogues or sulfonylureas (Giovannucci et al., 2010). In contrast, other anti-diabetic therapies, such as metformin and thiazolidinediones, do not attempt to directly target or mimic pancreatic insulin production to control blood glucose. Metformin decreases hepatic gluconeogenesis, while thiazolidinediones activate peroxisome proliferator-activated receptors (PPARs) to re-sensitize the body's tissues to insulin (Nathan et al., 2009). Both drugs often have a more durable effect on regulating blood insulin and glucose levels compared with other therapies (Nathan et al., 2009) and improve overall energy homeostasis in the body, which may also reduce cancer risk (Giovannucci et al., 2010).

## 1.4 Metformin

### *1.4.1. Metformin use for type-2 diabetes mellitus*

Metformin, marketed under trademark names such as Glucophage (Merck Santé), Glumetza (Valeant Pharmaceuticals), and Fortamet (Andrx Labs), was first synthesized in the 1920s at the University of Vienna but was not utilized clinically until decades later (Fischer & Ganellin, 2010). Metformin's parent compound (guanidine) was originally isolated from goat's rue (*Galega officinalis*), a plant that had been used in European folk-lore medicine for hundreds of years to treat symptoms of hyperglycemia. Guanidine proved too toxic for standard use - this prompted research into better-tolerated analogues of the drug (Fischer & Ganellin, 2010). Physician Dr. Jean Sterne demonstrated that one of these biguanide compounds, metformin, possessed significant efficacy in treating diabetes while minimizing general toxicity, and worked to develop metformin as a clinical treatment (Sterne, 1959). Metformin was first approved for use in France in 1979 and was adopted into diabetes treatment regimens worldwide over the following decades (Fischer & Ganellin, 2010). Since it is both extremely cost-effective to manufacture and generally well tolerated, metformin was added to the World Health Organization's list of essential medicines (WHO, 1999) and has become the front-line drug to treat type 2 diabetes worldwide.

### *1.4.2 Metformin mechanism in diabetes*

Metformin's action in treating diabetes is to reduce hepatic gluconeogenesis while also stimulating glucose uptake in target tissues, thus resulting in a marked decrease of circulating blood glucose (Hundal et al., 2000; Stumvoll et al., 1995). This mechanism is

primarily the result of AMP-activated protein kinase (AMPK) activation through inhibition of oxidative phosphorylation, which results in decreased in glucose output by hepatocytes and increases glucose uptake in muscle cells (Zhou et al., 2001). AMPK activation also results in decreased lipogenesis and induction of hepatic fatty acid oxidation to reduce levels of circulating triglycerides (Zhou et al., 2001). Furthermore, metformin increases the activity of the insulin receptor (IR) and insulin receptor substrate 2 and increases the translocation of glucose transporters (e.g. GLUT1) to the plasma membrane, thus further enhancing glucose uptake (Pernicova & Korbonits, 2014).

### *1.4.3 Alternative uses of metformin*

Metformin has been evaluated for numerous alternative uses in recent decades. For example, metformin was shown to improve treatment outcomes for non-diabetic obese women with polycystic ovary syndrome (PCOS) (Nestler et al., 1998). This effect was linked to reduced insulin signalling causing alterations in gonadotrophin secretion and intra-ovarian androgen production (Nestler & Jakubowicz, 1996). Metformin has also been shown to improve appetite regulation and cause significant weight loss in both diabetic and non-diabetic overweight patients (Kay et al., 2001; Lee & Morley, 1998). This effect is associated with an AMPK-dependent reduction in ghrelin secretion (Gagnon, Sheppard, & Anini, 2013) and changes in circulating leptin levels in diabetic patients (Ida, Murata, & Kaneko, 2017).

### *1.4.4 Metformin and cancer risk*

The idea that metformin might have anti-neoplastic properties first came to light in 2005 when an observational, population-based study in Scotland found that diabetics

taking metformin had a 21% (95% C.I.: 7-33%) reduced lifetime cancer risk compared with diabetics receiving other treatments (Evans et al., 2005). Subsequent observational cohort studies and meta-analyses have shown that metformin treatment decreases overall cancer incidence by an estimated 31-37%, with effects trending towards a dose-dependent relationship (DeCensi et al., 2010; Libby et al., 2009; Noto et al., 2012). Even when stringent bias-prevention analyses are employed to account for confounding variables of metabolism, lifestyle, and general health, metformin use is still associated with a modest (10-18%) reduction in overall cancer incidence (Gandini et al., 2014).

However, no clear trends have yet been established when moving from observational cohort studies to controlled clinical trials. In a 4-year study following diabetic men, metformin use did not significantly change prostate cancer incidence (Feng et al., 2015). Other small, randomized, controlled clinical trials evaluating the benefits of metformin use and other diabetes medications showed no significant change in cancer incidence (Home et al., 2010; Home et al., 2009; Kahn et al., 2006). It is important to note that the clear majority of past clinical studies have been limited to small sample sizes, short study durations, and variable treatment regimens, making it difficult to determine how exactly metformin impacts overall cancer incidence.

It should also be noted that there is debate as to how metformin-associated cancer risk studies should be best interpreted. Specifically, the selection of appropriate control groups remains controversial. Since diabetes is inherently associated with increases in cancer risk, some researchers (Gandini et al., 2013) argue that the most appropriate comparisons remain within diabetic patient pools only, and that comparing diabetic metformin users with diabetic non-users is the best way to determine if metformin

treatment alone reduces cancer risk. Others (Chlebowski, Aragaki, & McTiernan, 2013) have argued that there is value in choosing non-diabetic patients as case-controls. In such studies, a decrease in cancer risk would not only account for the effects of proper disease management reducing the diabetes-associated cancer risk, but would indicate an overall protective effect beyond improved glucose and insulin regulation. While there is no clear consensus on this issue, either approach may provide useful information for researchers, as long as appropriate care is taken to interpret findings within the proper context (Chlebowski et al., 2013).

### *1.4.5 Metformin and breast cancer risk*

The organ-specific effects of metformin on cancer risk are not yet clearly established, especially when it comes to breast cancer models. Several longer-term observational studies have presented evidence that metformin may decrease the risk of invasive breast cancer in diabetics by 25-37% (Chlebowski et al., 2012; Col et al., 2012; Tseng, 2014). Remarkably, this protective effect is also observed when comparing diabetic metformin-users with non-diabetic individuals. In an observational study in post-menopausal women, Chlebowski *et al*. (2012) showed that diabetics not using metformin had an *increased* risk of invasive breast cancer (HR: 1.16, CI: 0.93-1.45) while metformin users had a *decreased* risk (HR: 0.75, CI: 0.57-0.99) compared to the non-diabetic control group. This supports the notion that metformin likely has anti-cancer properties *in vivo* independent of its ability to regulate hyperglycemia and hyperinsulemia. The same study observed that metformin treatment may have subtype-specific anti-breast cancer effects: the incidence of ER+/PR+ and HER2- breast cancers was reduced most dramatically (HR: 0.64, CI: 0.45-0.92 and HR: 0.58, CI: 0.40-0.84,

respectively), with risk of ER-/PR- cancers (most commonly triple-negative) also being reduced by an estimated 32%, although the small sample size limited statistical power of this observation. A similar study focusing on triple-negative breast cancer found a trend towards decreased risk of distant metastases in metformin users compared with both diabetic non-users and non-diabetics (Bayraktar et al., 2012).

In contrast, however, certain studies have shown that metformin may not robustly reduce overall breast cancer incidence and mortality in diabetics (DeCensi et al., 2010; Kowall et al., 2015). It is important to note the lack of consensus on this issue to emphasize the need for further research into the potential effects of metformin on breast cancer.

### *1.4.6 Metformin mechanism in cancer*

It is stipulated that the anti-cancer activity of metformin involves a complex interaction between direct and indirect effects. These are thought to manifest in both the protective qualities of metformin, reducing tumorigenesis and cancer incidence, as well as its acute anti-neoplastic activity to target existing disease. A more detailed discussion on current knowledge of each of these factors is outlined below.

#### *1.4.6.1 Indirect and global effects*

The same mechanisms that make metformin a highly effective anti-diabetic drug may also be responsible for some of its global anti-cancer action. Decreased hepatic gluconeogenesis and increased glucose uptake by muscle tissue reduces overall glucose concentration in the blood. Glucose is well-established as a pro-growth molecule that

fuels cancer metabolism and proliferation; a reduction in circulating glucose would thus be predicted to prevent carcinogenesis and inhibit tumour progression (Wahdan-Alaswad et al., 2013). Furthermore, the concurrent reduction of circulating insulin helps to alleviate activation of insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) signaling, which both lie upstream of several pro-growth pathways (Belfiore & Frasca, 2008; Pollak, 2008). The inhibitory effect of metformin on these growth-associated signaling axes leads to decreased cellular proliferation, biogenesis, and cancer growth, as will be further discussed in section 1.4.6.2.2.

Metformin has additionally been shown to modulate inflammation and tumour-associated immune signaling, which may further heighten its overall anti-cancer effects. In a murine inducible breast cancer model, metformin was shown to inhibit activation of nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and prevent inflammation-associated cellular transformation (Hirsch, Iliopoulos, & Struhl, 2013). In mice, metformin has been shown to reduce the activity of cytokine-producing helper T-cells (Zhao et al., 2015) and tumour-associated macrophages (TAMs) (Incio et al., 2015), thus attenuating their pro-cancer inflammatory signaling. Metformin was additionally shown to increase the quantity and effectiveness of $CD8^+$ tumour-infiltrating lymphocytes in mouse models by preventing their exhaustion and apoptosis (Eikawa et al., 2015) to support anti-cancer immune function.

Furthermore, recent studies have shown that metformin may also affect cancer growth by modulating aspects of the tumour microenvironment. Metformin decreases production of pro-angiogenic factors like vascular endothelial growth factor (VEGF) from various microenvironment-associated cells, resulting in decreased angiogenesis and

inhibited cancer progression (Kolb et al., 2016; Orecchioni et al., 2015; Qu & Yang, 2014).

Taken together, these indirect effects likely play a significant role in the anti-neoplastic activity of metformin and are important elements to consider moving forward.

#### *1.4.6.2 Direct effects on existing cancer cells*

Metformin is known to be shuttled into both non-transformed and cancer cells through organic cation transporters (OCTs), mainly OCT1, OCT2, and OCT3 (Checkley et al., 2017; Dresser et al., 2002; Wang et al., 2002). However, it is not yet clear exactly which transporters are the most important for cancer cells, as distribution varies depending on tissue type (Dowling et al., 2012). Once present in the cancer cell environment, the direct actions of metformin are diverse and multifaceted (Figure 1.1).

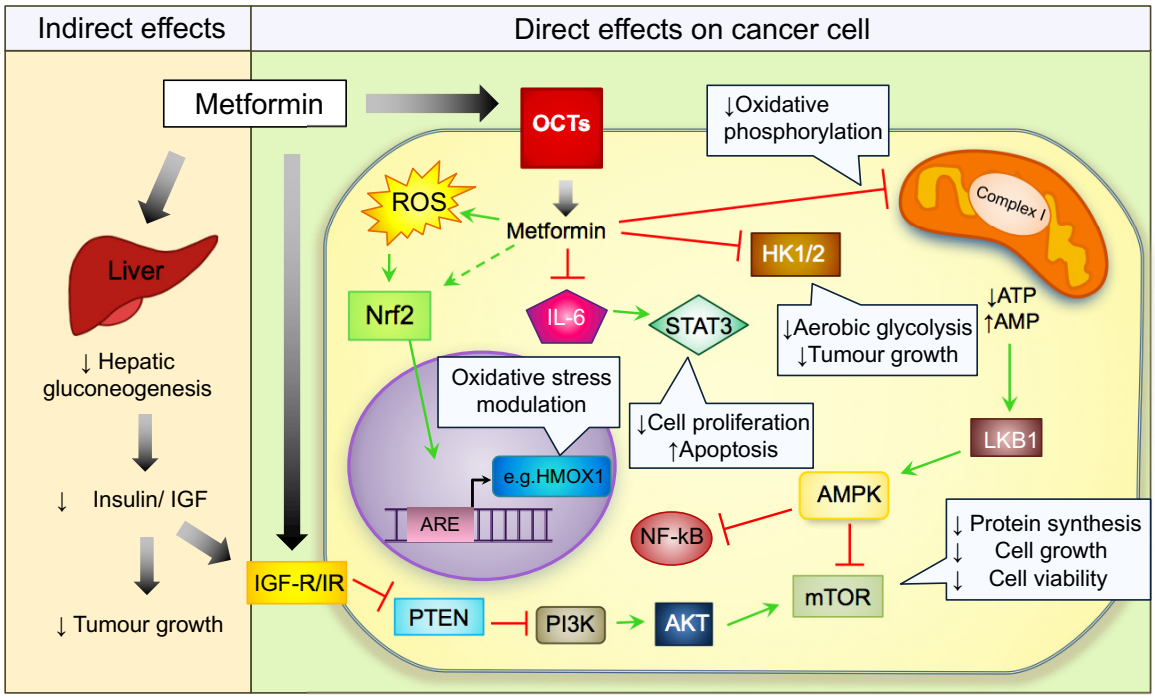



**Figure 1.1 Direct and indirect mechanisms of metformin anti-cancer activity.** *In vivo*, metformin inhibits hepatic gluconeogenesis to reduce blood glucose and insulin levels, resulting in suppression of insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF-1R) signalling. This effect is complemented by the direct action of metformin on the insulin receptor to inhibit phosphatidylinositol 3-kinase (PI3-K) via the phosphatase and tensin homolog (PTEN) tumour suppressor, which signals through protein kinase B (Akt) to regulate mammalian target of rapamycin (mTOR) activity and reduce overall tumour growth. Metformin is shuttled into cells via organic cation transporters (OCTs). It inhibits both mitochondrial complex I and hexokinase 1 and 2 (HK1/2) activity to suppress oxidative phosphorylation and glycolysis, respectively. The resultant increase in the intracellular adenosine monophosphate/ triphosphate (AMP/ATP) ratio results in the activation of AMP-activated protein kinase (AMPK) through mechanisms that are both dependent and independent of liver kinase B1 (LKB1). Activated AMPK suppresses nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity and further inhibits mTOR signalling to suppress protein synthesis, resulting in reduced cell growth and decreased viability. Metformin has also been shown to inhibit signal transducer and activator of transcription 3 (STAT3) signalling through suppressed interleukin 6 (IL-6) production, leading to decreased cell proliferation and increased apoptosis. Metformin increases oxidative stress in cancer cells through enhanced production of reactive oxygen species (ROS), an effect that can be both inhibitory or stimulatory. Through still unknown mechanisms, metformin signals through Nrf2 (encoded by nuclear factor erythroid-derived 2-like, NFE2L2) to transcriptionally regulate genes associated with anti-oxidant response elements (AREs), such as heme oxygenase 1 (HMOX1).

*1.4.6.2.1 AMP activated protein kinase (AMPK)-mediated effects*

Metformin's most direct mechanism of action in cancer is to reduce glucose metabolism and ATP production through inhibition of glycolysis and oxidative phosphorylation. Metformin blocks the hexokinase 1 and 2 (HK 1/2)-dependent sequestration of glucose into the glycolytic pathway, while simultaneously inhibiting complex I of the electron transport chain and inducing proton leak to uncouple mitochondrial respiration from ATP production (Marini et al., 2013; Salani et al., 2013; Wheaton et al., 2014). Given the metabolic reprogramming and high energy needs of many cancer cells, metformin effectively starves cells of energy and results in cancer-specific growth inhibition (Sanchez-Alvarez et al., 2013). Reduced oxidative phosphorylation also causes a rise in the intracellular adenosine monophosphate (AMP)/ adenosine triphosphate (ATP) ratio, which induces phosphorylation and activation of AMP-activated protein kinase (AMPK) through liver kinase B1 (LKB1)-dependent and independent means (Long & Zierath, 2006; Shaw et al., 2004; Zhou et al., 2001). This effect has been well established in numerous cancer models including breast cancer, where *in vitro* doses as low as 0.6 mM metformin may activate AMPK (Hadad et al., 2014). Once activated, AMPK regulates multiple pathways involved in cell proliferation and growth, with main targets in cancer being mammalian target of rapamycin (mTOR) and S6 kinase (Zakikhani et al., 2006). AMPK-mediated inhibition of these pathways results in reduced translation and global protein synthesis to cause decreased cell growth and proliferation (Dowling et al., 2007; Zakikhani et al., 2006). It is important to note, however, that certain cell lines, such as MDA-MB-231 triple-negative breast cancer cells,

lack LKB1 (Dowling et al., 2007). These cancers may be subject to alternative activation pathways of AMPK, as well as AMPK-independent avenues of metformin mechanism.

*1.4.6.2.2 Downstream effects of insulin signalling*

In addition to the indirect effects of metformin on insulin production *in vivo,* metformin also acts directly on the insulin receptor (IR), thus feeding into direct mechanistic pathways inside cancer cells (Dowling et al., 2012). Downstream of both the insulin receptor and the insulin-like growth factor 1 receptor (IGF-1R) lie several key pathways frequently dysregulated in cancer, including the phosphatidylinositol 3-kinase (PI3-K)/ protein kinase B (Akt)-mTOR axis (Huang & Houghton, 2003) and Ras-dependent mitogen-activated protein kinase (MAPK) cascade signalling (Ceresa & Pessin, 1998). The PI3-K-Akt-mTOR axis is normally regulated by the phosphatase and tensin homolog (PTEN) tumour suppressor, but this regulation mechanism is often lost in cancer (Cantley, 2002; Di Cristofano & Pandolfi, 2000). Metformin has been shown to inhibit activation of Akt to suppress mTOR activity in an AMPK-independent manner through suppression of IR/IGF-R1 activity to reduce protein biogenesis and cancer cell growth (Janjetovic et al., 2011; Zakikhani et al. 2010).

*1.4.6.2.3 Reversal of epithelial-to-mesenchymal transition (EMT)*

In triple-negative breast cancer models (MDA-MB-231 cells), 1 mM metformin was shown to suppress expression of key markers associated with epithelial-to-mesenchymal transition (EMT): zinc finger E-box-binding homeobox 1 (ZEB1), twist family BHLH transcription factor 1 (TWIST1), Slug (SNAI2), and transforming growth

factor beta 1 (TGF-ß1) (Vazquez-Martin et al., 2010). Suppression of the mesenchymal phenotype resulted in decreased migration ability and reduced capacity to form 3-dimensional mammospheres in culture. Similar EMT reversal effects are also seen in other cancer models (Li et al., 2014; Qu et al., 2014), indicating that metformin may target EMT markers to reduce cancer aggression and colony formation.

*1.4.6.2.4 Inflammatory cytokine interleukin 6 (IL-6) signalling*

In numerous cancer models, metformin has been shown to inhibit interleukin 6 (IL-6) signalling to prevent the phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) (Hirsch et al., 2013; Li et al., 2014). Reduced levels of IL-6 have been linked to metformin-dependent inhibition of NF-kB (nuclear factor kappa-light chain-enhancer of activated B cells), a positive regulator of IL-6 production (Hirsch et al., 2013; Iliopoulos, Hirsch, & Struhl, 2009; Kim et al., 2011). The effects of IL-6/STAT3 inhibition are particularly prominent in triple-negative breast cancer models, where reduced STAT3 activation significantly inhibits cell growth, induces apoptosis, and reduces expression of the epithelial-to-mesenchymal transition phenotype (Deng et al., 2012).

1.4.6.2.5 *Modulation of the cell cycle*

Apart from impacting cell growth and cell death as part of its anti-neoplastic activity, metformin also acts on the cell cycle to control proliferation. Metformin has been shown to inhibit cyclin D1 through an AMPK-dependent mechanism to induce cell cycle arrest (Zhuang & Miskimins, 2008). Metformin-mediated decreases in cyclin D1 expression have also been shown to occur through AMPK-independent mechanisms

(Ben-Sahra et al., 2008), thought to be in part through up-regulation of DNA-damage-inducible transcription 4 (DDIT4) and reduction of mTOR activity (Ben-Sahra et al., 2011). In breast cancer, it has been shown that some of these effects may be subtype-specific; certain effects on cyclins and cyclin-dependent kinases are only seen in models of luminal A/B and HER2+ cancers, whereas others appear to be triple-negative phenotype-specific (Liu et al., 2009).

*1.4.6.2.6 Modulation of oxidative stress*

Oxidative stress, categorized by an excess of free oxidative or nitrogen radicals, can cause cellular damage through numerous mechanisms that include lipid peroxidation, protein oxidation, and DNA damage (Sies, 1997). Aside from their direct harmful effects, these processes can also result in the formation of toxic by-products such as reactive aldehydes that further compound damage to normal cellular function (Esterbauer & Cheeseman, 1990). Metformin appears to have a complex relationship in modulating oxidative stress by changing intracellular levels of reactive oxygen species (ROS). In pancreatic cancer and glioma cell models, metformin treatment was suggested to attenuate ROS production (Cheng & Lanza-Jacoby, 2015; Janjetovic et al., 2011), which is similar to its metabolic cyto-protective effects in non-cancerous tissues (Cahova et al., 2015; Kocer, Bayram, & Diri, 2014). In contrast, studies in melanoma and breast cancer show that levels of oxidative stress are increased as a result of metformin treatment (Janjetovic et al., 2011; Marinello et al., 2016; Queiroz et al., 2014). This was shown to be a key mechanism of metformin action in both triple-negative and luminal A breast cancer cells, where increased levels of ROS resulted in DNA damage accumulation and apoptosis (Marinello et al., 2016; Queiroz et al., 2014). Mechanistically, this effect has

been linked to suppression of transcription factor activity by Nrf2 (encoded by the gene NFE2L2, nuclear factor erythroid-derived 2-like 2) which regulates key mediators of cellular redox and oxidative stress protection (Truong Do et al., 2014). It is evident that the impacts of metformin on oxidative stress in cancer may depend on the model of study.

### *1.4.7 Metformin anti-cancer action in experimental studies*

Metformin has been shown to have broad anti-neoplastic effects *in vitro* in numerous cancer types, as will be discussed further in 1.4.7.1. Some findings have been replicated in *in vivo* models (section 1.4.7.2), with the most promising moving into early clinical trials as outlined in section 1.4.7.3.

#### *1.4.7.1 In vitro models*

##### *1.4.7.1.1 All cancers*

Metformin has been shown to have potent anti-neoplastic effects in a variety of *in vitro* models. Endometrial cancer cells are some of the most sensitive, with significant cell death seen in doses as low as 1 mM (Cantrell et al., 2010). Numerous studies have shown metformin to be highly effective at targeting prostate cancer cells, notably while leaving normal prostate tissues unaffected (Ben-Sahra et al., 2010; Whitburn, Edwards, & Sooriakumaran, 2017).

Metformin also significantly attenuates cell proliferation, migration, and invasiveness in pancreatic cancer (Bao et al., 2012) as well as in ovarian cancer (Lengyel et al., 2015) models, which also show synergistic effects between metformin and

paclitaxel, carboplatin, and doxorubicin (Erices et al., 2013; Lengyel et al., 2015). Metformin has been shown to be effective in targeting non-small cell lung cancer (NSCLC) both as a monotherapy and in combination with other treatments like sorafenib, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKI), and radiation (Li et al., 2017).

#### *1.4.7.1.2 Breast cancer*

Metformin has been studied extensively for its breast cancer-specific anti-neoplastic effects *in vitro*. As a monotherapy, metformin targets a broad variety of subtypes to varying degrees (Dowling et al., 2012). More recently, researchers have been evaluating the use of metformin in combination therapies. There is significant evidence of synergistic action when using metformin with 2-deoxyglucose (2-DG) (Cheong et al., 2011), 5-fluorouracil (5-FU), paclitaxel, and doxorubicin (Qu et al., 2014), among others (Dowling et al., 2012; Hirsch et al., 2009). Interestingly, metformin specifically may target cancer stem cells (CSCs) in numerous breast cancer subtypes, which may have an impact on re-sensitizing treatment-refractory breast cancer to chemotherapy (Hirsch et al., 2009).

### *1.4.7.2 In vivo models*

#### *1.4.7.2.1 All cancer*

When working *in vivo,* it is not possible to achieve some of the highly supra-physiological concentrations of metformin that may have been effective against cancer cells *in vitro* (Dowling et al., 2016). Nonetheless, some *in vitro* findings have been successfully translated into *in vivo* models, primarily by using xenografts of human cell

lines or patient-derived tumours. Some examples include studies in colon (Wheaton et al., 2014), liver (Zhao et al., 2015), pancreatic (Kisfalvi et al., 2013), and prostate cancer (Ben-Sahra et al., 2008). Many *in vivo* successes have also come from using metformin in combination therapies (Dowling et al., 2012; Quinn et al., 2013). For example, treatment-refractory lung cancer xenografts were partially re-sensitized to EGFR-TKIs through adjuvant metformin therapy (Li et al., 2014), and combination treatment of doxorubicin and metformin significantly reduced relapse in lung and prostate cancer xenograft models compared with either drug as a monotherapy (Iliopoulos, Hirsch, & Struhl, 2011).

#### *1.4.7.2.2 Breast cancer*

Some of metformin's monotherapeutic action seen in *in vitro* breast cancer models translates into *in vivo* studies as well, although the anti-neoplastic effects tend to be less pronounced and are often difficult to predict (Dowling et al., 2012). Specific observations, like the finding that metformin preferentially targets breast cancer-stem cells with the $CD44^{+}CD24^{-/low}$ immunophenotype, have been successfully replicated in multiple *in vivo* models (Cufí et al., 2012; Hirsch et al., 2009). Metformin has also been shown to induce significant tumour shrinkage and longer disease-free survival in breast cancer xenograft models when combined with doxorubicin, 2-deoxyglucose (2-DG), or trastuzumab compared with each individual monotherapy (Cheong et al., 2011; Cufí et al., 2012; El-Ashmawy et al., 2017; Hirsch et al., 2009). Although *in vivo* studies mark an important step towards human applications, controlled clinical studies are needed to make any definitive observations about the efficacy of metformin treatment in breast cancer.

*1.4.7.3 Pre-clinical studies and clinical trials*

Some of the more promising *in vivo* findings on metformin and cancer have been investigated for their direct human applications through clinical studies. The results have been variable and largely depend on the model studied. For example, a metformin neoadjuvant study in endometrial cancer patients showed that pre-operative metformin treatment significantly decreased tumour proliferation markers and had a Ki-67 response rate of 65% (Schuler et al., 2015). A randomized early clinical trial in non-diabetics showed that over the span of one month, low-dose metformin significantly reduced the number of aberrant colon crypt foci compared to the control group, indicating that metformin may be effective at preventing carcinogenesis in colon tissue (Hosono et al., 2010). Another study showed that diabetic metformin users with non-metastatic pancreatic cancer had significantly improved median survival when adjusting for other clinical predictors (Sadeghi et al., 2012). However, a double blind, randomized, placebo-controlled clinical trial evaluating metformin in combination with erlotinib and gemcitabine in patients with advanced pancreatic cancer showed no significant benefit (Kordes et al., 2015). Additionally, a recent review of clinical studies combining metformin and radiation found that results were highly variable, with no clear benefits in treatment outcome or survival observed (Samsuri, Leech, & Marignol, 2017). Given that many metformin clinical trials have been done on late-stage cancers that are already highly refractory to therapy, more studies are needed to determine if metformin may provide a clinical benefit at earlier stages of disease.

#### *1.4.7.3.1 Clinical studies in breast cancer*

Of the 139 active clinical trials currently listed on ClinicalTrials.gov that are evaluating metformin use in the context of cancer therapy, 25 of these involve a focus on breast cancer. These include long-term studies, like a 10-year phase III clinical trial with over 3,600 patients recruited (NCT01101438), as well as studies involving metformin in combination with other therapies that include atorvastatin (NCT01980823), erlotinib (NCT01650506), and doxycycline (NCT02874430).

Studies for which results are available show mixed benefits for metformin use in breast cancer. It was found that metformin co-treatment increased the effectiveness of radiotherapy in diabetic breast cancer patients compared with both diabetic non-users and non-diabetics (Ferro et al., 2013). In another study, metformin use was associated with a near 3-fold higher pathologic complete response rate to chemotherapy compared with control subjects not taking metformin (Jiralerspong et al., 2009). This result was independent of diabetes status, body mass index, and clinical and molecular tumour characteristics. Other clinical studies, however, have shown no significant anti-neoplastic effect associated with metformin use in breast cancer (Bonanni et al., 2012).

Given these variable results, more controlled neoadjuvant studies are needed, especially to evaluate metformin use in non-diabetic patients. One such study was completed in non-diabetic women with early stage breast cancer. Pre-operative metformin treatment was shown to significantly decrease tumour proliferation markers and increase indicators of apoptosis by more than 2-fold (Niraula et al., 2012). This trial also found that metformin is well-tolerated in non-diabetic individuals, inducing only mild adverse effects such as nausea, bloating, and gastrointestinal upset (Niraula et al.,

2012). These results suggest that metformin may have beneficial clinical applications in breast cancer, especially during early stages of the disease.

### *1.4.8 Biomarkers for optimizing metformin treatment*

Biomarkers are signatures of DNA, RNA, or protein that are associated with a specific clinical outcome or diagnosis. The era of increasingly cost-effective molecular screening techniques has brought biomarker identification and validation to the forefront of cancer therapy. *Predictive* cancer biomarkers have disease-associated expression patterns that may either: 1) indicate a specific prognostic pattern such as increased cancer invasiveness or poor survival, or 2) predispose a cancer to sensitivity or resistance to a given treatment. Such information allows clinicians to better tailor cancer therapies for their patients so as not to waste time or resources on ineffective treatment. *Response* biomarkers may also be used in the clinic to monitor cancer recurrence, or to track a cancer's active response to therapy (Lord et al., 2015).

Several studies have attempted to identify biomarkers for the monitoring and prediction of metformin response in cancer. A clinical study in endometrial cancer patients suggested that phospho-Akt, phospho-AMPK, phospho-S6, phospho-eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP-1), and estrogen receptor (ER) may all be metformin response markers, as they are significantly down-regulated with treatment (Schuler et al., 2015). Using paired core tumour biopsies from non-diabetic breast cancer patients that had received neoadjuvant metformin, it was demonstrated that phosphodiesterase 3B (PDE3B), signal sequence receptor subunit 3 (SSR3), tumour protein 53 (TP53), and coiled-coil domain containing 14 (CCDC14) transcripts, as well

as protein levels of phospho-Akt, were significantly down-regulated with metformin treatment (Hadad et al., 2015). Interestingly, phospho-AMPK was shown to be significantly up-regulated with metformin in this breast cancer study (Hadad et al., 2015), whereas the opposite effect was seen in a clinical endometrial cancer study where phospho-AMPK levels were decreased (Schuler et al., 2015).

Insulin-like growth factor 1 receptor (IGF-1R) has been previously investigated as a metformin biomarker with clinical significance (Quinn et al., 2013). Around this time, a team with the British Columbia Cancer Agency determined that a patient enrolled in their Personalized Onco-Genomics trial (Laskin et al., 2015) with recurrent stage IV breast cancer had a 75-copy gain in IGF-1R. Believing their patient may benefit from metformin therapy, they suggested a combination treatment approach of standard anti-estrogen chemotherapy and metformin. Within several months of commencing this treatment, the cancer became undetectable and has been in remission for 4 years (unpublished data – personal correspondence, Dr. Janessa Laskin). This study further speaks to the potential benefits of utilizing metformin biomarkers for guiding metformin-based treatment in oncology practice.

### 1.5 Rationale

The re-purposed anti-diabetic drug metformin has been shown to exhibit broad *in vitro* and *in vivo* anti-neoplastic activity in various cancer types, including breast cancer. These promising results have resulted in several clinical trials that are currently underway to evaluate metformin use in breast cancer therapy. However, some *in vivo* and early

clinical results are showing that metformin response varies greatly between patients, with outcomes ranging from complete pathological response to zero benefit.

Validated genetic or protein biomarkers isolated from a tumour biopsy can indicate if that cancer will respond readily to a chosen treatment. Furthermore, some biomarker expression patterns can indicate if a cancer already under treatment is readily responding. Except for IGF-1R, there are no targeted biomarkers currently in clinical use to predict a cancer's sensitivity to metformin, or to monitor patient response during and after metformin treatment.

Metformin biomarkers validated for use in breast cancer would allow clinicians and researchers to make more informed decisions about their patient's treatment regimen. This is of special relevance for the treatment of triple-negative breast cancers, which have no targeted therapies and are often difficult to treat successfully before progressing to terminal stage. The first step in identifying biomarkers for metformin sensitivity and treatment response in breast cancer is to better understand the molecular pathways that underlie metformin's mechanism of action. Once candidate genes are identified, further studies can be undertaken to validate their use as potential clinical biomarkers.

### *1.5.1 Previous work - quantitative proteomics on metformin-conditioned triple-negative breast cancer cells*

To identify the pathways involved in metformin response in triple-negative breast cancer, our lab recently completed a quantitative proteomics analysis of metformin-treated cells using an *in vitro* TNBC model. Three biological replicates of MDA-MB-231 cells (Met-231A, Met-231B, and Met-231C ) were conditioned for 12 weeks to 1 mM

metformin (Bentley, 2014). This was previously shown to be a physiologically-relevant dose *in vitro* to induce a genetic response in MDA-MB-231 cells (Dowling et al., 2016). Furthermore, a 1 mM concentration of metformin in other *in vitro* cancer models has been shown to cause intracellular metformin accumulation equivalent to the blood plasma concentration of intraperitoneally-injected mice (Dowling et al., 2016). This further increases the *in vivo* relevance of the chosen dose.

Metformin-conditioned cells were analyzed for their quantitative proteomic profile against wild-type MDA-MB-231 cells using isobaric labeling of peptides with tandem mass tags (Ross et al., 2004; Thompson et al., 2003) and a triple-stage mass spectrometry (MS3) strategy for quantification (Ting et al., 2011). Further methodology, including statistical analysis of rough data as completed by Dr. Jayme Salsman (unpublished data – Salsman, Murphy, Bentley, Dellaire.), has been outlined in Murphy et al., 2015 (Murphy et al., 2015). A total of 4241 unique peptides were quantified in this analysis (unpublished data). Of these, a total of 12 proteins were up-regulated a minimum of 2-fold within 1 standard deviation of the mean (Figure 1.2).

#### *1.5.1.1 Known functions of the 12 most up-regulated proteins*

The 12 most up-regulated proteins in metformin-conditioned MDA-MB-231cells identified through our proteomics study are highly diverse in their known functions, although none have been previously implicated in metformin mechanism in breast cancer. As is further discussed in section 3.1.2 (*Knock-down of AKR1C3*), aldo-keto-reductase family 1 member C3 (AKR1C3) is primarily involved in sex hormone metabolism (Penning et al., 2000) but has also been shown to confer cancer resistance to docetaxel,

abiraterone, doxorubicin, and radiation therapy (Heibein et al., 2012; Liu et al., 2017; Matsunaga et al., 2016; Xiong et al., 2014). Ancient ubiquitous protein 1 (AUP1) has only been studied in non-cancerous tissues, and has been implicated in integrin signalling, protein quality control, and mitophagy regulation in response to energy deprivation (Journo, Mor, & Abeliovich, 2009; Kato et al., 2002; Klemm, Spooner, & Ploegh, 2011; Mueller et al., 2008). Connective tissue growth factor (CTGF) has sweeping functions in fibroblast maintenance and proliferation, cell adhesion, and promotion of angiogenesis (Moussad & Brigstock, 2000; Song et al., 2017; Wang et al., 2017; Yang et al., 2016). CTGF was previously shown to be suppressed with metformin treatment in murine renal fibroblast model (Lu et al., 2015).

Little is known about ectonucleotide pyrophosphatase (ENPP4), although it has been shown to promote platelet aggregation, is expressed on tumour-associated macrophages, and is associated with a metastatic phenotype in osteosarcoma (Albright et al., 2012; Miretti et al., 2008; Yan et al., 2016). High mobility group nucleosome binding domain 5 (HMGN5) functions as a tissue-specific transcriptional regulator and has been shown to promote autophagy and invasive phenotypes in cancer (King & Francomano, 2001; Meng et al., 2017; Shirakawa et al., 2000; Weng et al., 2015; Yang et al., 2014), while also suppressing expression of hexokinase I (HK1) (Ciappio et al., 2014). Insulin like growth factor binding protein 7 (IGFBP7) binds to insulin like growth factor (IGF) to regulate its signalling activity, and has been suggested to function as a tumour suppressor in part through regulating oncogene-induced senescence and apoptosis (Horikawa et al., 2017; Wajapeyee et al., 2008; Wen-jing et al., 2006). Microtubule associated protein RP/EB family member 2 (MAPRE2) shares significant homology to the tumour suppressor gene

adenomatous polyposis coli (APC) and is involved in regulating mitotic progression and genome stability (Goldspink et al., 2013; Iimori et al., 2016; Su & Qi, 2001).

Phosphoglycerate dehydrogenase (PHGDH) regulates the early steps of L-serine synthesis, an essential process for breast cancer cell growth and proliferation (Klomp et al., 2000; Locasale et al., 2011; Possemato et al., 2011). Although L-serine starvation has been shown to potentiate the anti-neoplastic activity of metformin in other cancer models, PHGDH depletion alone was shown to not affect metformin sensitivity in murine colon cancer cells (Gravel et al., 2014). Mitochondrial RNA polymerase (POLRMT) is required for the successful transcription of mitochondrial genes and is hence essential for mitochondrial reprogramming in response to energy stress, such as may be caused by metformin (Blomain & Mcmahon, 2012; Gaspari et al., 2004; Salem et al., 2012). SH3-domain binding glutamate rich protein like (SH3BGRL) is a thioredoxin fold protein that is associated with cellular transformation and is upregulated in TNBC (Majid et al., 2006; Muñiz et al., 2014; Yin et al., 2005). Few studies have been conducted on sperm associated protein on the X chromosome B1 (SPANXB1); current knowledge suggests it is involved in sperm development and may be an ectopically-expressed tumour antigen that is recognized by cytotoxic T cells (Almanzar et al., 2009; Frank et al., 2008; Westbrook et al., 2000). Finally, titin (TTN) is a giant protein that helps maintain muscle elasticity and ensures proper contraction. TTN has been shown to be frequently mutated in cancer, including TNBC and other breast cancers (Göhler et al., 2017; Kim et al., 2013; Lips et al., 2015).

Given that none of these proteins have been previously implicated in metformin response in breast cancer, they may collectively present a unique opportunity to explore novel pathways involved in metformin mechanism of action.

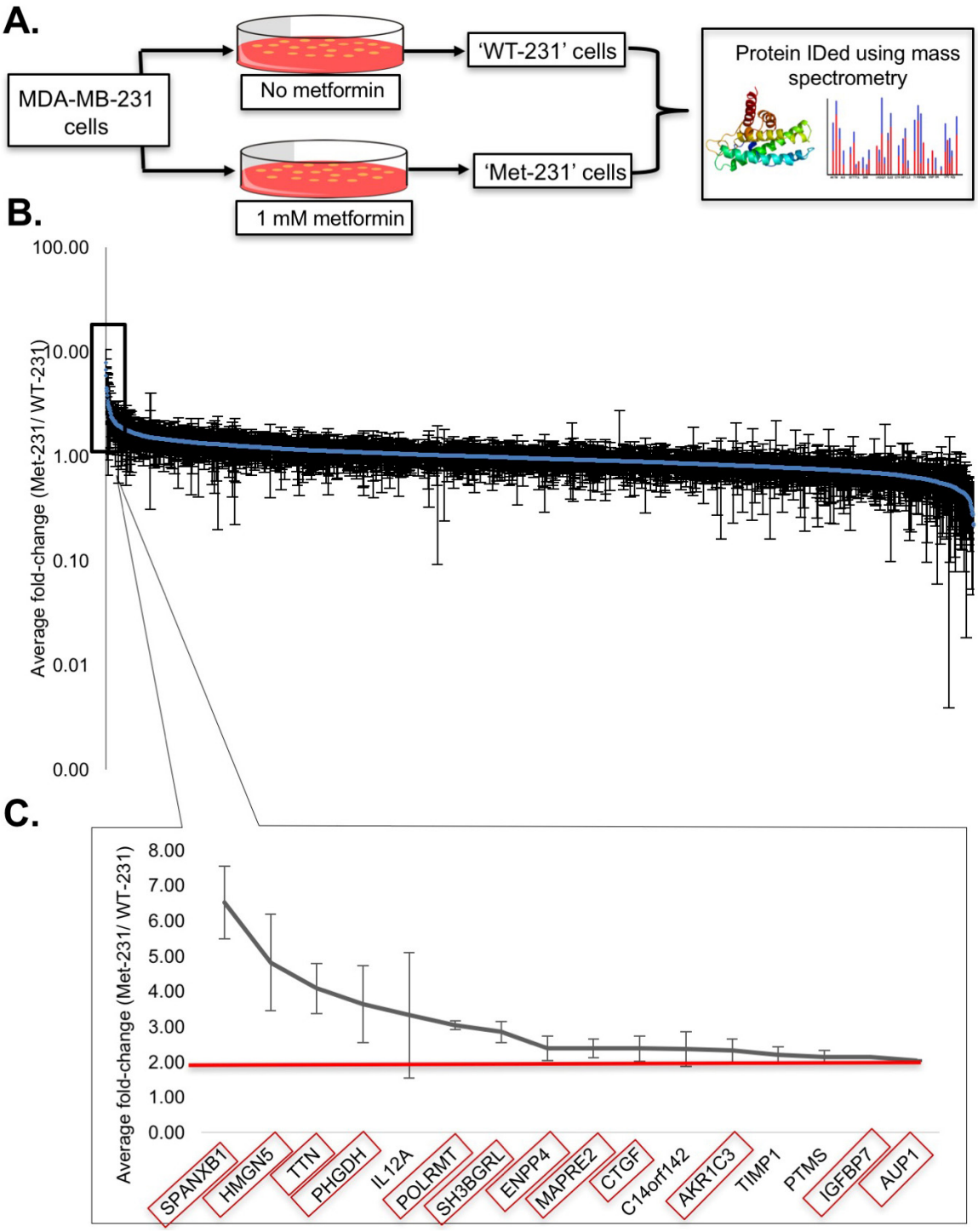



**Figure 1.2 Methodology and results of quantitative proteomics profiling of metformin-conditioned triple-negative breast cancer cells. A.** Experimental set-up of proteomics screen. MDA-MB-231 cells were cultured for 12 weeks in the presence or absence of 1 mM metformin to create "Met-231" and "wild type (WT)-231" cell lines, respectively. N= 3 biological replicates. To quantify protein changes occurring with metformin conditioning, peptides were labeled with tandem mass tags and identified

using triple-stage mass spectrometry. **B.** 4241 unique peptides were quantified between Met-231 and WT-231 cells. **C.** A subset of 12 genes, indicated with red boxes, were up-regulated in Met-231 cells a minimum of 2-fold within 1 standard deviation of the mean. For quality control, all changes had to be significant ($p<0.05$) and proteins must have been quantified with at least 2 independent mass tags to ensure the best clarity and reproducibility of analyses going forward.

## 1.6 Hypothesis

I hypothesize that the genes encoding the 12 most up-regulated proteins in our metformin-conditioned MDA-MB-231 cells, as identified through quantitative proteomics (Figure 1.2), are putative modifiers of metformin response and are involved in metformin's mechanism of action. I hypothesize that knocking down or over-expressing these genes to mimic their naturally-occurring up- or down-regulation in tumours will alter metformin sensitivity in breast cancer cells, especially in triple-negative breast cancer. I further hypothesize that the basal expression levels of proteins encoded by these genes may predict metformin sensitivity of a breast cancer, and/or that the expression of these proteins may change before and after treatment as a "response" to metformin, indicating that they may have potential uses as clinical biomarkers.

# Chapter 2: Materials and Methods

## 2.1 Cell lines and tissue culture

All wild-type cell lines were obtained from the American Type Culture Collection (ATCC) except SUM149, which were sourced from Asterand Bioscience. Cells were cultured according to ATCC guidelines outlined in Table 2.1 in humidified 37°C incubators, and split on a 3-4 days basis once cells were 80-90% confluent. Dulbecco's Modified Eagle's Medium (DMEM – 11995, 11885, 11966), Roswell Park Memorial Institute medium (RPMI-1640 – 11875), Leibovitz's medium (L-15 - 11415), Minimal Essential Medium (MEM - 12492), fetal bovine serum (FBS – 10437, 12484), phosphate-buffered saline (PBS - 10010), 0.05% Trypsin-EDTA (25300), non-essential amino acids (NEAA – 11140), and sodium pyruvate (11360) were sourced from Gibco Cell Culture/ Thermo Fisher. Glucose-free DMEM was supplemented with 4.5 mM sterile-filtered galactose (Sigma). Human insulin (I9278), bovine insulin (I0516), and glutathione (G6013) were sourced from Sigma-Aldrich. Early passage cells were frozen in growth media containing 7.5 % dimethyl sulfoxide (DMSO – D2650, Sigma) and stored in liquid nitrogen.

**Table 2.1 *In vitro* media and culturing conditions for cell lines used.** Triple-negative breast cancer (TNBC) sub-classification taken from Lehmann *et al.* 2011: mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR), basal-like 1 (BL1), basal-like 2 (BL2), unknown (Unk.). Terminology: Dulbecco's Modified Eagle's Medium (DMEM), Leibovitz's medium (L-15), Roswell Park Memorial Institute Medium (RPMI-1640), fetal bovine serum (FBS), non-essential amino acids (NEAA), carbon dioxide ($CO_2$).

| Cell Line | ATCC# | Subtype (Lehmann *et al.* 2011) | Media | Additives | Incubation Conditions | Passaging |
|---|---|---|---|---|---|---|
| **MDA-MB-231** | HTB-26 | TNBC-MSL | DMEM | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **MDA-MB-436** | HTB-130 | TNBC - MSL | L-15 | 10% FBS, 10 µg/mL human insulin, 16 µg/mL glutathione | 0% $CO_2$ | Scraped |
| **MDA-MB-453** | HTB-131 | TNBC - LAR | L-15 | 10% FBS | 0% $CO_2$ | 0.05% Trypsin |
| **MDA-MB-468** | HTB-132 | TNBC - BL1 | L-15 | 10% FBS | 0% $CO_2$ | 0.05% Trypsin |
| **HCC1143** | CRL-2321 | TNBC – BL1 | RPMI-1640 | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **HCC1806** | CRL-2335 | TNBC – BL2 | RPMI-1640 | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **HCC70** | CRL-2315 | TNBC – BL2 | RPMI-1640 | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **Hs578t** | HTB-126 | TNBC - MSL | DMEM | 10% FBS, 10 µg/mL bovine insulin | 5% $CO_2$ | 0.05% Trypsin |
| **BT-20** | HTB-19 | TNBC – Unk. | MEM | 10% FBS, 1x NEAA, 1mM sodium pyruvate | 5% $CO_2$ | 0.05% Trypsin |
| **BT-549** | HTB-122 | TNBC - M | RPMI-1640 | 10% FBS, 0.8 µg/mL human insulin | 5% $CO_2$ | 0.05% Trypsin |
| **SUM149** | - | TNBC – Unk. | DMEM | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **MCF7** | HTB-22 | ER+, PR+ (low) | DMEM | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **T47D** | HTB-133 | ER+, PR+ (high) | DMEM | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |
| **HEK293T** | CRL-3216 | Embryonic kidney | DMEM | 10% FBS | 5% $CO_2$ | 0.05% Trypsin |

## 2.2 Drugs

Metformin hydrochloride (MW: 165.62g/mol, D150959, Sigma) was re-suspended in sterile dd$H_2O$ and diluted into 200 mM working stocks with cell-specific media. Aliquots were frozen at -20°C, protected from light and with freeze/thaw cycles avoided.

## 2.3 RNAi

MDA-MB-231 cell lines with stable gene knock-down were generated using lentivirus-delivered pGIPZ-based short hairpin RNAs (Table 2.2, Thermo Fisher/ GE Dharmacon) obtained from the Dalhousie University Enhanced Gene Analysis and Discovery (EGAD) construct library. DNA was purified using Plasmid Mini and Midi Plus kits (27106 and 12945, Qiagen) and co-delivered into HEK293T cells with lentivirus packaging vectors pMD2.G, pCMV-8.92, and pCMV-8.93 via calcium phosphate transfection (Promega) according to manufacturer's protocol. After 48 hr, lentivirus-containing media was removed from cells and filtered at 0.45 μm to remove cellular debris. This stock was diluted 50:50 with 10% FBS-containing DMEM and placed on MDA-MB-231 cells for 48 hr. Transduced cells recovered in fresh media for 24 hr before a selection period of 72 hr with 1.5 μg/mL puromycin (A11138, Gibco). Cells were maintained in 0.25 μg/mL puromycin until further experimental manipulation. Successful gene knock-down was confirmed using quantitative reverse-transcription PCR, and protein immunoblotting in the case of AKR1C3.

**Table 2.2 pGIPZ-based short hairpin RNA constructs used to knock down genes of interest.** All clones were sourced from Thermo Fisher/ GE Dharmacon. Details shown on given name of short hairpin (sh)RNA for use in this study in format of sh(target gene)_#. Control shRNA is not known to target any mammalian gene. Accessions targeted indicates how many isoforms are targeted by the mature antisense shRNA sequence (5'-3'), as well as location of binding. ORF: open reading frame, 3'UTR: 3' untranslated region.

| Clone name (shTARGET_#): | Clone ID: | Accessions targeted | Mature antisense sequence |
|---|---|---|---|
| **shControl ((non-targeting)** | pGIPZ-RHS4349 (Thermo-Fisher) | N/A | CTTACTCTCGCCCAAGCGAGAG |
| **shAKR1C3_1** | V2LHS_24056 | 2/3 - ORF | AAATCTAGCAATTTACTCC |
| **shAKR1C3_2** | V3LHS_372664 | 2/3 – 3'UTR | TAGCTTTACACACTGGTGT |
| **shAKR1C3 _3** | V3LHS_410279 | 2/3 - ORF | AAAAATTTATTGTCTTTCT |
| **shAUP1_1** | V2LHS_25203 | 4/4 – ORF and non-coding | TCATATAGTGCTTGCTTGC |
| **shAUP1_2** | V2LHS_240485 | 4/4 – non-coding and ORF | ACTTCCTTGACTCTCTGAG |
| **shCTGF_1** | V2LHS_151004 | 1/1 - ORF | AATCATAGTTGGGTCTGGG |
| **shCTGF_2** | V2LHS_151007 | 1/1 – 3'UTR | ATGGTGTTCAGAAATTGAG |
| **shENPP4_1** | V2LHS_96157 | 1/1 - ORF | TAGTTCTTCAGATAATCAG |
| **shENPP4_2** | V2LHS_96158 | 1/1 - ORF | ATGGGTACATCAGTACCAG |
| **shHMGN5_1** | V2LHS_116727 | 4/4 – 3'UTR | ATTATGAAACTACATAGGG |
| **shHMGN5_2** | V3LHS_346762 | 4/4 - ORF | AGTCTTCTTCAACAACTGC |
| **shIGFBP7_1** | V3LHS_302132 | 2/2 - ORF | TGCACACGCACACGCCGCT |
| **shIGFBP7_2** | V3LHS_400018 | 1/2 – 3'UTR | AAATTTTTGTAGATAGTCT |
| **shMAPRE2_1** | V2LHS_197313 | 6/7 – ORF, non-coding | TACTATGTCATTAACCCAT |
| **shMAPRE2_2** | V2LHS_5968 | 6/7 – ORF, non-coding | TAACCCATGCAATGATGTC |
| **shPHGDH_1** | V2LHS_91258 | 7/7 – 3'UTR | TGCTGTACTACAGGGTCAG |
| **shPHGDH_2** | V2LHS_91260 | 7/7 - ORF | TAGAAGTGGAACTGGAAGG |
| **shPOLRMT_1** | V2LHS_239681 | 2/3 - 3'UTR | TATTTACACACTGACAAGG |
| **shPOLRMT_2** | V2LHS_239699 | 3/3 - ORF | TTTGCTTGACCTTGGAGTC |
| **shSH3BGRL_1** | V2LHS_152878 | 3/3 – 3'UTR | TCTATGTTGGTTATTTAGG |
| **shSH3BGRL_2** | V3LHS_316091 | 3/3 - ORF | AGGAAACCAAGCACATCTT |
| **shSPANXB1_1** | V3LHS_305733 | 1/1 - ORF | TTGCTGACTTTCTGGATTT |
| **shSPANXB1_2** | V3LHS_305737 | 1/1 - ORF | ATCTTTATTGCTGACTTTC |
| **shTTN_1** | V2LHS_171635 | 11/11 - ORF | TTCCACAGGACATCAATTC |
| **shTTN_2** | V2LHS_171632 | 11/11 - ORF | TTGTAATAAAGTAAGGCGC |

## 2.4 AKR1C3 cDNA expression

AKR1C3 cDNA (clone ID: 2988160, accession: BC001479.2, GE Dharmacon) was cloned into the pClover-J1 vector, which is based on the pEGFP-C1 plasmid (AddGene) and contains a modified multiple cloning site. Successful integration of the AKR1C3 cDNA sequence was validated using restriction digest and DNA sequencing. Competent Escherichia coli (E. coli) bacteria were transformed with pClover-AKR1C3 using heat-shock technique and plasmid DNA was isolated using a Plasmid Midi Plus kit (12945, Qiagen) according to manufacturer's instructions. Target cell lines were transfected via electroporation using the Neon Transfection System (Life Technologies) with parameters outlined in Table 2.3. Transfected cells were immediately plated into fresh DMEM + 10% FBS and left to recover for 24 hr prior to beginning metformin treatment.

**Table 2.3 Electroporation protocol for transfection of pClover plasmid into breast cancer cells.** Adapted from manufacturer's protocol, Neon Transfection System (Life Technologies). DNA is purified Clover-Control or Clover-AKR1C3 plasmid.

| Cell Line | Reaction volume (µl) | Cells per reaction | DNA per reaction (µg) | Voltage (V) | Pulse width (ms) | Pulses |
|---|---|---|---|---|---|---|
| **MDA-MB-231** | 100 | $2 \times 10^6$ | 20 | 1,400 | 10 | 4 |
| **MCF-7** | 100 | $5 \times 10^6$ | 30 | 1,100 | 30 | 2 |

## 2.5 Cell counting and viability

### *2.5.1 Cell counting*

General cell counts and approximate cell death measurements were obtained using Type S cassettes (MXC002, Orflo Technologies) with the Moxi Z Mini Automated Cell

Counter (Orflo Technologies) and Trypan blue (T8154, Sigma) exclusion dye with a manual haemocytometer. Cells were trypsinized and re-suspended in pre-warmed media to be counted within 15 min.

*2.5.2 Cell viability assay*

Cell viability was measured using the alamarBlue fluorogenic assay. Cells were seeded in 96-well plates at concentrations of 2-5 x $10^4$ cells/ mL depending on cell line growth rate (consistent between replicates). After a 24 hr settling period, cells were treated with experimental media +/- metformin treatment for 72 hr in technical quadruplicate. Following treatment, media was aspirated and replaced with pre-warmed cell-line specific media (Table 2.1) containing 10% alamarBlue reagent (DAL1100, Invitrogen). After 3-8 hr incubation, dye conversion was quantified using the Infinite 200 Pro plate reader (Tecan) set at 560 nm excitation and 590 nm emission wavelength. Please see Appendix A for further description of alamarBlue methodology.

## 2.6 **Protein immunoblotting**

Adherent cell cultures were washed thoroughly with PBS and collected by scraping. Cell pellets were obtained by centrifugation at 4ºC (1,200 × *g* for 15 min) and re-suspended in ice-cold lysis buffer consisting of radioimmunoprecipitation (RIPA) buffer (R0278, Sigma) and 1x protease inhibitor (P8340, Sigma). After 20 min, samples were centrifuged, 14,000 × *g* at 4ºC, to precipitate cellular debris. Supernatants were removed and protein concentrations were determined using the spectroscopic Bradford protein assay (50000, Bio-Rad) according to manufacturer's instructions. Samples were diluted to 1x with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

protein sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 0.7135 M (5%) β-mercaptoethanol, 10 % glycerol – all Sigma-Aldrich) and were boiled for 5 minutes to denature proteins. Lysates were either immediately utilized in SDS-PAGE or were stored at -80ºC to be re-boiled prior to use. After wet-transfer of samples, nitrocellulose membranes (88018, Thermo Fisher) were blocked using 4% milk (Nestlé Carnation) in PBS for 1 hr, washed 3 x 5 min with PBS and incubated with primary antibody against rabbit anti-AKR1C3 (PA5-28065, Thermo Fisher, 1:1,000), mouse anti-ß-Tubulin (T7816, Sigma, 1:50,000), or rabbit anti-Vinculin (18058**,** Abcam, 1:1,000), in 4% milk overnight on a 4ºC roller. Membranes were washed 4 x 5 min with PBS, followed by 45 min incubation at room temperature with secondary antibody: goat anti-rabbit-HRP (A120-101P, Bethyl, 1:5,000) or sheep-anti-mouse-HRP (ab6808, Abcam, 1:5,000). Following 4 x 5 min PBS washes, Western blots were developed using Clarity Western ECL Blotting Substrates (17050, Bio-Rad) and imaged using a VersaDoc molecular imager (Bio-Rad) with optimized exposure settings.

**2.7 Immunofluorescence Microscopy**

Cells grown on glass coverslips were washed 2 x 5 min with PBS and fixed in 2% paraformaldehyde-PBS solution (15710, Electron Microscopy Sciences) for 10 min. Following 3 x 5 min PBS wash, cells were permeabilized using 0.5% Triton-X100 (Sigma) in PBS for 5 min, PBS-rinsed 3 x 5 min, and blocked in 4% bovine serum albumin (BSA-PBS, A2058, Sigma) in a humidified chamber for 30 min. Cells were immunoblotted with primary antibody rabbit anti-AKR1C3 (ab137545, Abcam, 1:100) for 1 hr, PBS-rinsed 3 x 5 min and blotted with fluorescently-labeled secondary antibody:

goat anti-rabbit DyLight 488- conjugated (A120-101D2, Bethyl, 1:100) or goat-anti-rabbit DyLight 550-conjugated (A120-101D3, Bethyl, 1:100), for 30 min protected from light. After 1 x 5 min wash, cells were stained with DAPI (D9564, Sigma, 1:1000) in PBS for 10 min to visualize nuclei. After 1 x 5 min PBS wash, coverslips were mounted on frosted glass slides (12-550-15, Fisher Scientific) using fluorescent mounting medium (S3023, Dako). Fluorescent micrographs were captured using a custom-built Zeiss Cell Observer Microscope (Intelligent Imaging Innovations, 3i), equipped with a solid-state Light Engine (Lumencor) and an HQ2 CCD camera (Photometrics) using a 63x 1.4 NA immersion oil objective lens. Slidebook capture software v. 6.0.12 (3i) was used and final images were exported as 16 or 8-bit TIFs, with linear adjustments made to the contrast and levels of each channel (red, green, blue) using Adobe Photoshop CS5.

### 2.8 RNA extraction and RT-qPCR

Cells cultured in 10 cm dishes were lysed using 2 mL TRIzol reagent (15596, Invitrogen) and stored in 1 mL aliquots at -80ºC until further use. Total RNA was extracted according to manufacturer's protocol using the PureLink RNA Mini Kit (12183, Ambion) with on-column deoxyribonuclease (DNase) treatment (12185, Invitrogen) to prevent genomic DNA contamination. In concordance with the MIQE publication guidelines for quantitative PCR (Bustin et al., 2009), RNA quality and quantity were assessed using spectrophotometry (Eppendorf) and agarose gel electrophoresis. Complementary DNA (cDNA) was generated from RNA using iScript Reverse Transcription Supermix (17088, Bio-Rad) according to manufacturer's protocol. Quantitative PCR was conducted for each sample in technical quadruplicate using the

CFX96 Touch real-time PCR detection system (Bio-Rad) with SsoAdvanced Universal SYBR Green Supermix (17252**,** Bio-Rad) and gene-specific primers (Integrated DNA Technologies). Primers were designed using the Basic Local Alignment Search Tool (BLAST, NCBI-NIH) to target maximum number of gene isoforms and were evaluated for efficiency according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009). Relative expression levels were calculated between experimental conditions by normalizing target gene expression to 2 reference genes, HMBS and HUWE1 (target stability values: M <0.5, CV< 25%), using the ΔΔCq method in CFX Manager v. 3.1 (Bio-Rad).

**2.9 Statistical analysis**

The 12 proteomics peptides identified for further investigation were chosen based on a minimum 2-fold average up-regulation within 1 standard deviation of the mean, at a significance level of p=0.05. Excel 2016 (Microsoft) was used for basic statistical analysis, including: calculation of means and standard deviations, linear regressions, Spearman's correlation coefficients, and student's T-Test (2-tailed, unpaired, assuming unequal variances). For cell viability assays, experimental condition values were normalized to the mean of the untreated control (0 mM) for each cell line to assess viability remaining out of 1. Two-way repeated measures ANOVAs (**An**alyses **O**f **Va**riance) with Bonferroni post-hoc correction were conducted using SPSS Statistics (build 1.0.0.580, IBM) to test for differences between AKR1C3 knock-down/ over-expression and control cells.

Protein density quantification from Western blotting was determined using the rectangular and circular volume functions and global background subtraction on unsaturated, raw VersaDoc images, using Quantity One (v. 4.6.6) and Image Lab (v.5.2.1) software (Bio-Rad).

The cBioPortal platform was used to create Kaplan-Meyer survival curves using the TCGA breast cancer dataset (Cerami et al., 2012; Gao et al., 2013).

# Chapter 3: Results

## 3.1 Evaluation of genes potentially modulating metformin sensitivity

The proteins that were the most significantly up-regulated in metformin-conditioned (Met-231) MDA-MB-231 cells in our previous proteomics study (Figure 1.2) may potentially be involved in modulating metformin sensitivity in triple-negative breast cancer. Along this line of investigation, the genes for the top 12 up-regulated proteins (Figure 1.2, panel C) were stably knocked down in individual pools of MDA-MB-231 cells using lentivirus-delivered pGIPZ-based shRNAs, as annotated in Table 2.2. The pool of MDA-MB-231 cells transduced with shPOLRMT_1 and shPOLRMT_2 displayed extensive cell death before and during puromycin selection. Quantitative RT-PCR showed that the remaining puromycin-resistant population of cells did not have effective POLRMT knock-down, confirming previous studies that indicate POLRMT is likely an essential gene (Hart et al., 2015). Quantitative RT-PCR showed that shIGFBP7_2, shENPP4_2, and shTTN_2 did not result in efficient knock-down of their respective target transcripts (data not shown); cells expressing these constructs were hence excluded from further analysis. Cell lines with AKR1C3 knock-down are discussed separately in 3.1.2.

### *3.1.1 Short hairpin (shRNA)-mediated depletion of gene expression of putative metformin response genes*

AlamarBlue cell viability studies showed that effects of knock-down on metformin sensitivity depended on the target gene, and sometimes changed with glucose-availability and extent of gene knock-down. Under high-glucose (25 mM glucose) culturing conditions, knock-down of all 10 remaining genes (AUP1 - Figure 3.1.1, CTGF - Figure 3.1.2, ENPP4 - Figure 3.1.3, HMGN5 - Figure 3.1.4, IGFBP7 - Figure 3.1.5, MAPRE2 - Figure 3.1.6, PHGDH - Figure 3.1.7, SH3BGRL - Figure 3.1.8, SPANXB1 - Figure 3.1.9, TTN – Figure 3.1.10) resulted a trend towards higher sensitivity at 25 mM metformin, with some effects also seen at 10 mM and 50 mM doses.

At 25 mM metformin, average sensitivity was further increased by a minimum of 35% (shSH3BGRL_1, Figure 3.1.8) and a maximum of 53% (shTTN_2, Figure 3.1.10) compared with control. At 10 mM metformin, knock-down of HMGN5, IGFBP7, MAPRE2, SPANXB1, and TTN each resulted in a trend of increased sensitivity, with a maximum effect of 42% increased average sensitivity (shSPANXB1_2, Figure 3.1.9). At 50 mM tested dose, knock-down of CTGF, ENPP4, MAPRE2, and TTN showed possible sensitization effects compared to control to a maximum of 30% further loss of viability (shTTN_1, Figure 3.1.10). Significance was not tested for these trends as only a single biological replicate of each assay was completed.

In certain cases, the possible sensitizing effect of gene knock-down was reproduced under glucose deprivation conditions (trace glucose, + 4.5 mM galactose). IGFBP7 knock-down resulted in a trend of 31% further-decreased cell viability compared with control at 1 mM metformin (Figure 3.1.5). Knock-down of MAPRE2 (shMAPRE2_1, Figure 3.1.6), PHGDH (Figure 3.1.7), SH3BGRL (shSH3BGRL_2, Figure 3.1.8), SPANXB1 (shSPANXB1_1 – Figure 3.1.9), and TTN (Figure 3.1.10) also

each showed possible trends towards increased sensitivity at 1 mM metformin under glucose-deprived conditions.

In other cases, the trends seen with glucose deprivation contrasted those seen under high-glucose conditions. Knock-down of AUP1 (Figure 3.1.1), CTGF (Figure 3.1.2), ENPP4 (Figure 3.1.3), HMGN5 (Figure 3.1.4), PHGDH (Figure 3.1.7), and SH3BGRL (Figure 3.1.8) sometimes resulted in lower average metformin sensitivity, especially at higher doses (5 mM+). These results suggest that the effects of gene knock-down on metformin sensitivity in triple-negative breast cancer may be glucose-dependent.

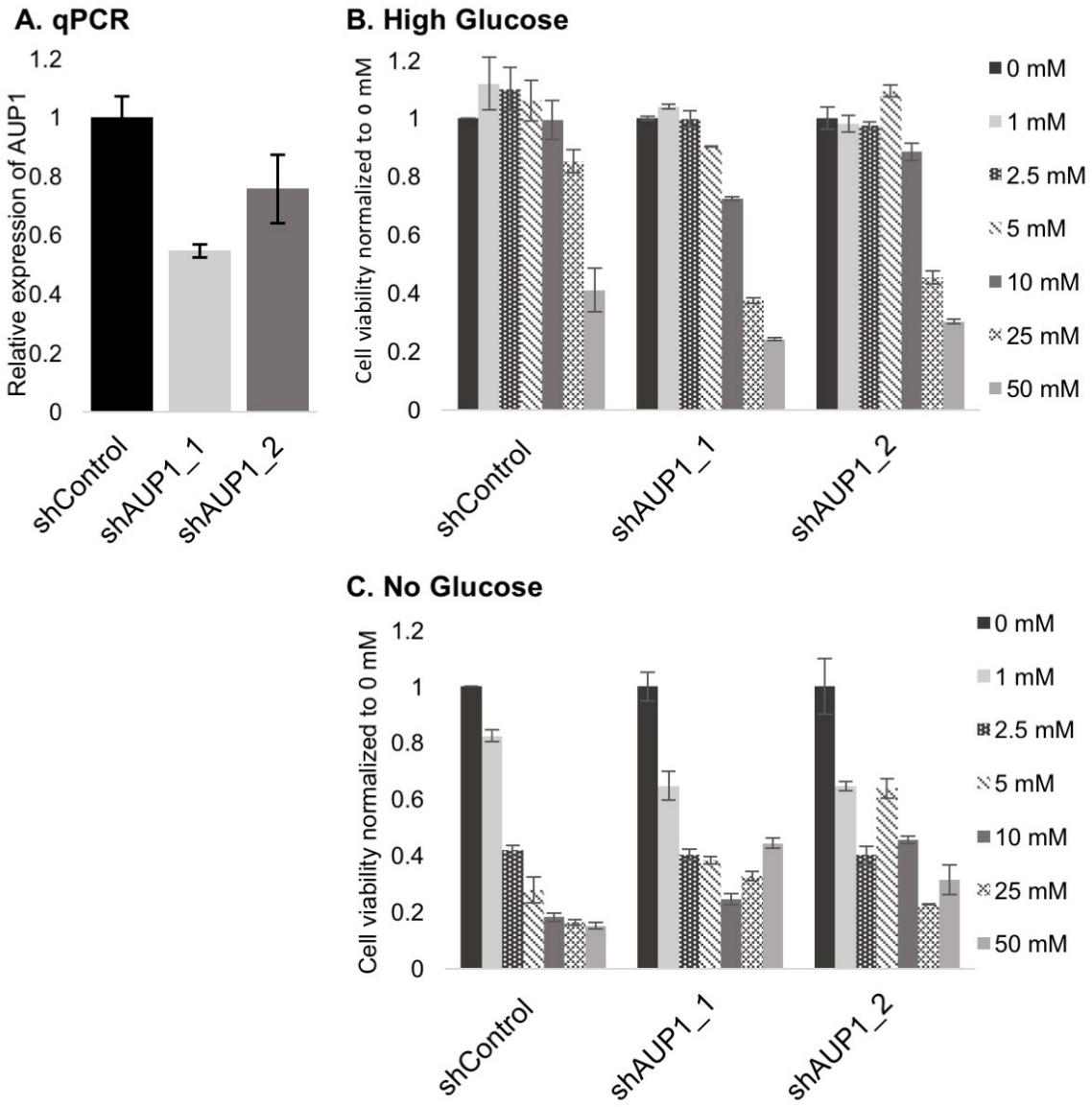



**Figure 3.1.1 Effects of AUP1 knock-down on metformin sensitivity relative to glucose concentration. A.** Effective knock-down of AUP1 transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 25 mM dosing was seen under high-glucose media conditions. **C.** Under glucose-deprived conditions, AUP1 knock-down may result in increased resistance to some higher doses of metformin. MDA-MB-231 cells were transduced with lentivirus carrying each of two pGIPZ-based AUP1-targeting shRNAs (shAUP1_1 and shAUP1_2) or a non-targeting control (shControl). Cells were treated with indicated doses of metformin for 72 hr and cell viability was measured using an alamarBlue assay. ShAUP1_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

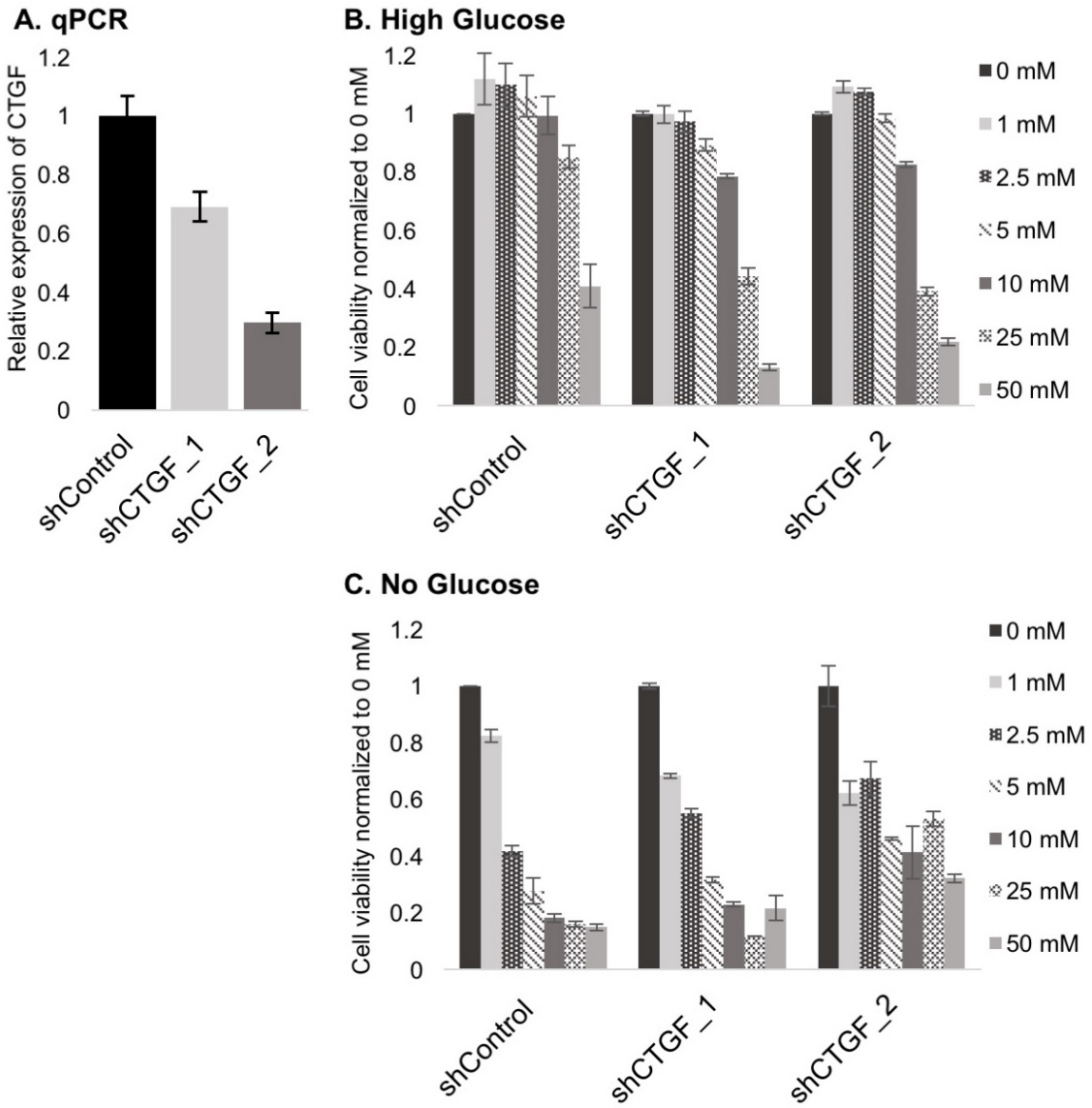



**Figure 3.1.2 Effects of CTGF knock-down on metformin sensitivity relative to glucose concentration. A.** Effective knock-down of CTGF transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 25 mM and 50 mM dosing was seen under high-glucose media conditions. **C.** Under glucose-deprived conditions, strong CTGF knock-down may result in increased resistance to some higher doses (2.5 mM and above) of metformin. MDA-MB-231 cells transduced with lentivirus carrying each of two pGIPZ-based shRNAs targeting CTGF (shCTGF_1 and shCTGF_2) or a non-targeting control (shControl). Cells were treated with indicated doses of metformin for 72 hr and cell viability was measured using an alamarBlue assay. ShCTGF_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

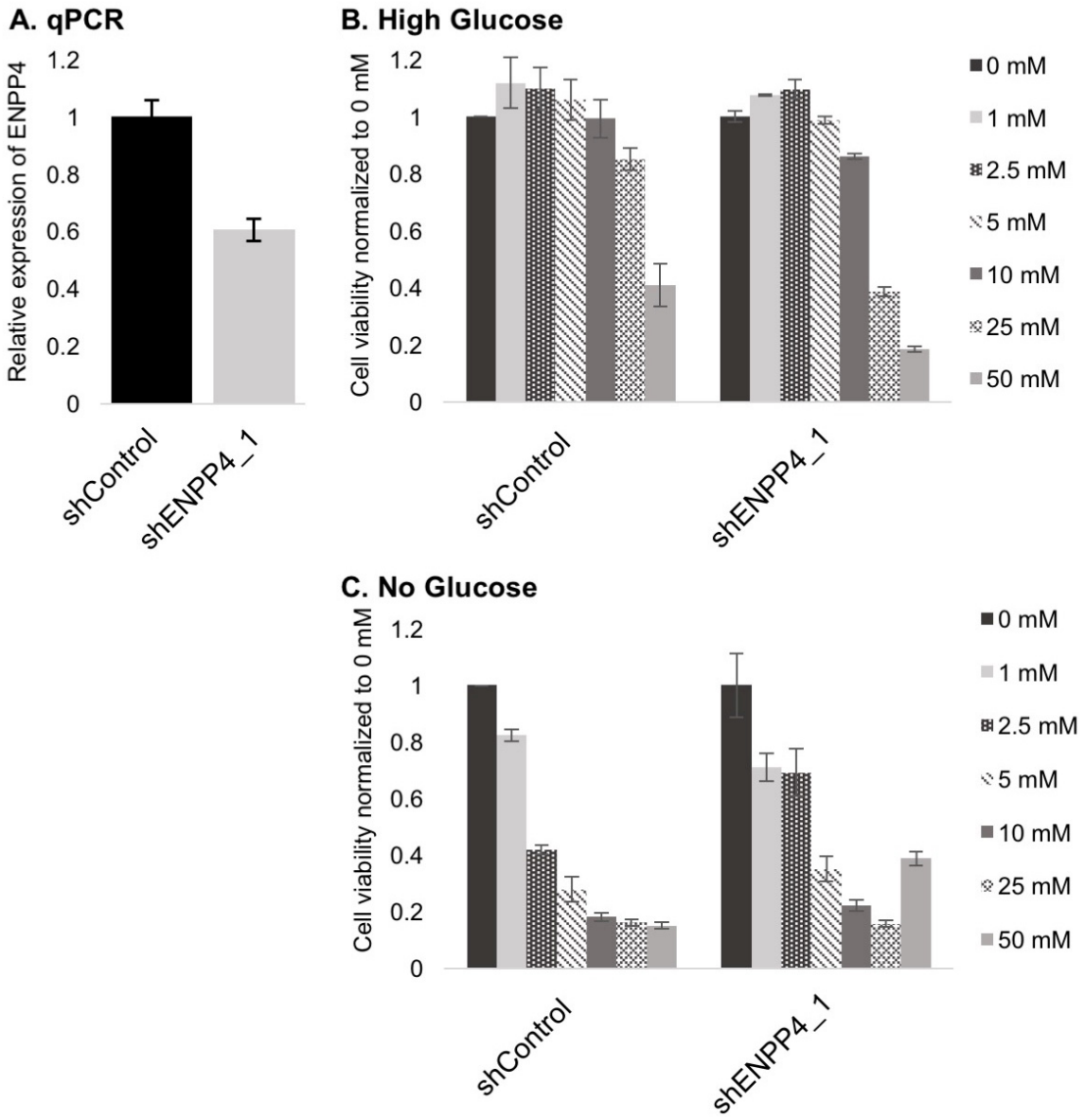



**Figure 3.1.3 Effects of ENPP4 knock-down on metformin sensitivity relative to glucose concentration. A.** Effective knock-down of ENPP4 transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 25 and 50 mM dosing was seen under high-glucose media conditions. **C.** Under glucose-deprived conditions, ENPP4 knock-down showed a weak trend towards increased resistance 2.5 mM and 50 mM metformin. MDA-MB-231 cells were transduced with lentivirus carrying a pGIPZ-based shRNA targeting ENPP4 or a non-targeting control (shControl). Cells were treated with indicated doses of metformin for 72 hr and cell viability was measured using an alamarBlue assay. ShENPP4_1: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

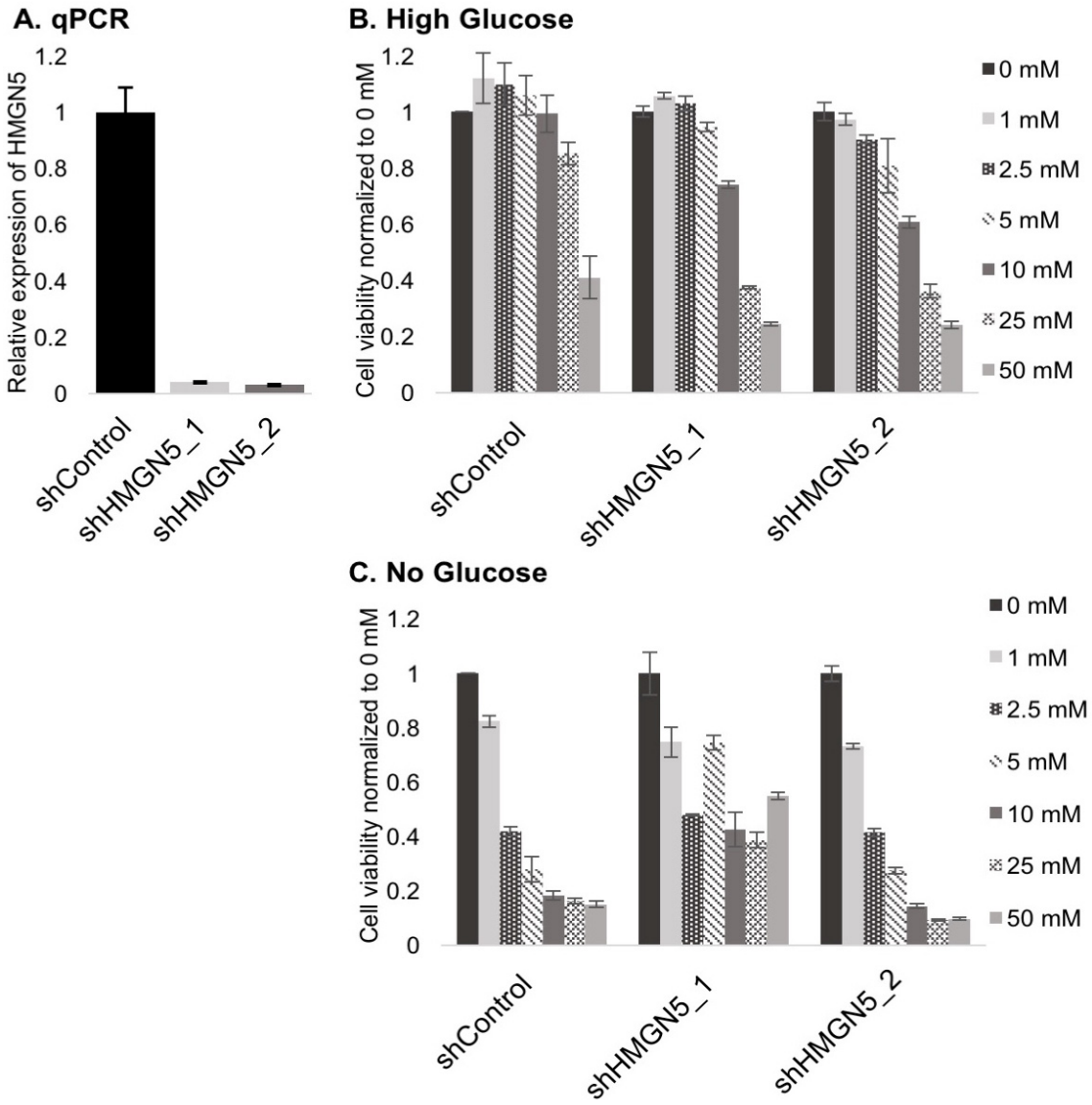



**Figure 3.1.4 Effects of HMGN5 knock-down on metformin sensitivity relative to glucose concentration.** **A.** Effective depletion of HMGN5 expression was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 10 and 25 mM dosing was seen under high-glucose conditions. **C.** Under glucose-deprived conditions, HMGN5 knock-down with shHMGN5_1 showed increased resistance to some higher doses (5 mM +) of metformin, but this effect was not reproduced with the second knock-down (shHMGN5_2). MDA-MB-231 cells were transduced with lentivirus carrying each of two pGIPZ-based HMGN5-targeting shRNAs (shHMGN5_1 and shHMGN5_2) or a non-targeting control (shControl). Cells were treated with metformin for 72 hr and cell viability was measured using alamarBlue assay. ShHMGN5_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

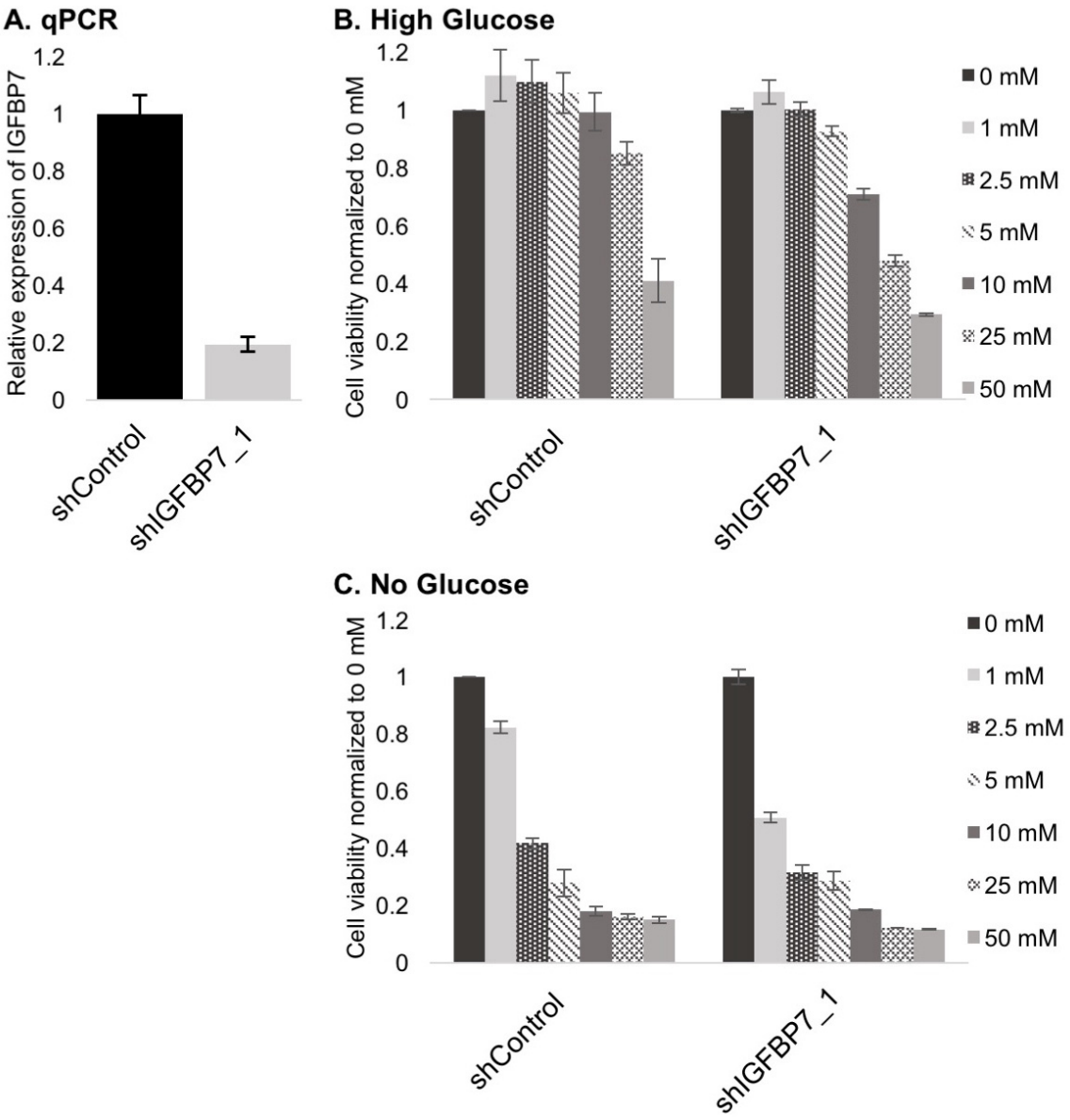



**Figure 3.1.5 Effects of IGFBP7 knock-down on metformin sensitivity relative to glucose concentration. A.** Effective knock-down of IGFBP7 transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 10 mM and 25 mM dosing was seen under high-glucose media conditions. **C.** Under glucose-deprived conditions, IGFBP7 knock-down resulted in a trend of increased sensitivity at 1 mM dose of metformin. MDA-MB-231 cells were transduced with lentivirus delivering a pGIPZ-based IGFBP7-targeting shRNA or a non-targeting control (shControl). Cells were treated with indicated doses of metformin for 72 hr and cell viability was measured using an alamarBlue assay. ShIGFBP7_1: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

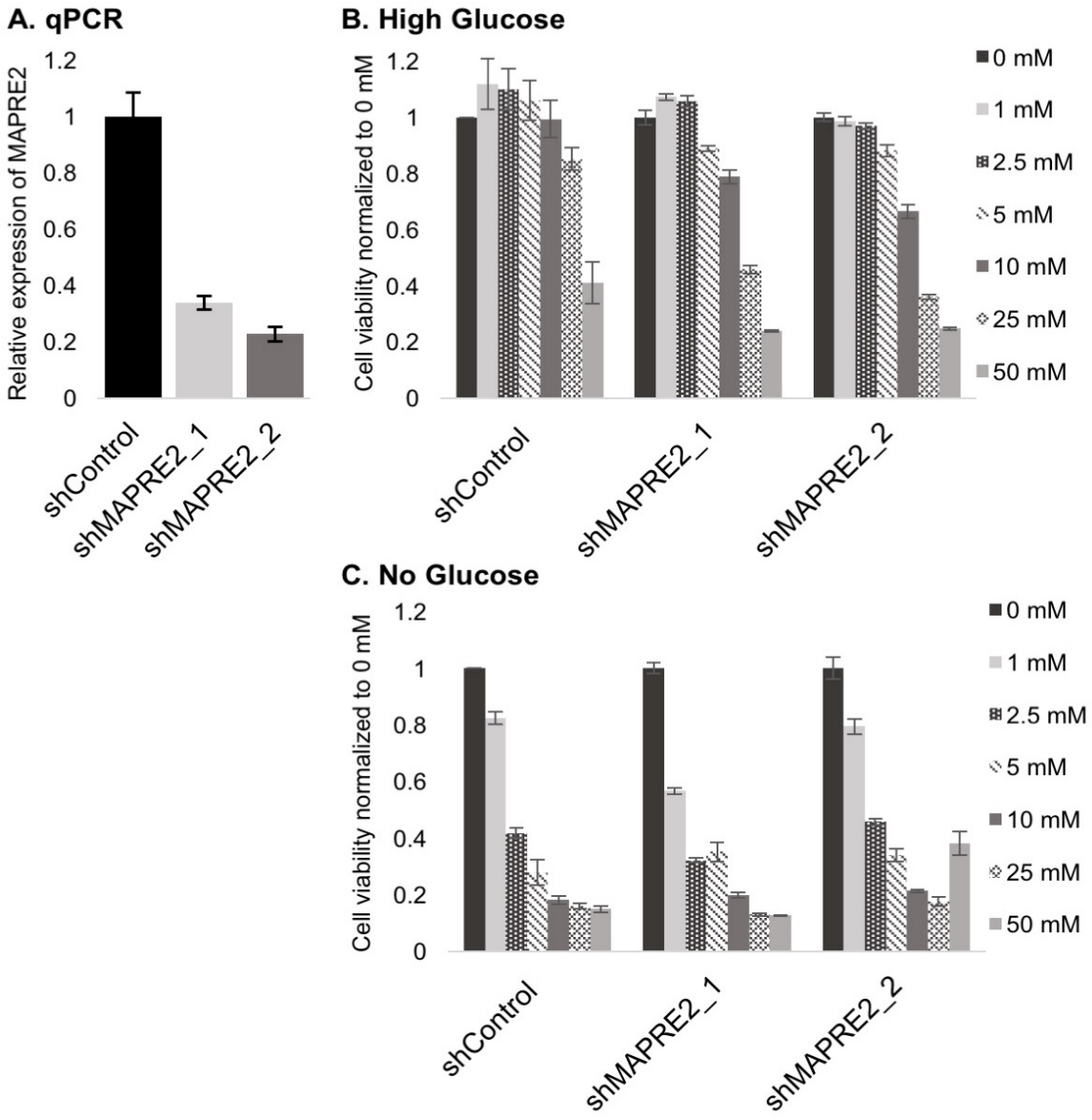



**Figure 3.1.6 Effects of MAPRE2 knock-down on metformin sensitivity relative to glucose concentration.** **A.** Effective depletion of MAPRE2 expression was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 10, 25, and 50 mM was seen in MAPRE2-knock-down cells under high-glucose media conditions. **C.** Under glucose-deprived conditions, MAPRE2 knock-down appeared to have no reproducible effect on metformin sensitivity. MDA-MB-231 cells were transduced with lentivirus containing one of two MAPRE2-targeting pGIPZ-based shRNAs (shMAPRE2_1 and shMAPRE2_2) or a non-targeting control (shControl). Cells were treated with metformin for 72 hr and cell viability was measured using alamarBlue. ShMAPRE2_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

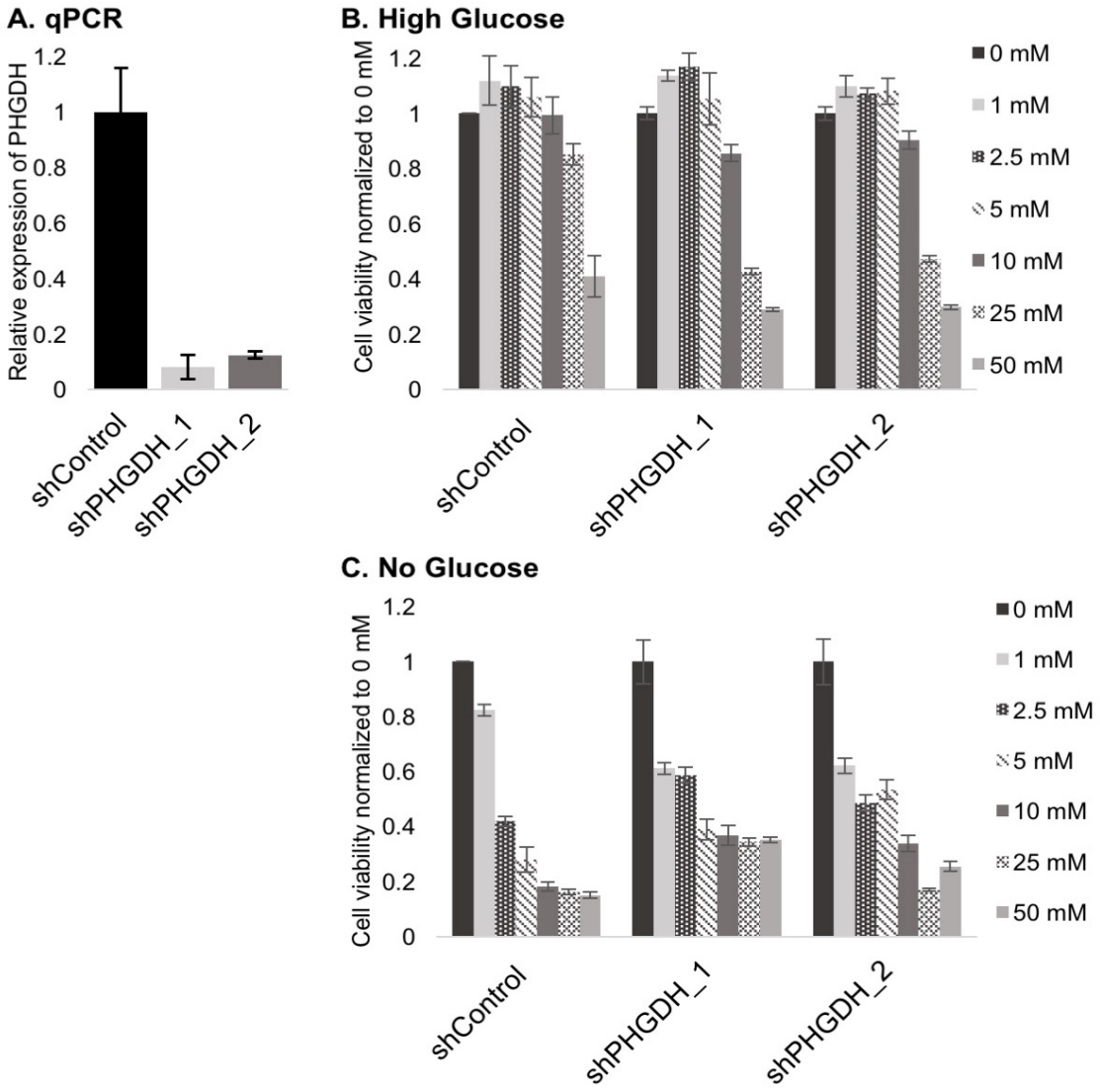



**Figure 3.1.7 Effects of PHGDH knock-down on metformin sensitivity relative to glucose concentration. A.** Effective knock-down of PHGDH transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased sensitivity at 25 mM metformin was seen in PHGDH-knock-down cells under high-glucose conditions. **C.** Under glucose-deprived conditions, PHGDH knock-down may show a weak trend of decreased metformin sensitivity at higher doses. MDA-MB-231 cells were transduced with lentivirus carrying one of two PHGDH-targeting pGIPZ-based shRNAs (shPHGDH _1 and shPHGDH _2) or a non-targeting control (shControl). Cells were treated with metformin for 72 hr and cell viability was measured using alamarBlue. ShPHGDH_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

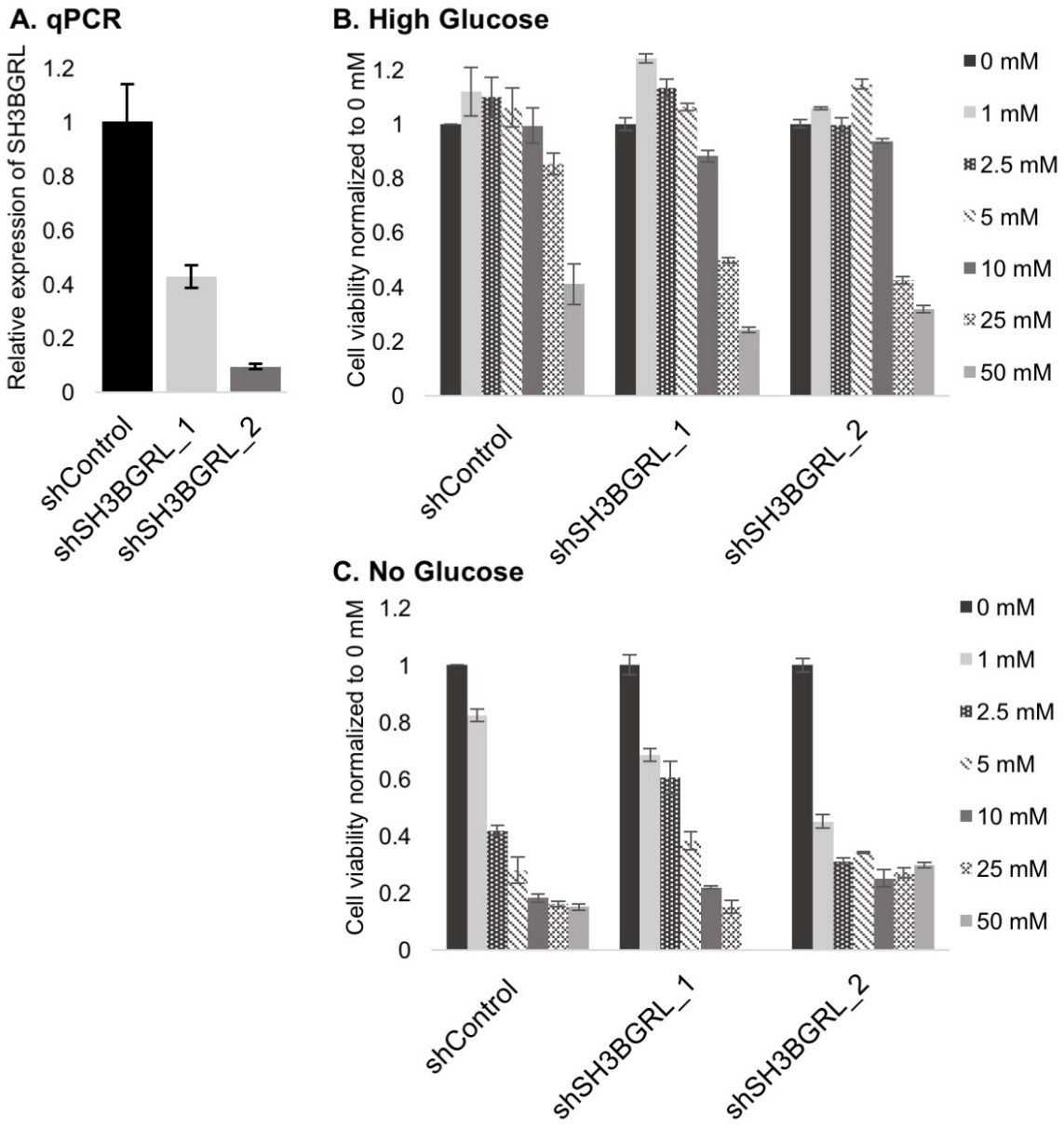



**Figure 3.1.8 Effects of SH3BGRL knock-down on metformin sensitivity relative to glucose concentration. A.** Effective depletion of SH3BGRL transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased metformin sensitivity at 25 mM was seen in SH3BGRL-knock-down cells with high-glucose conditions. **C.** Under glucose-deprived conditions, SH3BGRL knock-down appeared to have no reproducible effect on metformin sensitivity. MDA-MB-231 cells were transduced with lentivirus delivering one of two SH3BGRL-targeting pGIPZ-based shRNAs (shSH3BGRL_1 and shSH3BGRL_2) or a non-targeting control (shControl). Cells were treated with of metformin for 72 hr and cell viability was measured using alamarBlue assay. ShSH3BGRL_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

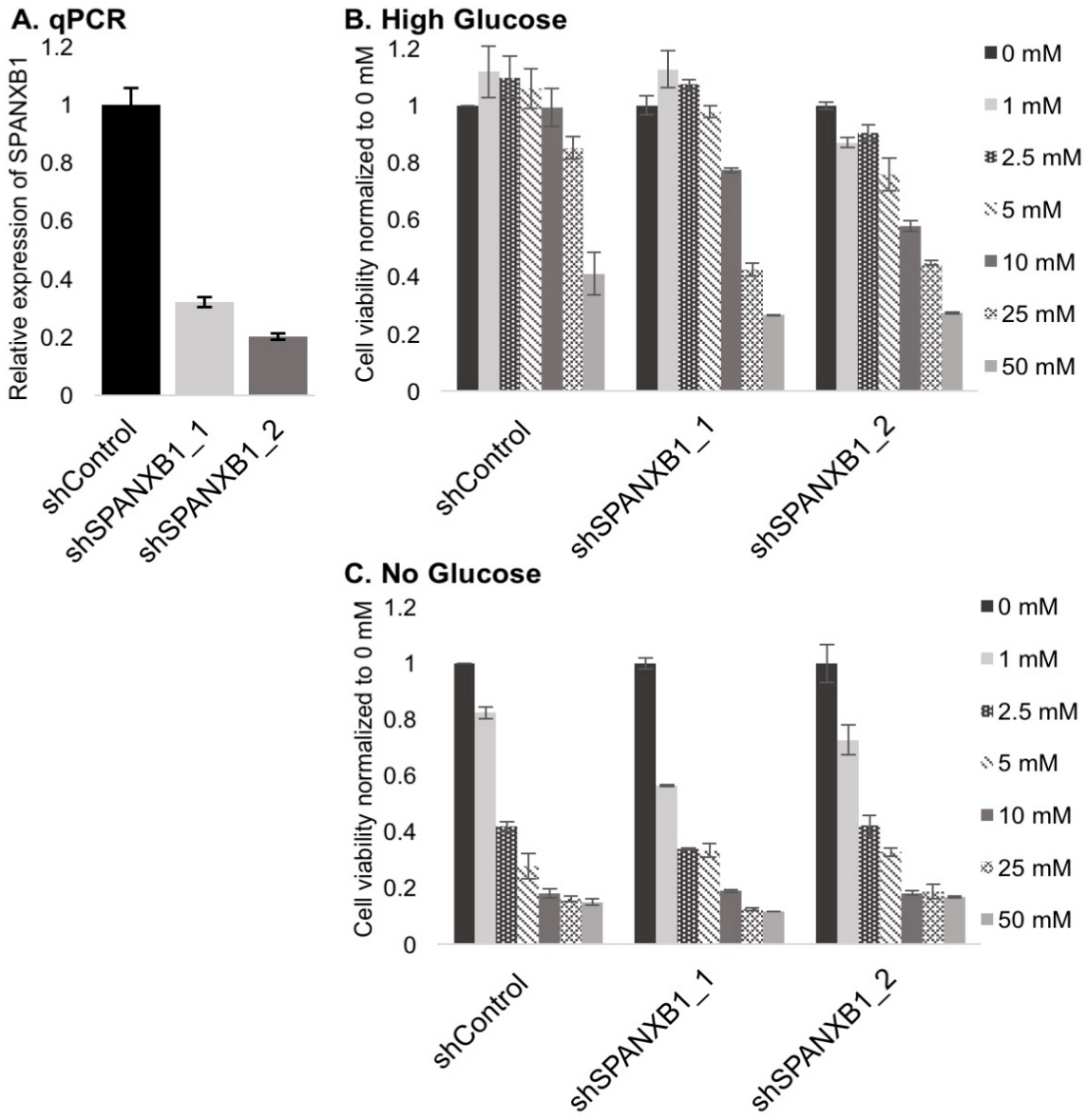



**Figure 3.1.9 Effects of SPANXB1 knock-down on metformin sensitivity relative to glucose concentration. A.** Effective depletion of SPANXB1 transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased sensitivity at 10, 25, and 50 mM metformin was seen in SPANXB1-knock-down cells under high-glucose conditions. **C.** Under glucose-deprived conditions, SPANXB1 knock-down appeared to have no reproducible effect on metformin sensitivity. MDA-MB-231 cells were transduced with lentivirus carrying one of two SPANXB1-targeting, pGIPZ-based shRNAs (shSPANXB1_1 and shSPANXB1_2) or a non-targeting control (shControl). Cells were treated with metformin for 72 hr and cell viability was measured using alamarBlue assay. ShSPANXB1_1/2: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

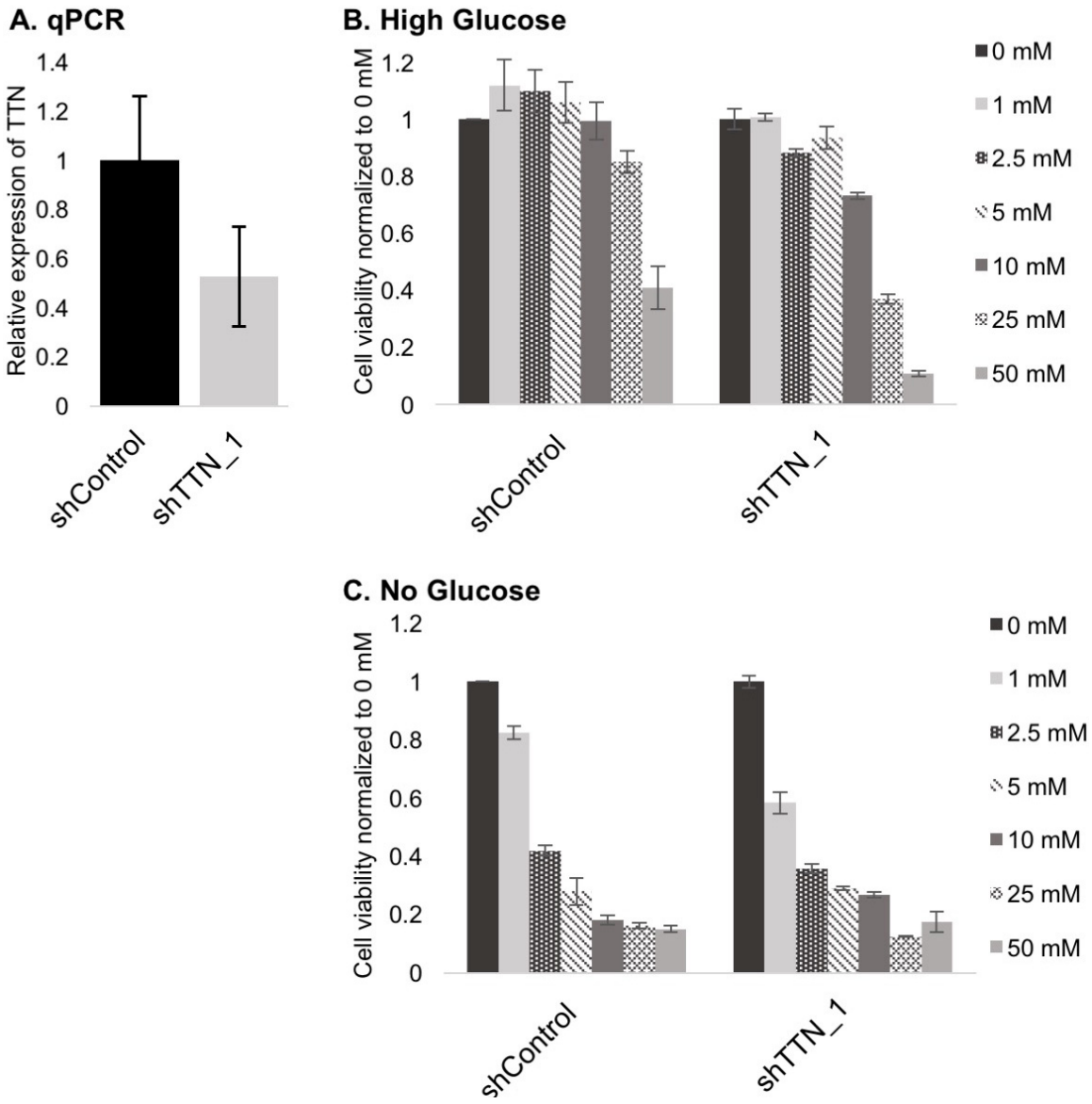



**Figure 3.1.10 Effects of TTN knock-down on metformin sensitivity relative to glucose concentration. A.** Effective knock-down of TTN transcript was confirmed using qPCR with expression normalized to control, N=1 and error bars = SEM. **B.** A trend towards increased sensitivity at 10 mM, 25 mM, and 50 mM metformin was seen in TTN-knock-down cells under high-glucose media conditions. **C.** Under glucose-deprived conditions, TTN knock-down appeared to have no impact on metformin sensitivity. MDA-MB-231 cells were transduced with lentivirus carrying a pGIPZ-based, TTN-targeting shRNA or a non-targeting control (shControl). Cells were treated with metformin for 72 hr and cell viability was measured using an alamarBlue assay. ShTTN_1: n=1, error bars = SEM (technical replicates). ShControl: n=3, error bars = SEM (biological replicates).

*3.1.2 Knock-down of AKR1C3*

Up-regulation of aldo-keto reductase family 1 member C3 (AKR1C3), as seen in the Met-231 proteomics analysis, has been implicated in resistance to numerous types of chemotherapy including docetaxel (Matsunaga et al., 2016), abiraterone (Liu et al., 2017), and doxorubicin (Heibein et al., 2012). Despite its usual role in sex hormone metabolism (Penning et al., 2000), AKR1C3 is also a known anti-oxidant-response gene transcriptionally regulated by Nrf2 (Chen et al., 2017), a transcription factor known to be modulated by metformin (Truong Do et al., 2014). Additionally, AKR1C3 up-regulation in cancer cells has been shown to confer radiation resistance by decreasing cytotoxic levels of reactive oxygen species (ROS) (Xiong et al., 2014). This was of interest to me since metformin is known to modulate oxidative stress in triple-negative breast cancer cells as part of its cancer-targeting mechanism (Marinello et al., 2016). Finally, it was found that AKR1C3 is one of the most up-regulated transcripts in metformin-refractory luminal A breast cancer (MCF-7) cells (Oliveras-Ferraros et al., 2014); together, these observations suggested that further investigation into the role of AKR1C3 in metformin response was warranted. Specifically, AKR1C3 may be a metformin resistance marker, in which case knock-down of the gene was hypothesized to induce metformin sensitivity.

AKR1C3 was stably knocked down in MDA-MB-231 cells using 3 separate shRNA clones (Figure 3.1.11, panel A). Messenger RNA transcript levels of AKR1C3 were reduced by 4-fold, 5-fold, and 9-fold in shAKR1C3_1, shAKR1C3_2, and shAKR1C3_3, respectively (Figure 3.1.11, panel B). Using densitometry analysis of Western blots, it was determined that AKR1C3 protein levels were reduced to 30-35% that of expression in control cells (Figure 3.1.11, panels C and D).

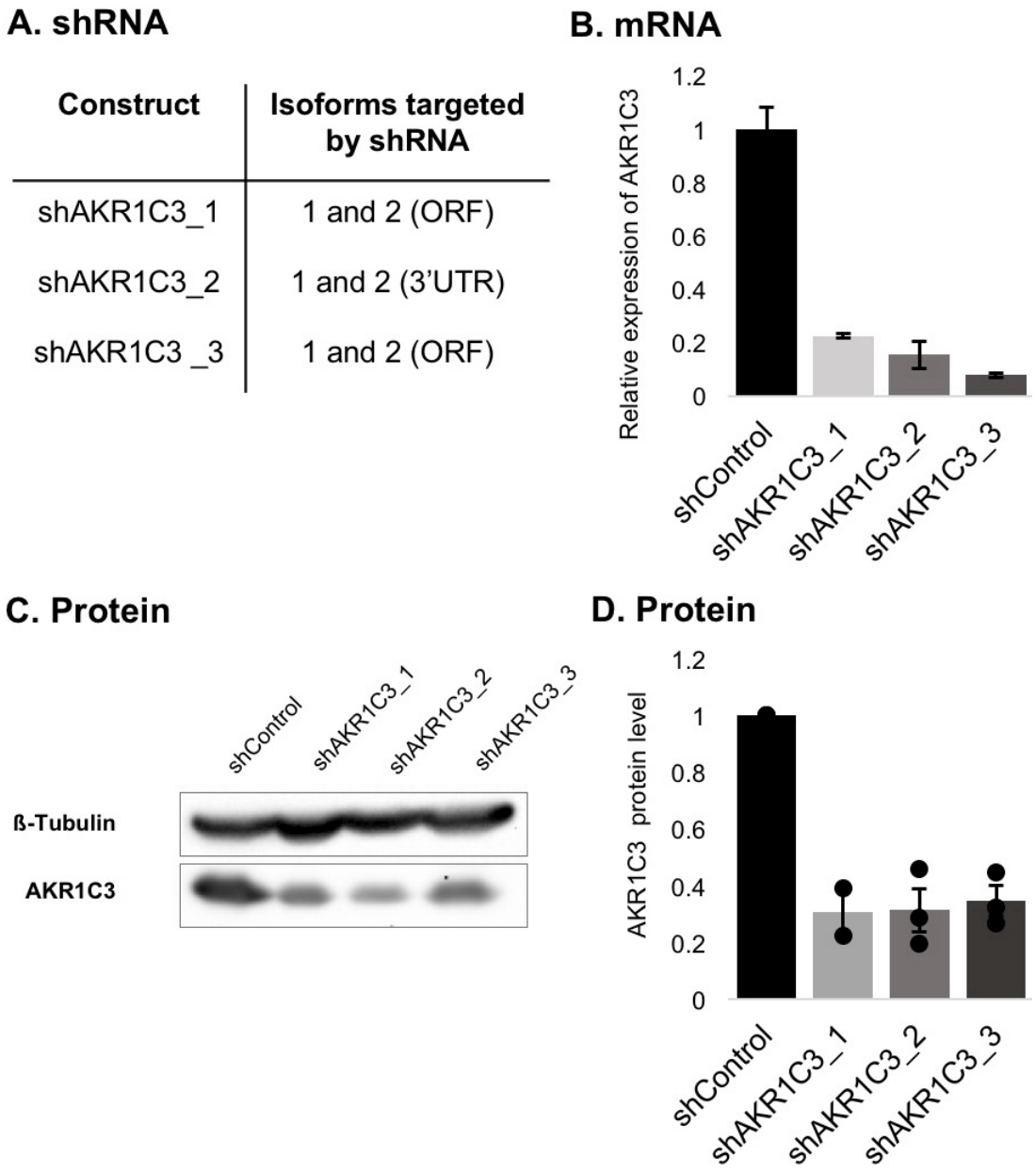


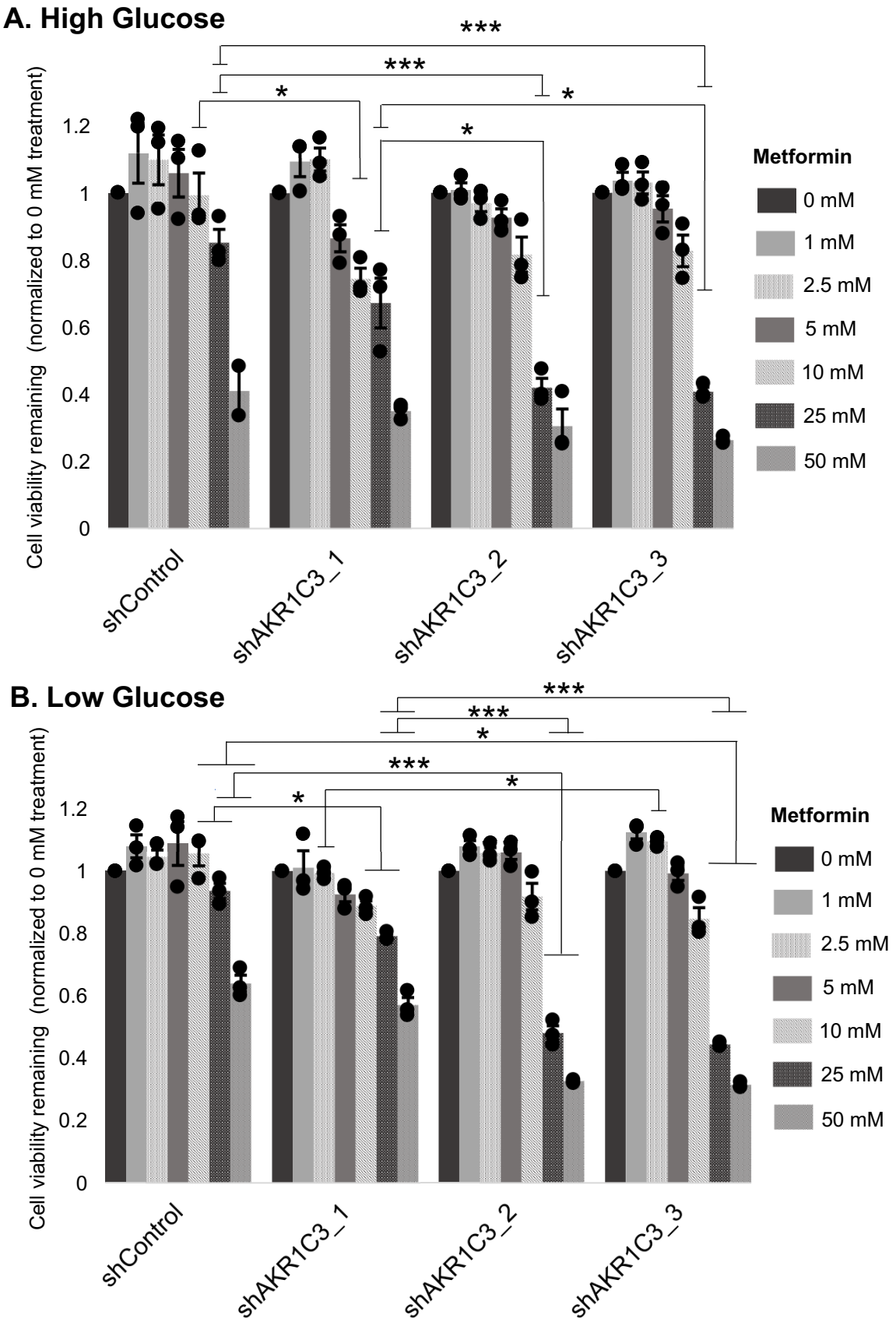

**Figure 3.1.11 Characterization of AKR1C3 depletion and generation of stable knock-down MDA-MB-231 cells. A.** Three separate pGIPZ-based shRNA constructs were used to knock down AKR1C3 in MDA-MB-231 cells via lentivirus delivery. All three shRNAs targeted the two main functional isoforms of AKR1C3. **B.** Messenger RNA transcript levels were shown to be reduced effectively in all three AKR1C3 shRNA-bearing cell lines via quantitative RT-PCR. $\Delta\Delta C_q$ values are shown normalized to control shRNA-bearing cells. N=1, error bars = SEM (4 technical replicates). **C.** AKR1C3 protein levels were shown significantly reduced by Western blot. Protein levels were quantified (**D.**) using densitometry. Individual means are indicated by black dots. N=3, error bars = SEM.

Under high-glucose (25 mM glucose) media conditions, AKR1C3 knock-down significantly increased metformin sensitivity of MDA-MB-231 cells at specific doses, an effect that was dependent on the shRNA construct used and the degree of gene knock-down (Figure 3.1.12, panel A). One shRNA (shAKR1C3_1) resulted in a 25% loss of cell viability at 10 mM metformin, a significant increase in sensitivity compared with the non-targeting control shRNA (no loss of viability). Cells targeted with the remaining two shRNAs, shAKR1C3_2 and shAKR1C3_3, were significantly more sensitive to 25 mM metformin compared with control cells, with a near 4-fold increase in metformin sensitivity at that dose.

Under low-glucose (5 mM glucose) conditions, sensitization effects with AKR1C3 knock-down were seen (Figure 3.1.12, panel B) like those under high-glucose conditions. Depending on the shRNA used, cells became significantly more sensitive to metformin at 10 mM, 25 mM, and/or 50 mM doses. The most pronounced effect occurred when using shAKR1C3_3, which resulted in an 8-fold increase in metformin sensitivity at 25 mM dosing, and a 2-fold increase in sensitivity at 50 mM (Figure 3.1.12, panel B).

Under glucose-deprived conditions (trace glucose + 4.5 mM galactose), AKR1C3 knockdown with shAKR1C3_1 had no effect on metformin sensitivity, while shAKR1C3_2 and shAKR1C3_3 resulted in the opposite effect as seen under high- and low-glucose conditions (Figure 3.1.12, panel C). Cells carrying the shAKR1C3_2 construct were significantly more resistant to metformin at doses of 2.5 mM, 5 mM, and 10 mM. Similarly, AKR1C3 knock-down with the shAKR1C3_3 shRNA resulted in increased metformin resistance at 2.5 mM, 5 mM, 10 mM, and 25 mM. These effects

indicate that the effects of AKR1C3 knock-down on metformin sensitivity *in vitro* are dependent upon glucose availability.


A. High Glucose
Cell viability remaining (normalized to 0 mM treatment)
0
0.2
0.4
0.6
0.8
1
1.2
***
***
*
*
*
shControl
shAKR1C3_1
shAKR1C3_2
shAKR1C3_3
Metformin
0 mM
1 mM
2.5 mM
5 mM
10 mM
25 mM
50 mM
B. Low Glucose
Cell viability remaining (normalized to 0 mM treatment)
0
0.2
0.4
0.6
0.8
1
1.2
***
***
*
***
*
*
shControl
shAKR1C3_1
shAKR1C3_2
shAKR1C3_3
Metformin
0 mM
1 mM
2.5 mM
5 mM
10 mM
25 mM
50 mM

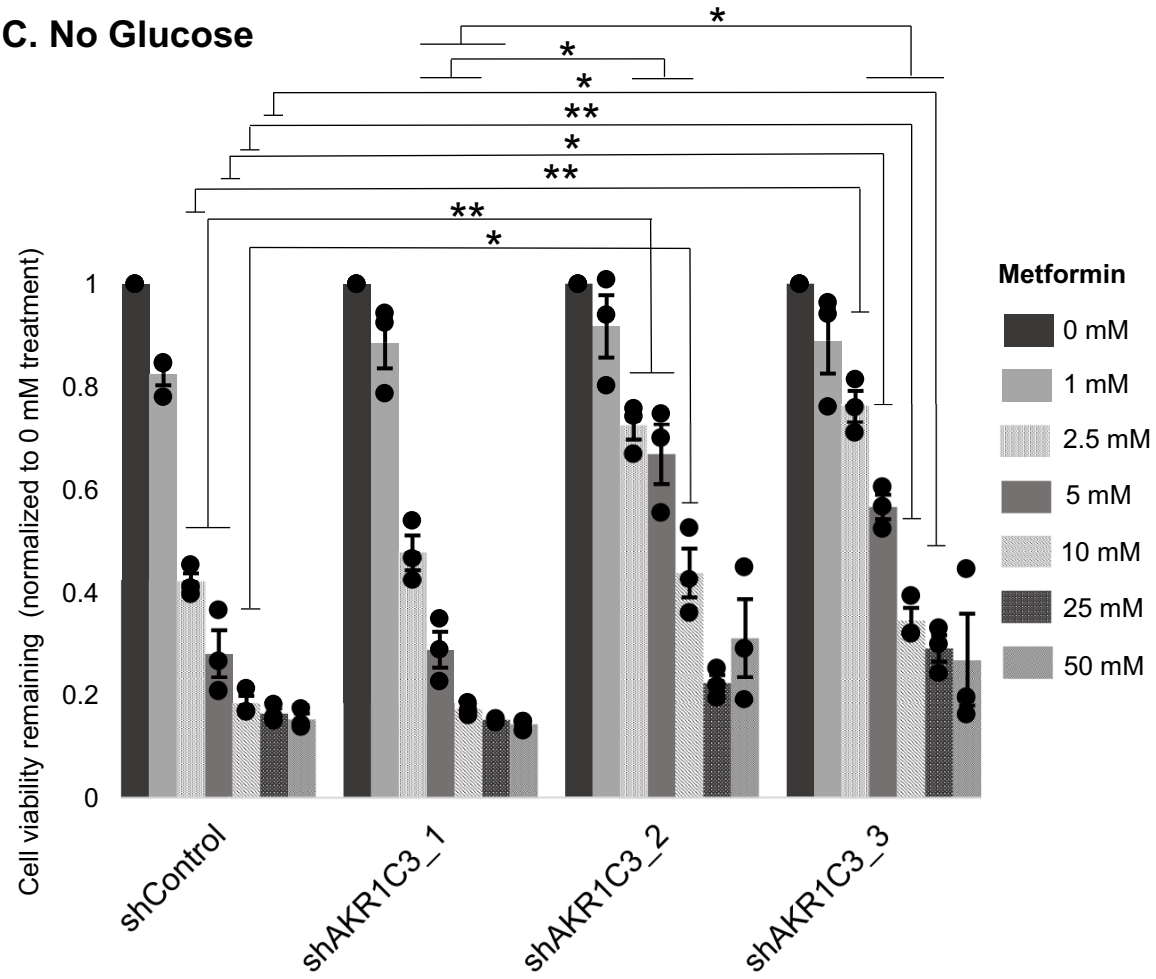



**Figure 3.1.12 Effects of AKR1C3 depletion on metformin sensitivity under various energy conditions. A.** Under high-glucose conditions, AKR1C3 knock-down increases metformin sensitivity at 10 mM and 25 mM doses, with some variability between the shRNA constructs used. **B.** Under low-glucose conditions, AKR1C3 knock-down further sensitizes cells to high doses of metformin (10, 25, and 50 mM), which was largely consistent between the three knock-down populations. **C.** Under no-glucose conditions, metformin sensitivity is decreased for multiple doses (2.5-25 mM) in AKR1C3-knock-down cells carrying shAKR1C3_2 and shAKR1C3_3 constructs. For all experiments, cells were treated with indicated doses of metformin for 72 hr and cell viability was measured using an alamarBlue assay. Values are given normalized to 0 mM control for each cell line. Individual means are indicated by black dots. N=3 biological replicates, error bars = SEM. Significance determined using ANOVA with Bonferroni correction. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

## 3.2 AKR1C3 cDNA expression and metformin sensitivity

### *3.2.1 Effect of AKR1C3 over-expression on metformin sensitivity in a triple-negative breast cancer model*

Analysis of breast cancer gene expression studies from the The Cancer Genome Atlas (TCGA) using the cBioPortal platform revealed that AKR1C3 is over-expressed in 7-11% of basal-like breast cancers, which are enriched in the triple-negative breast cancer phenotype (Cerami et al., 2012; Gao et al., 2013). Therefore, we sought to determine if increased expression of AKR1C3 might confer resistance to metformin in MDA-MB-231 triple-negative breast cancer cells. AKR1C3 was over-expressed as a green fluorescent protein (GFP)-AKR1C3 fusion peptide through transient transfection with pClover-AKR1C3 expression vector (Figure 3.2.1, Panels A, B, E). Clover is a monomeric version of GFP that is brighter than original green fluorescent protein (Lam et al., 2012), allowing for easy tracking of transfected cells by fluorescence microscopy while still ensuring proper folding of AKR1C3 protein. Clover expression indicated a high level of transfection efficiency (Figure 3.2.1, Panels C and D) with AKR1C3 being expressed approximately 49-fold (± 20, SEM) over basal expression in MDA-MB-231 cells.

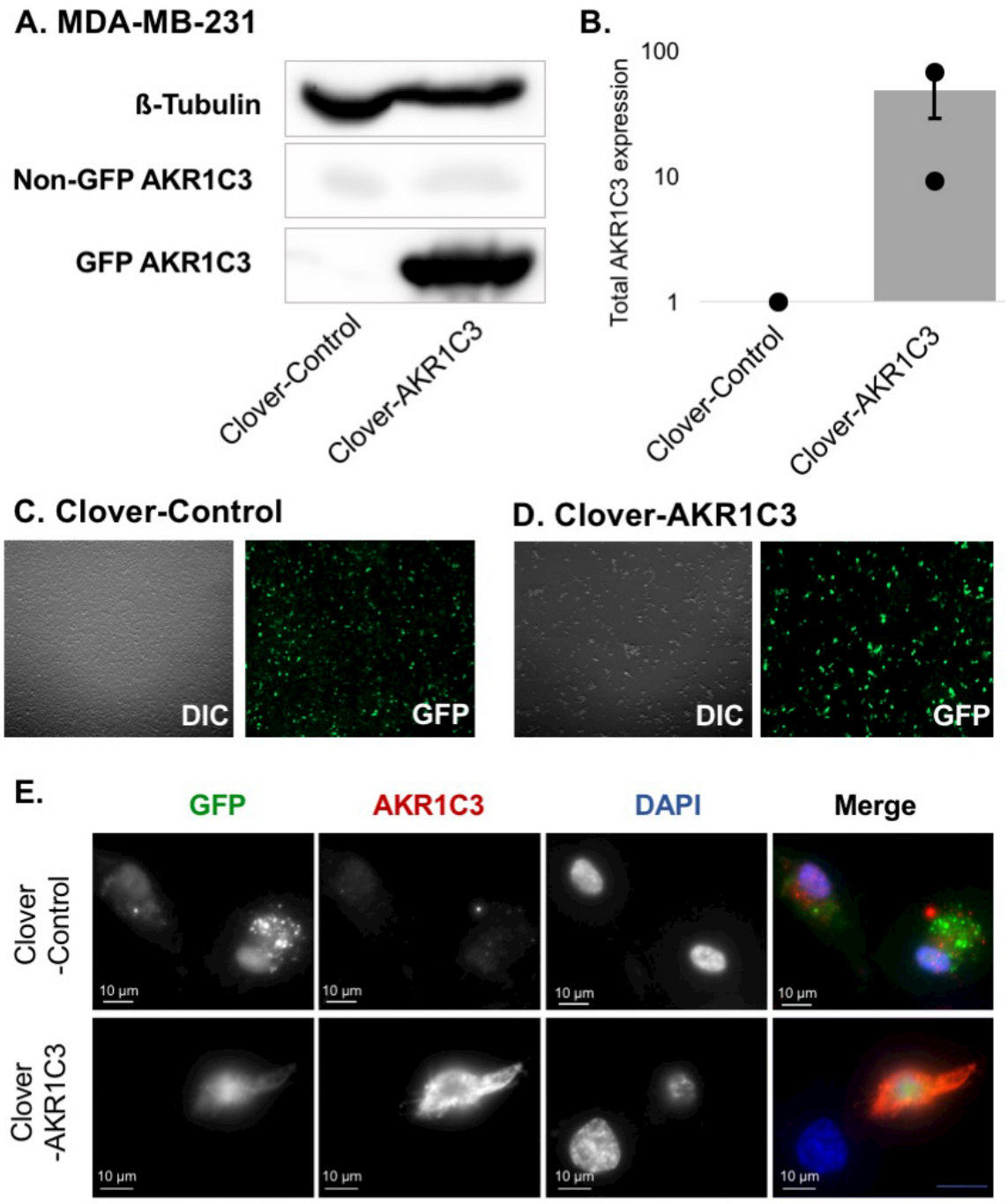
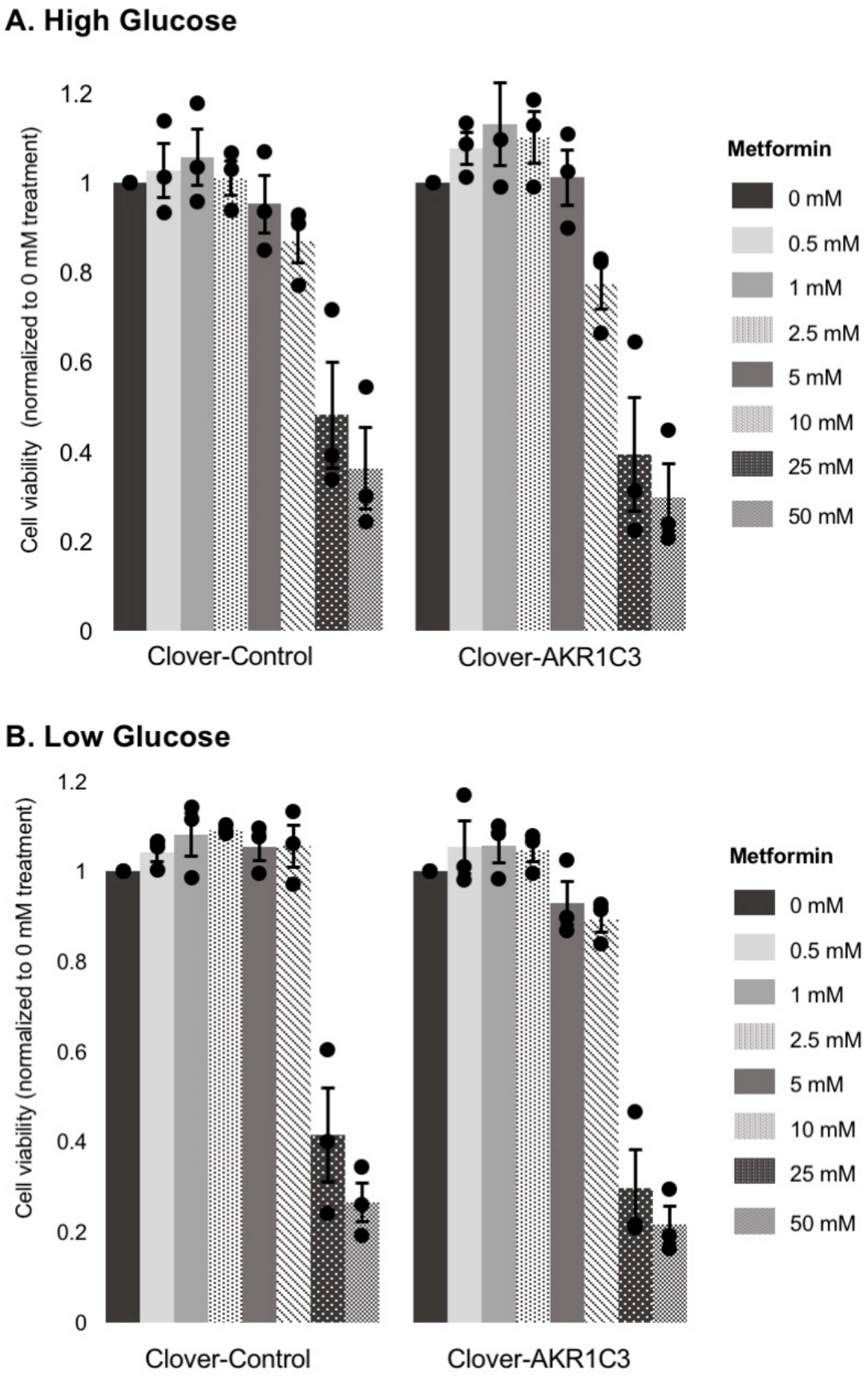



**Figure 3.2.1 Characterization of AKR1C3 over-expression and transfection efficiency in MDA-MB-231 triple-negative breast cancer cells.** Cells were transiently transfected with a pClover (EGFP)-AKR1C3 and empty pClover vector via electroporation. **A.** Western blot analysis of pooled cells 48 hours post-transfection showed significant AKR1C3 over-expression with Clover-AKR1C3 vector compared to control. **B.** Quantification of Western blots showed an average 49-fold (± 20) up-regulation of AKR1C3 with Clover-AKR1C3 vector. Individual means are indicated by

black dots. N=3, error bars = SEM. Contrast white light (differential interference contrast, DIC) and green fluorescent protein (GFP) microscopy of cells transfected with Clover-Control (**C.**) and Clover-AKR1C3 **(D.**) shows high transfection efficiency. **E.** Immunofluorescence imaging of Clover-Control and Clover-AKR1C3 transfected MDA-MB-231 cells (GFP-positive, green) shows concentrated expression of AKR1C3 (red) in Clover-AKR1C3-bearing cells, but not in Clover-Control cells. Cells were grown on coverslips for 48 hr, fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton-X, and stained with DAPI (blue), rabbit-anti-AKR1C3 primary antibody, and fluorescent goat-anti-rabbit secondary antibody (red).

AKR1C3 over-expression significantly altered metformin sensitivity in triple-negative breast cancer cells in glucose-deprived (0 mM + 4.5 mM galactose) conditions, but not under high glucose (25 mM) or low glucose (5 mM) conditions (Figure 3.2.2) at the p=0.05 significance level. Under glucose-deprived conditions (Figure 3.2.2, panel C), AKR1C3 over-expression made cells highly resistant to 0.5 mM, 1 mM and 2.5 mM metformin (normalized cell viability: 1.06 ± 0.07, 1.08 ± 0.07, and 0.93 ± 0.02, respectively), compared with control cells which exhibited 18% loss of viability (0.82 ± 0.09) at 0.5 mM metformin, 24% loss of viability (0.76 ± 0.08) at 1 mM, and 43% loss of viability (0.57 ± 0.05) at 2.5 mM metformin. Similar trends were seen at higher metformin doses, although they were not found to be statistically significant. These results indicate that the effects of AKR1C3 over-expression on metformin sensitivity *in vitro* depend on glucose availability and media conditions.


A. High Glucose
Cell viability (normalized to 0 mM treatment)
1.2
1
0.8
0.6
0.4
0.2
0
Clover-Control
Clover-AKR1C3
Metformin
0 mM
0.5 mM
1 mM
2.5 mM
5 mM
10 mM
25 mM
50 mM
B. Low Glucose
Cell viability (normalized to 0 mM treatment)
1.2
1
0.8
0.6
0.4
0.2
0
Clover-Control
Clover-AKR1C3
Metformin
0 mM
0.5 mM
1 mM
2.5 mM
5 mM
10 mM
25 mM
50 mM

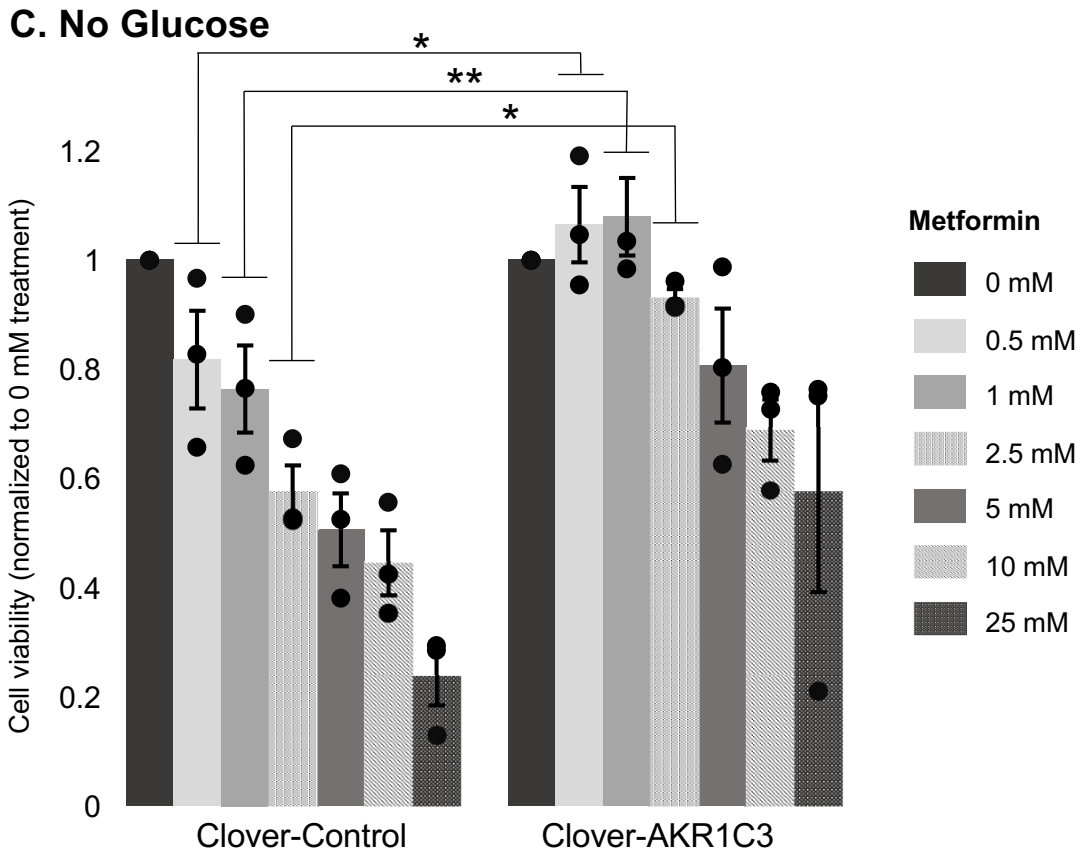



**Figure 3.2.2 Effects of AKR1C3 over-expression on metformin sensitivity of triple-negative breast cancer cells under various glucose conditions.** MDA-MB-231 cells were transiently transfected with pClover (GFP) and AKR1C3 cDNA-containing pClover-AKR1C3 constructs using electroporation. 24 hr post-transfection, cells were treated with varying doses of metformin and remaining cell viability was measured after 72 hr using an alamarBlue assay. Cell viability is normalized to untreated control (0 mM) of each experimental group. Under both high glucose (25 mM) and low glucose (5.5 mM) conditions, shown in panel **A.** and **B.** respectively, AKR1C3 cDNA expression showed no significant effect on metformin sensitivity. **C.** Under glucose deprivation conditions (supplemented with 4.5 mM galactose), AKR1C3 over-expression significantly increased metformin resistance at 1, 10, and 25 mM, with trends towards significance at all other doses. Individual means are indicated by black dots. N=3 for all experiments, error bars = SEM. Significance tested using two-way ANOVA with repeated measures and Bonferroni correction. * $p<0.05$, ** $p<0.01$.

*3.2.2 Luminal A (ER+/PR+) breast cancer model*

To assess if the effects of AKR1C3 over-expression on metformin sensitivity are universal to other subtypes of breast cancer, AKR1C3 protein was also over-expressed in luminal A (ER+/PR+) MCF-7 cells. AKR1C3 was over-expressed in these cells by an average of 91-fold (± 42, SEM) compared with basal expression in the form of a GFP-AKR1C3 fusion peptide, produced from transient transfection with pClover-AKR1C3 construct (Figure 3.2.3, Panels A, B, C).

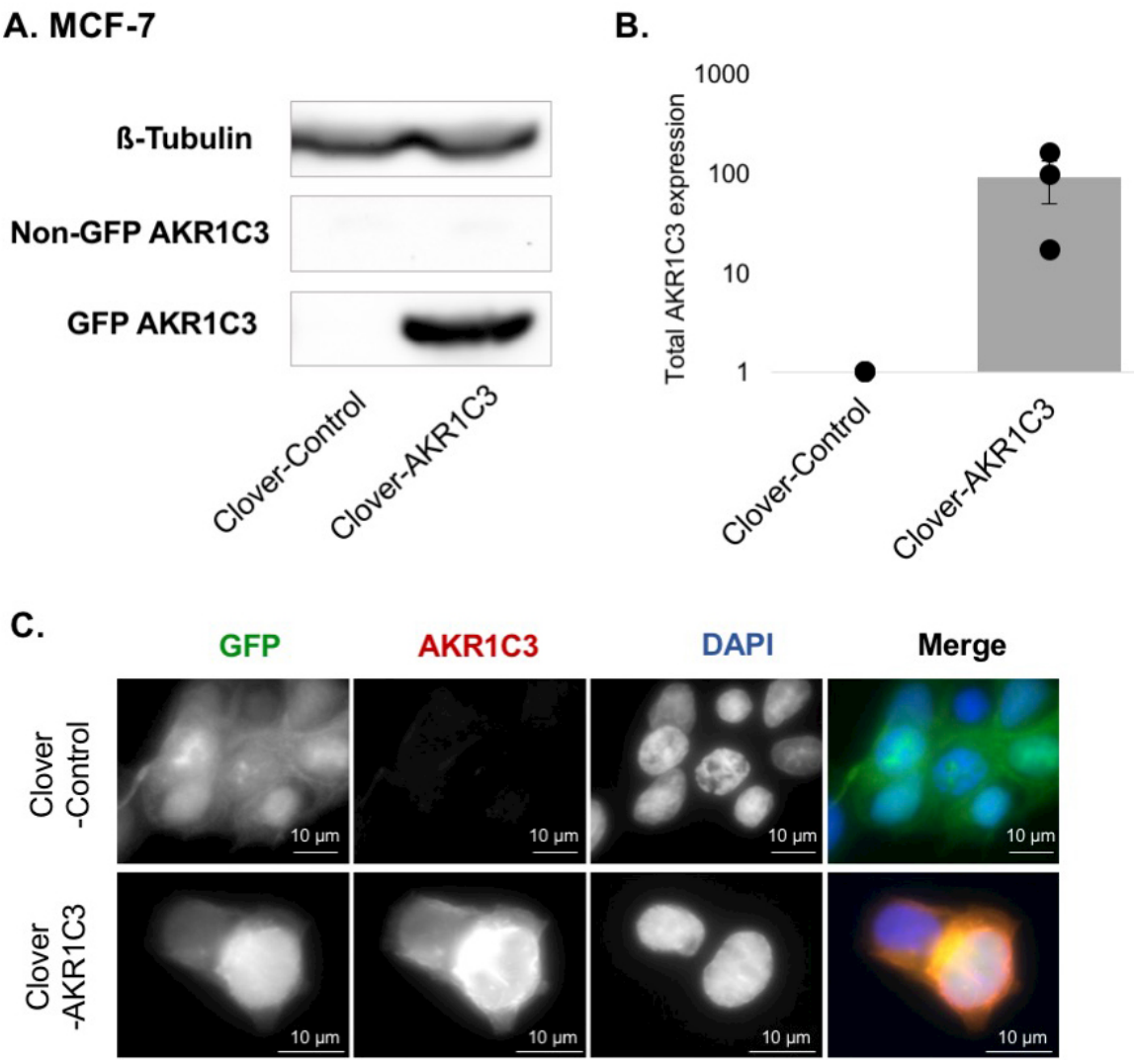



**Figure 3.2.3 Characterization of AKR1C3 over-expression in MCF-7 luminal A breast cancer cells.** Cells were transiently transfected with a pClover (EGFP)-AKR1C3 and empty pClover vector via electroporation. **A.** Western blot analysis of pooled cells 48 hr post-transfection showed significant AKR1C3 over-expression with Clover-AKR1C3 vector compared to control. **B.** Quantification of Western blots showed an average 91-fold (± 42) up-regulation of AKR1C3 with Clover-AKR1C3 vector. Individual means are indicated by black dots. N=3, error bars = SEM. **C.** Immunofluorescence imaging of Clover-Control and Clover-AKR1C3 transfected MCF-7 cells (GFP-positive, green) shows concentrated expression of AKR1C3 (red) in Clover-AKR1C3-bearing cells, but not in Clover-Control cells. Cells were grown on coverslips for 48 hr, fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton-X, and stained with DAPI (blue), rabbit-anti-AKR1C3 primary antibody, and fluorescent goat-anti-rabbit secondary antibody (red).

In contrast with MDA-MB-231 models, AKR1C3 over-expression in MCF-7 luminal A breast cancer cells did not significantly alter metformin sensitivity under either high glucose (25 mM) or glucose-deprived (0mM + 4.5 mM galactose) conditions (Figure 3.2.4). A trend was observed of a ~20% increase in metformin sensitivity at 2.5 mM and 5 mM doses under no-glucose conditions (Figure 3.2.4, panel C), but these were found to not be significant at p=0.05 significance level. These results suggest that AKR1C3 over-expression affects metformin sensitivity predominantly in TNBC models.

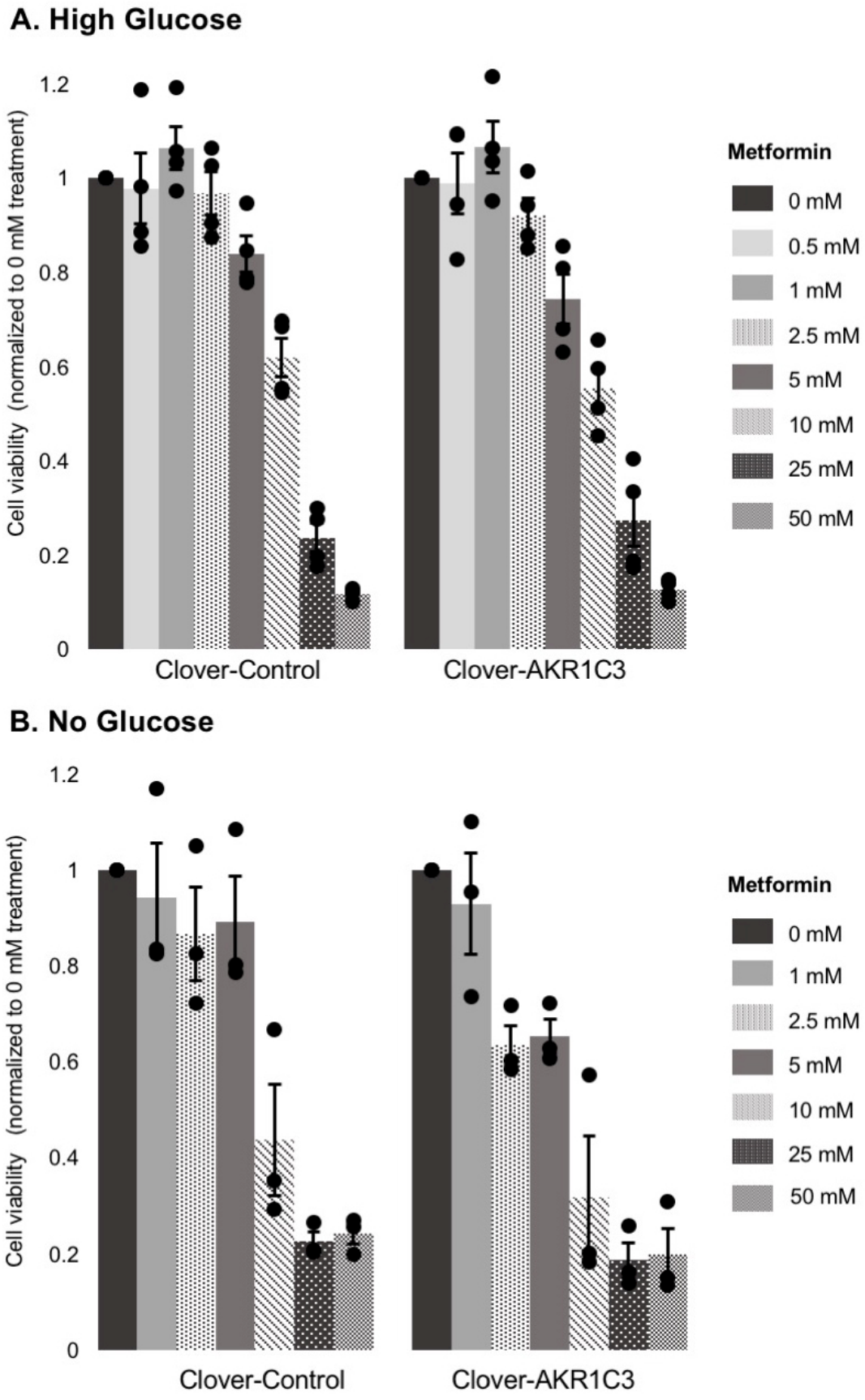

A. High Glucose
Cell viability (normalized to 0 mM treatment)
1.2
1
0.8
0.6
0.4
0.2
0
Clover-Control
Clover-AKR1C3
Metformin
0 mM
0.5 mM
1 mM
2.5 mM
5 mM
10 mM
25 mM
50 mM
B. No Glucose
Cell viability (normalized to 0 mM treatment)
1.2
1
0.8
0.6
0.4
0.2
0
Clover-Control
Clover-AKR1C3
Metformin
0 mM
1 mM
2.5 mM
5 mM
10 mM
25 mM
50 mM

**Figure 3.2.4 Effects of AKR1C3 over-expression on metformin sensitivity of MCF-7 luminal A breast cancer cells under various energy conditions.** ER+ MCF-7 cells were transiently transfected with pClover (EGFP) and AKR1C3 cDNA-containing pClover-AKR1C3 constructs using electroporation. 24 hr post-transfection, cells were treated with varying doses of metformin and remaining cell viability was measured after 72 hr using an alamarBlue assay. Cell viability is normalized to untreated control (0 mM) of each experimental group. Under both high glucose (25 mM) and no glucose (+ 4.5 mM galactose) conditions, shown in panel A. and B. respectively, AKR1C3 cDNA expression did not significantly change metformin sensitivity. Individual means are indicated by black dots. N=4 and n=3 for high and no glucose experiments, respectively. Error bars = SEM. Significance tested at $p<0.05$ using a two-way ANOVA with repeated measures and Bonferroni correction.

### 3.3 AKR1C3 as a metformin response marker

Since AKR1C3 was found to be up-regulated with long-term metformin conditioning in our MDA-MB-231 cells, I chose to investigate if short-term metformin treatment also affected AKR1C3 expression. I observed that acute (72 hr) metformin exposure increased AKR1C3 protein levels in MDA-MB-231 cells in a dose-dependent manner (Figure 3.3). AKR1C3 was up-regulated 1.3-fold (± 0.3) at 1 mM metformin, 1.6-fold (± 0.2) at 5 mM, and 4-fold (± 2.5) at 25 mM metformin, indicating that AKR1C3 is a metformin response marker in triple-negative breast cancer.

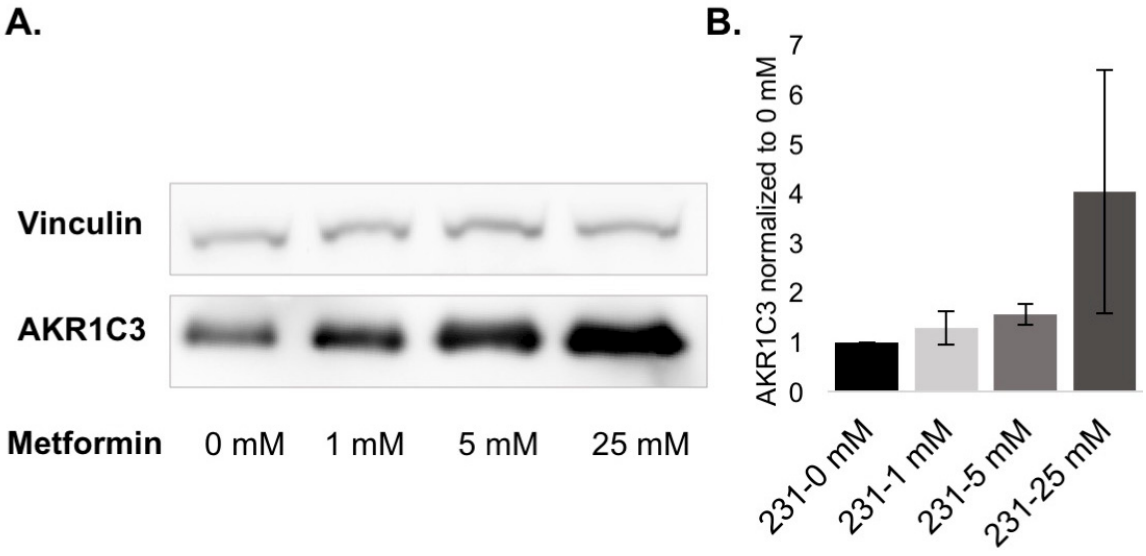



**Figure 3.3 Evaluating AKR1C3 as an acute metformin response marker in triple-negative breast cancer cells.** MDA-MB-231 cells were treated with indicated doses of metformin for 72 hr. **A.** Immunoblotting of AKR1C3 protein showed up-regulated with metformin treatment in a dose-dependent manner. Vinculin was used as a reference for total sample protein. **B.** Levels of AKR1C3 were quantified using densitometry (Image Lab), adjusted for background, and normalized to vinculin reference. N=3, error bars = SEM.

## 3.4 AKR1C3 as a predictive biomarker

### *3.4.1 Clinical data and genomic profiling*

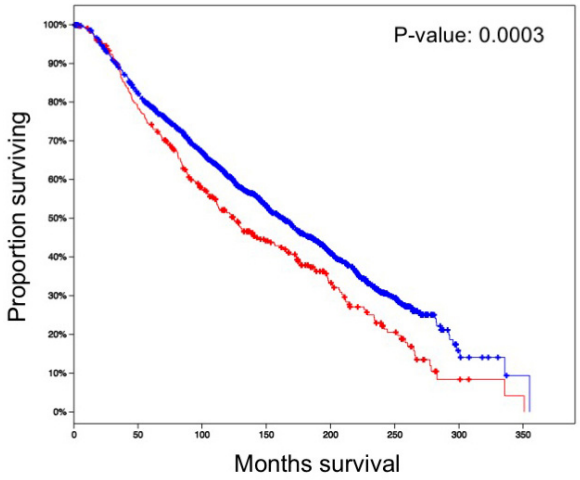
Survival analyses and profiling of AKR1C3 expression in a large cohort of breast cancer patients (METABRIC – Curtis et al. 2012, Pereira et al. 2016) with various tumour subtypes showed that AKR1C3 transcript levels may help predict survival outcome (Figure 3.4.1). The cBioPortal platform was used to create Kaplan-Meyer curves (Cerami et al., 2012; Gao et al., 2013).

Down-regulation of AKR1C3 expression was defined as a mRNA z-score smaller than the value that is 1 standard deviation **below** the mean z-score of the cohort. Cancers with down-regulated AKR1C3 expression were associated with a significantly worse survival outcome for patients (p=0.0003 – Figure 3.4.1, panel A). The median survival in cases of down-regulated AKR1C3 expression was 124.8 months, compared with 163.1 months for those patients whose cancers were not AKR1C3-down-regulated.

Conversely, up-regulation of AKR1C3 expression resulted in a significantly better survival outcome for breast cancer patients (p=0.0081, Figure 3.4.1, panel B). Up-regulation was defined as an AKR1C3 mRNA z-score larger than the value that is 0.75 standard deviations **above** the mean for the cohort. Patients with cancers that were up-regulated for AKR1C3 had a median survival of 185.7 months, whereas patients with no up-regulation had a median survival of 148.8 months.

Collectively, these results indicate that AKR1C3 expression in breast cancer may help predict survival outcomes, with high expression being associated with better outcomes and low expression being indicative of worse outcomes.

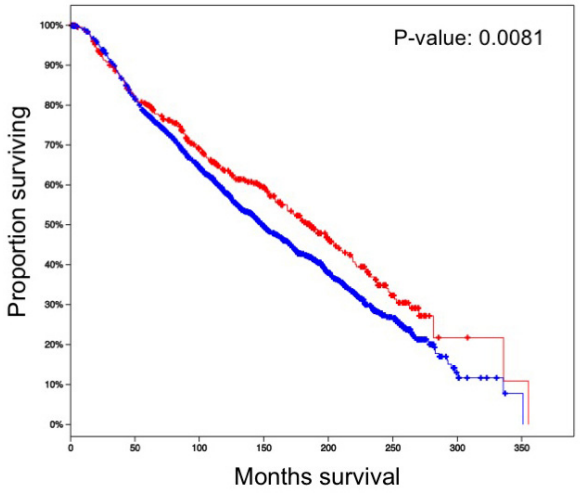
**A. AKR1C3 Downregulation**




AKR1C3 expression < (1 SD below the mean)
AKR1C3 expression ≥ (1 SD below the mean)

| | AKR1C3 < (mean- 1 SD) | AKR1C3 ≥ (mean- 1 SD) |
|---|---|---|
| Total # cases | 331 | 1649 |
| Cases # deceased | 226 | 917 |
| Median months survival | 124.8 | 163.1 |

**B. AKR1C3 Upregulation**




AKR1C3 expression > (0.75 SD above the mean)
AKR1C3 expression ≤ (0.75 SD above the mean)

| | AKR1C3 > (mean+ 0.75 SD) | AKR1C3 ≤ (mean+ 0.75 SD) |
|---|---|---|
| Total # cases | 430 | 1550 |
| Cases # deceased | 218 | 925 |
| Median months survival | 185.7 | 148.8 |

**Figure 3.4.1 AKR1C3 expression and survival statistics from a breast cancer genomics data set.** Clinical data and AKR1C3 mRNA expression values were obtained from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset as published in Nature (Curtis et al., 2012) and Nature Communications (Pereira et al., 2016) and analyzed using the cBioPortal platform for Cancer Genomics (Cerami et al., 2012; Gao et al., 2013). Analyses included all profiled samples (all breast cancer subtypes). **A.** AKR1C3 down-regulation was assigned as an expression value of 1 standard deviation (SD) or more below the mean expression of the reference population (mRNA z-score). AKR1C3 down-regulation was associated with significantly poorer median survival for the breast cancer patients profiled. **B.** AKR1C3 up-regulation was assigned as an expression value of 0.75 standard deviations (SD) or more above the mean expression of the reference population (mRNA z-score). AKR1C3 up-regulation was associated with significantly better median survival for the breast cancer patients assessed.

*3.4.2 In vitro modeling*

Since AKR1C3 may be a predictive survival prognostic marker in breast cancer, I wished to test if basal (pre-metformin) levels of AKR1C3 are also predictive of metformin sensitivity. To do this, I profiled a panel of 13 breast cancer cell lines for metformin sensitivity (Figure 3.4.2) and basal AKR1C3 protein expression (Figure 3.4.3). The panel consisted of 11 triple-negative breast cancer cell lines as well as 2 non-TNBCs (luminal A, ER+/PR+) to test the universality of findings with special emphasis on TNBCs.

It was observed that metformin sensitivity as well as AKR1C3 expression showed significant diversity among cell lines tested. Using rank-order (Spearman's) correlation of metformin resistance to AKR1C3 expression, it was shown that basal AKR1C3 levels are not strongly associated with metformin sensitivity at 1 mM, 5 mM, 10 mM, or 25 mM doses (Figure 3.4.4). An inverse trend (Spearman's correlation coefficient: -0.43) was observed when comparing AKR1C3 levels with cell viability at 25 mM metformin, suggesting that high AKR1C3 expression may be associated with metformin sensitivity, and low expression may be associated with resistance. However, this trend was not statistically significant.

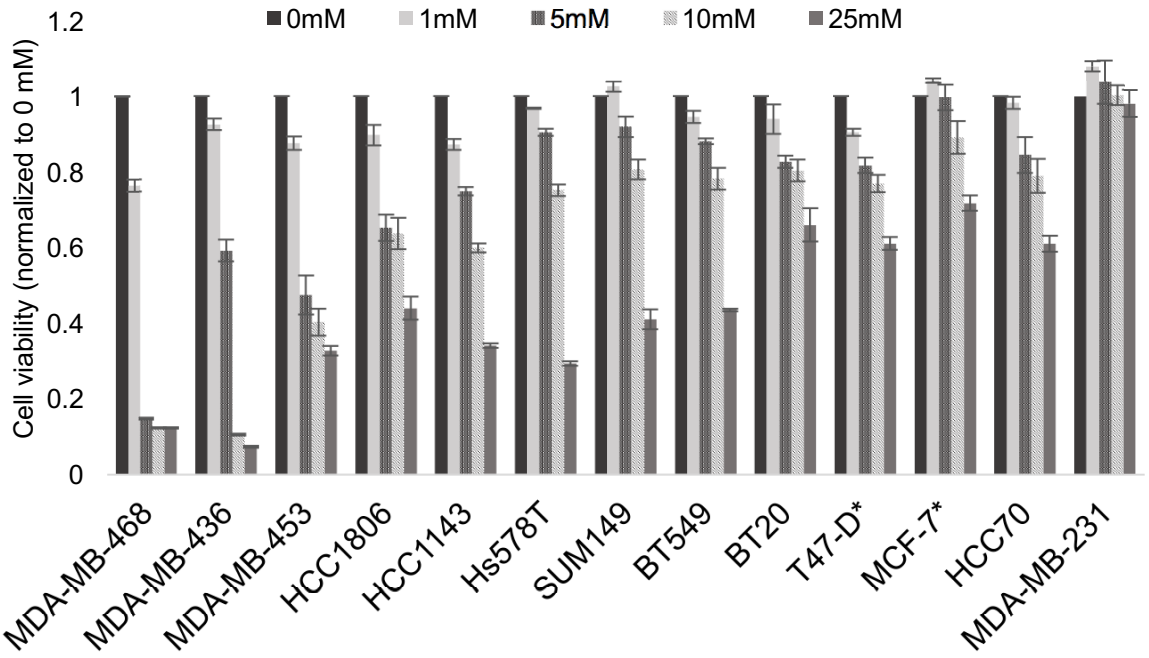



**Figure 3.4.2 Evaluating metformin sensitivity across a panel of 13 breast cancer cell lines of various subtypes.** Metformin sensitivity was screened over a panel of 11 triple-negative breast cancer cell lines of various sub-classifications, as well as 2 non-triple-negative breast cancer models (as indicated with *), MCF-7 and T47-D (ER+/PR+). Cells were treated with indicated dose of metformin under cell line-specific culturing conditions (see Methods Table 2.1) for 72 hr. Cell viability was assessed using alamarBlue and viability normalized to 0 mM treatment for each group. N=3, error bars = SEM.

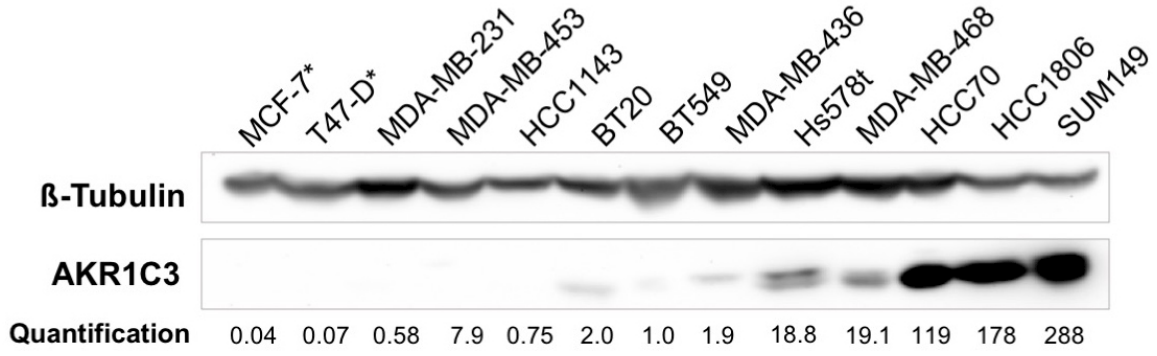



**Figure 3.4.3 Characterizing basal AKR1C3 expression across a panel of 13 breast cancer cell lines of diverse subtypes.** Basal levels of AKR1C3 protein were determined via Western blotting in a panel of 11 triple-negative and 2 non-triple-negative (*, ER+/PR+, MCF-7 and T47-D) breast cancer cell lines. Populations were grown under cell line-specific culturing conditions (see Methods Table 2.1). AKR1C3 protein levels were quantified using densitometry (Image Lab), adjusted for background, and normalized to ß-tubulin reference. Rough values were normalized to BT549 (1.0) for proportional comparison. N=3 for AKR1C3 quantification.

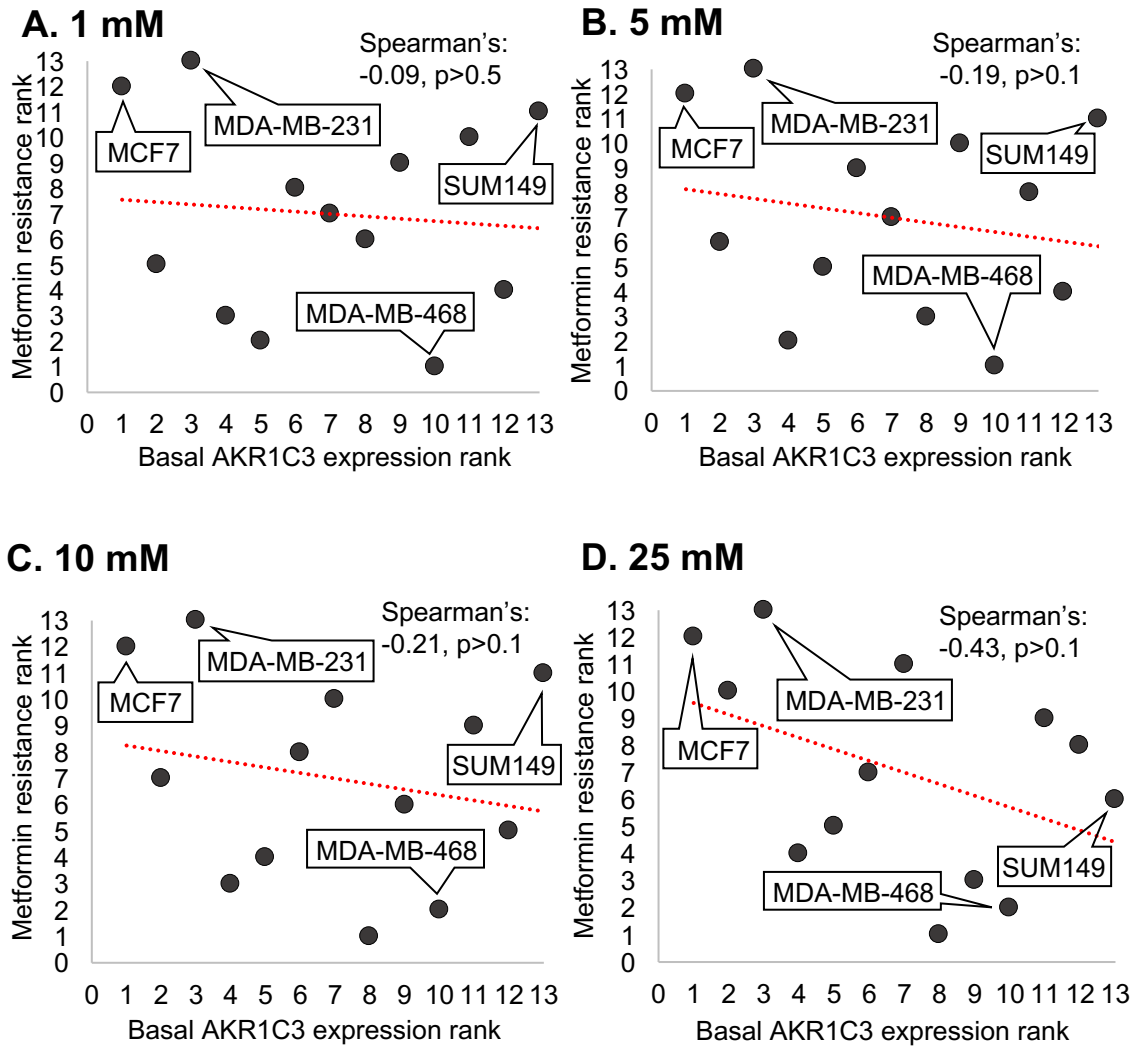



**Figure 3.4.4 Rank-order correlation of basal AKR1C3 expression and metformin sensitivity in a panel of 13 breast cancer cell lines.** The panel contained 11 triple-negative breast cancer cell lines, as well as 2 non-triple-negative (ER+/PR+) models. Cell lines were rank-ordered lowest-to-highest according to their metformin resistance at each indicated dose, with 1 being the most sensitive and 13 being the most resistant (Figure 3.4.2, n=3). Metformin resistance rank was then correlated with basal AKR1C3 protein expression, also ranked lowest-to-highest, with 1 being the lowest expression and 13 being the highest (Figure 3.4.3, n=3). Spearman's rank-order correlation coefficients were calculated and p-values were determined to be >0.05 in all cases – **A.** 1 mM metformin, **B.** 5 mM metformin, **C.** 10 mM metformin, **D.** 25 mM metformin.

### 3.5 Summary

In this study, 11 novel genes have been implicated in modifying metformin response in MDA-MB-231 triple-negative breast cancer cells *in vitro*: AKR1C3, AUP1, CTGF, ENPP4, HMGN5, IGFBP7, MAPRE2, PHGDH, SH3BGRL, SPANXB1, and TTN. Most of the metformin response-modifying effects of these genes depended on glucose availability. Under high glucose conditions, gene depletion sensitized cells to high dose metformin by as much as 53% compared to control, whereas depletion increased metformin resistance by as much as 3-fold under glucose-deprived conditions. Of these novel modifiers of metformin response, AKR1C3 was the most robust modifier of metformin sensitivity in TNBC; knock-down increased metformin sensitivity under high energy conditions and increased resistance under glucose-deprived conditions, whereas over-expression led to increased resistance exclusively in low-energy culturing conditions. The effects of AKR1C3 overexpression on metformin response could not be replicated in luminal A breast cancer cells, suggesting AKR1C3 may predominantly play a role in triple-negative breast cancer. AKR1C3 was also shown to be a novel metformin response marker *in vitro* and was up-regulated with metformin treatment in a dose-dependent manner. AKR1C3 was evaluated as a predictive biomarker for metformin response in breast cancer - an inverse non-significant trend was found between basal AKR1C3 expression and metformin sensitivity at 25 mM. These results suggest that high basal AKR1C3 expression *may* predict sensitivity to metformin, whereas low expression *may* predict resistance, although further investigation is needed to elucidate this trend.

# Chapter 4: Discussion

## 4.1 Identifying novel modifiers of metformin response in triple-negative breast cancer

The re-purposed anti-diabetic drug metformin has been shown to have significant anti-cancer properties *in vitro* and *in vivo*, and has promising potential as a clinical therapeutic for the treatment of breast cancer. One aim of this project was to identify novel genes involved in modifying metformin response in breast cancer with specific focus on triple-negative breast cancer, an aggressive subtype currently lacking targeted therapies. With a better understanding of the mechanistic pathways underlying metformin action, we may better address the instances of metformin resistance frequently seen in clinical studies.

I have characterized 11 novel genes as potential modifiers of metformin response in the triple-negative breast cancer model MDA-MB-231. Ten of these genes (AUP1, CTGF, ENPP4, HMGN5, IGFBP7, MAPRE2, PHGDH, SH3BGRL, SPANXB1, TTN) were evaluated in tandem through shRNA-mediated depletion of expression and showed differential effects. The existing knowledge on the function of these genes in cancer is limited. Only a few (CTGF, ENPP4, and PHGDH) have been previously connected to metformin (Goding, Grobben, & Slegers, 2003; Lu et al., 2015; Wairagu et al., 2015), although none have been implicated in direct modification of metformin response in cancer models.

Knock-down of the 10 genes listed above showed trends towards increasing metformin sensitivity at high doses (10 mM, 25 mM and/or 50 mM) when cultured in high-glucose media. The extent of sensitization under these conditions depended on

which gene was being depleted. In some cases, the sensitization was as much as 53% increased loss of cell viability at a high dose of metformin compared with control (TTN - Figure 3.1.10). However, some of these sensitization effects were reversed under glucose deprivation conditions, and in certain cases gene depletion even appeared to increase metformin resistance. As I will discuss below, the phenomenon of differential metformin response under varying glucose concentrations is well-characterized.

It has been previously established that glucose concentrations greatly impact metformin sensitivity of *in vitro* cancer models. Cells can be completely resistant to metformin in media with supra-physiological glucose but are significantly sensitized when glucose is decreased to plasma-equivalent concentrations (~5 mM) or below, especially when an alternative energy source (such as galactose) is provided (Cheng & Lanza-Jacoby, 2015; Menendez et al., 2012; Wahdan-Alaswad et al., 2013). This is likely due to metformin's combined impact on oxidative phosphorylation and glycolysis. Since metformin uncouples mitochondrial respiration from ATP production (Wheaton et al., 2014), cells must shift their reliance for energy to glycolysis. Metformin also inhibits glycolysis by competing with glucose for the substrate binding site of hexokinase 1 and 2 (HK 1/2), which are normally required to 'trap' glucose in the glycolytic pathway as glucose-6-phosphate (Marini et al., 2013; Salani et al., 2013). Under high-glucose conditions, I speculate that the abundance of glucose out-competes metformin for the HK 1/2 binding sites, allowing the cell to overcome metformin-imposed energy deprivation to continue fueling cellular functions with the products of glycolysis. This effect would be lessened under low-glucose conditions, where the concentrations of glucose are no longer sufficient to overcome the anti-glycolytic effects of metformin. Finally, I speculate that

metformin's anti-neoplastic activity is dramatically amplified in no-glucose/ galactose-supplemented media because of a double insult to glycolysis; in addition to metformin inhibiting HK1/2 from binding trace glucose from serum, it is known that galactose must be processed into glucose-6-phosphate through an alternative pathway that yields no net ATP production from glycolysis (Marroquin et al., 2007). The cell would therefore have an increased reliance on oxidative phosphorylation for energy, making it especially susceptible to metformin treatment. Overall, these glucose-dependent effects should not be disregarded, and should guide further investigation into the mechanistic action of metformin in breast cancer. Additional experimental and statistical limitations of this aim of the study will be discussed in section *4.4.1 (Limitations of experimental replicates and statistical significance)*, with additional detail on proposed future experiments outlined in section *4.5 (Future directions)*.

The gene AKR1C3 was investigated separately and presented the most robust evidence of being a genetic modifier of metformin response, indicating it may be directly involved in metformin's mechanism of action in triple-negative breast cancer. It was shown that AKR1C3 knock-down significantly increased metformin sensitivity with doses as low as 10 mM and as high as 50 mM depending on the quantity of glucose present in the culturing media (Figure 3.1.11). The most profound effect was under low-glucose (5 mM glucose) conditions, where AKR1C3 depletion resulted in an 8-fold increase in metformin sensitivity at 25 mM. As was observed with some of the other genes evaluated in this study, the sensitization effect of AKR1C3 knock-down was reversed under glucose-deprivation conditions, where AKR1C3 depletion made MDA-MB-231 cells *more* resistant to metformin (Figure 3.1.11, panel C).

Unexpectedly, this effect was mirrored in subsequent over-expression studies, where ectopic expression of AKR1C3 *also* significantly increased metformin resistance under glucose-deprived conditions (Figure 3.2.2). However, there are limitations to these over-expression studies that beg caution in their interpretation. First, qualitative observations showed an initial die-back of 25-30% of Clover-AKR1C3-expressing cells during the 24 hr post-transfection recovery period (data not shown); this phenomenon was not seen in Clover-Control cells, and lowering total Clover plasmid concentrations with or without a non-expression vector (to augment total DNA concentration) significantly sacrificed transfection efficiency while still inducing death in about 20-30% of GFP-positive (Clover-AKR1C3-transfected) cells. Secondly, the resistance-inducing effect of AKR1C3 over-expression was specific to the MDA-MB-231 TNBC cell line and was not replicated in luminal A (ER+/PR+) MCF-7 cells (Figure 3.2.4). These findings indicate that the role of AKR1C3 in modifying metformin response may be specific to TNBC, underlining that metformin has subtype-specific anti-cancer effects. Although the precise pathways involved in how AKR1C3 expression modifies metformin sensitivity are yet to be elucidated, I speculate on the mechanistic connection between AKR1C3 and metformin in section **4.2.**

### 4.2 Characterizing AKR1C3 as a novel metformin response marker *in vitro*

A secondary aim of this study was to evaluate genes identified as putative modifiers of metformin sensitivity for their potential applications as metformin response biomarkers. AKR1C3 was shown to be up-regulated with acute (72 hr) metformin treatment in a dose-dependent manner in MDA-MB-231 triple-negative breast cancer

cells (Figure 3.3), confirming it as a metformin response marker *in vitro*. Given that both AKR1C3 depletion and over-expression changed metformin sensitivity in TNBC, the cellular up-regulation of AKR1C3 with metformin treatment likely has a direct mechanistic impact on pathways that alter metformin response. Whether this up-regulation is promoting or hindering cell survival is not yet clear.

One possible hypothesis for these collective observations is that AKR1C3 is being up-regulated by metformin-induced oxidative stress. As discussed in section **4.1,** metformin uncouples mitochondrial respiration from ATP production through inhibition of complex I and increased proton leak (Birsoy et al., 2014; Salani et al., 2013). Thus, endogenous reactive oxygen species (ROS) continue to be produced through mitochondrial respiration although no ATP is being generated. Furthermore, cells are prompted to supplement their energy demands through glycolysis (Cantrell et al., 2010), causing a rapid depletion of glucose and further generation of endogenous oxidative radicals (Song & Lee, 2003). The accumulation of oxidative stress causes the dissociation of Kelch-like ECH-associated protein (KEAP1) from Nrf2 to allow its translocation to the nucleus to up-regulate genes involved in the anti-oxidant response element (ARE)-directed detoxification process (Onken & Driscoll, 2010; Truong Do et al., 2014). AKR1C3 has been shown to be one of these Nrf2-regulated anti-oxidant enzymes (Chen et al., 2017). AKR1C3 is known to reduce reactive aldehyde products of lipid peroxidation, such as 4-hydroxynonenal (4-HNE), into less-reactive alcohols (Matsunaga et al., 2016) in addition to decreasing levels of reactive oxygen species in cancer cells through still unknown mechanisms (Chen et al., 2017; Xiong et al., 2014).

The up-regulation of AKR1C3 could therefore be cyto-protective to a certain threshold level, supporting the observations that AKR1C3 is up-regulated in a dose-dependent manner in MDA-MB-231 cells, that knock-down under high- and low-glucose conditions sensitizes cells further to metformin, and that over-expression via Clover-AKR1C3 increases metformin resistance. Additionally, pilot experiments (data not shown) indicated that breast cancer cell lines with low basal AKR1C3 (Figure 3.4.3) were able to up-regulate AKR1C3 with metformin (similar to MDA-MB-231 cells) and simultaneously displayed comparatively higher metformin resistance.

Beyond a certain threshold of expression, I speculate that AKR1C3 may be harmful to the cell. This may be due to its other cellular functions in hormone metabolism, especially in synthesizing 5-α pregnanes and prostaglandins that are involved in proliferative and apoptotic signalling in breast cancer (Blanco Jr et al., 2017; Chewchuk, Guo, & Parissenti, 2017; Penning et al., 2000). This threshold-effect hypothesis would be consistent with observations that 20-30% of Clover-AKR1C3-transfected cells die within 24 hr (data not shown), indicating they may already have exceeded a manageable level of AKR1C3 expression and leave only cells with below-threshold/ cyto-protective quantities of AKR1C3. Additionally, I observed in pilot experiments that TNBC cell lines with high endogenous AKR1C3 expression (Figure 3.4.3) were the most sensitive to metformin treatment, and failed to up-regulate AKR1C3 beyond basal levels when exposed to metformin (data not shown). These observations may indicate that these cells were already near a threshold of AKR1C3 expression, and that further up-regulation with metformin resulted in pro-death signalling.

It is also important to note that cells under glucose deprivation already generate significant quantities of endogenous reactive oxygen species (Song & Lee, 2003). I speculate that this may be due to increased reliance on oxidative phosphorylation when cells are supplemented with galactose, which yields no net ATP production via glycolysis (Marroquin et al., 2007). Since metformin also uncouples mitochondrial respiration from ATP production through proton leak and complex I inhibition, the cell may be increasing levels of respiration to make up for reduced ATP output, thus augmenting production of endogenous ROS. Under such energy-deprived conditions, increased oxidative stress would likely signal through Nrf2 to increase basal levels of cyto-protective AKR1C3. Metformin treatment may act as a second metabolic and oxidative insult to the stress already imposed by glucose starvation, which may push AKR1C3 over the threshold to induce the significant cell death seen (Figure 3.1.12). Cells with AKR1C3-depletion would thus be kept below the threshold of expression and would be expected to be more resistant to metformin under glucose-deprived conditions, as was seen in Figure 3.1.12, panel C. Future studies to investigate this hypothesis further are outlined in section **4.5**.

Importantly, one must also consider the implications of AKR1C3 up-regulation if metformin is to be combined with other therapies for treating cancer. For example, AKR1C3 is known to reduce doxorubicin into its inactive metabolite, doxorubicinol (Heibein et al., 2012). Metformin and doxorubicin have been proposed as a combination therapy for breast cancer; if metformin up-regulates AKR1C3, it may directly oppose doxorubicin action and render treatment ineffective.

## 4.3 Evaluation of AKR1C3 as a predictive biomarker to metformin response in breast cancer

The final major aim of this study was to evaluate candidate genes for their use as predictive biomarkers for metformin sensitivity. In a panel of 13 breast cancer cell lines, a trend was observed of an inverse correlation between basal AKR1C3 expression and metformin sensitivity at high dosing (Figure 3.4.4). However, this trend was found to not be statistically significant when considering the entire data set. Notably, an inverse correlation between basal expression and metformin resistance would fit into the AKR1C3 expression-threshold model outlined in section **4.3.** Cells that express low AKR1C3 would be able to upregulate it to cyto-protective levels with metformin treatment, making them more resistant, while cells that already have high AKR1C3 levels would be pushed over the up-regulation threshold to induce loss of cell viability. Pilot studies (data not shown) appear to support this hypothesis, indicating that further investigation is merited, as outlined in section **4.5.**

## 4.4 Limitations and pitfalls

### *4.4.1 Limitations of experimental replicates and statistical significance*

In all, 11 genes were evaluated for their effect on metformin sensitivity through shRNA-mediated depletion in MDA-MB-231 cells. Except for AKR1C3, for which 3 biological replicates were completed, only one replicate experiment was conducted for each of these genes. This represents a major statistical weakness in the interpretation of observed trends that suggest these genes may be modifiers of metformin response. Statistical testing could not be done, and the data was confirmed to not be normally

distributed at 25 mM metformin dosing in high-glucose conditions, or for most doses in glucose deprivation conditions (Shapiro-Wilk Normality test, 1965). Findings should therefore be interpreted with caution, and should merely be used to guide future experiments as outlined in section **4.5**.

### *4.4.2 Limitations of in vitro modeling*

Since the experiments outlined in this study were completed exclusively *in vitro*, it cannot be speculated how findings might translate into *in vivo* and pre-clinical settings. As outlined in detail in Chapter 1: section **1.2.5**, *in vitro* models of breast cancer fail to account for various factors affecting tumour growth and metformin response. These elements include: *in vivo* homeostasis of glucose, cytokines, and growth factors, the biological impacts of a 2-dimensional versus 3-dimensional growth matrices (Kenny et al., 2007; Lee et al., 2007), accessory cells in the tumour microenvironment (Vargo-Gogola & Rosen, 2007), and a vast array of immunological considerations (Fantozzi & Christofori, 2006). The studies described here thus only represent a first step in the bigger scope of validating candidate genes as clinical metformin biomarkers or putative genetic modifiers of metformin sensitivity.

### *4.4.3 Limitations of physiological relevance of metformin and glucose concentrations*

*In vitro* studies of metformin in cancer have been repeatedly scrutinized for their use of supra-physiological metformin doses that could never be attainable in a patient (Cantrell et al., 2010; Quinn et al., 2013; Whitburn et al., 2017). The exact definition of what is 'supra'-physiological depends on the hypothesized method of clinical administration. Daily oral dosing in diabetics results in very low plasma concentrations,

approximately 10-20 μM (Dowling et al., 2016, 2012; Erices et al., 2013; Owen, Doran, & Halestrap, 2000). However, intraperitoneal injections of metformin in mice have been shown to increase plasma concentrations to 145 μM without major toxicity (Dowling et al., 2016), and metformin has been predicted to accumulate up to 1000-fold in the mitochondrial matrix (Owen et al., 2000). Interestingly, only about 10-15% of the *in vitro* dose of metformin actually enters cancer cells (Dowling et al., 2016). An *in vitro* concentration of up to 1.4 mM may thus be considered physiologically-relevant with regards to an intra-peritoneal injection model. Even though intra-tumoural injections may bring the physiological concentration even higher, the elevated doses of metformin used in this study still present a significant limitation. It should be noted that foundational studies on the mechanism of action of metformin conducted under supra-physiological conditions still have value; many of the pertinent signalling pathways appear to be amplified with higher metformin dosing, thus making them easier to detect and study for clinical relevance (Oliveras-Ferraros et al., 2014).

The effects of metformin on cancer *in vitro* are also strongly dependent on glucose concentrations (Menendez et al., 2012; Song & Lee, 2003; Wahdan-Alaswad et al., 2013), indicating that *in vitro* experiments should be performed in the most physiologically-relevant energy conditions possible to stand the best chance at *in vivo* translation. It is worth noting that even though many *in vitro* studies define physiological glucose to be ~5 mM, in-depth metabolomics analyses in colon and stomach cancers have shown that intra-tumoural glucose concentrations may in fact be as low as 130-430 μM (Hirayama et al., 2009). The no-glucose media in my experiments contained 10% normal (rather than dialyzed) fetal bovine serum (FBS), which contains an average of 0.85-1.5

mg/mL glucose (Thermo Fisher). Therefore, the final glucose concentration of my 'glucose-deprived' media (476-840 μM) may actually be close to the real intra-tumoural, physiological range. Nonetheless, FBS-supplemented media is also very high in growth-factors and other supra-physiological components, indicating that the conditions of my *in vitro* experiments may not mimic *in vivo* environments for metformin action. It should also be noted that the timespan in which experiments were conducted may have caused depletion of glucose in low-glucose (5 mM) media to the point of glucose deprivation, and may have reduced the trace glucose in galactose-supplemented media down to zero. Most experiments in this study were conducted over a 72 hr period. Using data from numerous sources outlining glucose consumption rates, I have estimated that the volume of low-glucose media added to cultures was exactly sufficient to sustain cellular glucose consumption for 72 hr, while high-glucose media provided 5x excess glucose. It would be advisable to carefully monitor glucose concentrations over the experimental time-course moving forward to control for the confounding variable of fluctuating energy availability, especially when attempting to make judgements on what is the most relevant condition to mimic *in vivo* glucose homeostasis.

## 4.5 Future directions

### *4.5.1 Further evaluation of putative modifiers of metformin response*

Further studies should be performed to confirm the roles of the candidate genes outlined in **4.1** in modifying metformin sensitivity. First, more experimental replicates must be completed to confirm the statistical significance of the glucose-related effects seen with depletion of AUP1, CTGF, ENPP4, HMGN5, IGFBP7, MAPRE2, PHGDH, SH3BGRL, SPANXB1, and TTN in MDA-MB-231 cells. Given that depletion of these

genes may sensitize cells to metformin under certain conditions, it would be advantageous to evaluate if they can be targeted as resistance markers clinically through chemical inhibition. It would also be advisable to test for the effects of over-expression of these candidate genes in MDA-MB-231 cells, as was done for AKR1C3, since it may help further elucidate their functional significance in conferring metformin resistance or sensitivity in target cells.

Secondly, it would be beneficial to test the universality of putative modifiers of metformin response by replicating all depletion and over-expression experiments in other triple-negative breast cancer cell lines, as well as breast cancers of other subtypes, such as luminal A, luminal B, and HER2-enriched. Additionally, it would be advisable to evaluate the effects of gene depletion and over-expression on non-cancerous mammary epithelial models, such as MCF10A cells, to determine if these pathways are unique to cancer.

Thirdly, an important step in validating the mechanistic roles of these genes in modulating metformin sensitivity is to test the effects of depletion or over-expression *in vivo*, most likely through xenotransplantation studies in mice and/or zebrafish. This would possibly be the most beneficial study to evaluate whether these genes are directly involved in the mechanism of action of metformin in cancer. *In vivo* models would account for physiologically-relevant glucose and growth-factor concentrations that were not represented in our *in vitro* studies, and would provide a 3-dimensional framework for tumour growth and microenvironmental interactions.

Lastly, it would be favourable to also evaluate the genes encoding the most *down*-regulated proteins in the Met-231 proteomics analysis (data not shown), as these may

additionally be expected to modulate metformin sensitivity in triple-negative breast cancer models. It is important to note that no investigation was done to determine if the up- and down-regulated proteins in the proteomics data set were functionally linked through common pathways. This may be a valuable avenue for further investigation, as is elucidated with speculation in section *4.5.3* on a common signalling axis dependent on Nrf2.

*4.5.2 Validation of AKR1C3 as a metformin response marker*

Given that AKR1C3 was confirmed as a metformin response marker in MDA-MB-231 cells, it should be assessed for a similar response in other triple-negative breast cancer cells, as well as in other breast cancer subtypes such as luminal A, luminal B, or HER2-enriched. Such studies would determine whether AKR1C3 upregulation in response to metformin treatment is a universal phenomenon or unique to the MDA-MB-231 cell model. One could also use excised patient-derived tumours and treat these with metformin *ex vivo* to assess effects on AKR1C3 expression.

Regardless of the model, it is also important to assess how long AKR1C3 remains up-regulated after metformin exposure. Time-course experiments at both the transcript and protein level would also help elucidate any cycles in expressional regulation of AKR1C3, which would be an essential consideration if AKR1C3 is to be used clinically as a biomarker to monitor metformin response. Given AKR1C3's role in reducing doxorubicin into its inactive metabolite, it should also be investigated if AKR1C3 remains up-regulated in a cancer upon cessation of metformin treatment. If this is the case, metformin-induced up-regulation of AKR1C3 may interfere with the anti-neoplastic activity of doxorubicin in future anti-cancer therapy.

Most importantly, AKR1C3's role as a metformin response marker must be confirmed *in vivo*. An important consideration is the contrast between physiologically-attainable concentrations of metformin versus the supra-physiological dose used in my *in vitro* studies. Considering that a modest increase in AKR1C3 expression was seen in doses as low as 1 mM, there is nonetheless a realistic expectation that AKR1C3 may respond to metformin therapy *in vivo*. One excellent method for confirming *in vivo* relevance would be to use paired core tumour biopsies from breast cancer patients, before and during neoadjuvant metformin therapy (Niraula et al., 2012), to monitor changes in AKR1C3 expression.

### *4.5.3 Mechanistic studies of AKR1C3 and metformin response*

Focused studies should be conducted to determine the exact mechanism(s) underlying metformin's modulation of AKR1C3 expression. If metformin-induced AKR1C3 upregulation is hypothesized to be due to increased levels of oxidative stress, future experiments must include the addition of oxidative radicals (hydroxides, peroxides, or superoxides) or reactive aldehydes (4-HNE) to cell culture to monitor AKR1C3 expression levels. Conversely, the addition of anti-oxidants (such as N-acetyl cysteine [NAC] or glutathione) to cells undergoing metformin treatment may be expected to attenuate metformin cytotoxicity as well as AKR1C3 up-regulation. To complement these studies, a reliable oxidative stress-detection assay (such as CellROX from Thermo Fisher) should be adapted to these experiments to monitor changing levels of ROS and reactive aldehydes.

An accurate time-coursing may also be able to tease apart the sequence of important events regarding metformin mechanism, especially to elucidate if AKR1C3 responds to oxidative stress, and if oxidative stress is changed by AKR1C3. This approach may also assist in evaluating the threshold hypothesis of AKR1C3 expression. If the hypothesis is true, one would expect to see AKR1C3 expression increase to a certain level, upon which the cell begins to display characteristics of cell cycle arrest or early apoptosis. Inducible models of AKR1C3 expression (such as a Tet-inducible vector) may be beneficial to help titrate AKR1C3 levels, whereas fluorescence microscopy, fluorescence-activated cell sorting (FACS) for early apoptosis markers (annexin V), or the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay would help elucidate impacts on cell viability as AKR1C3 levels fluctuate.

Additionally, since AKR1C3 up-regulation in response to metformin was hypothesized to occur in a Nrf2-dependent manner, it would be highly beneficial to evaluate the potential mechanistic connection between metformin, oxidative stress, Nrf2, and Nrf2-regulated genes. Notably, AKR1C3 was not the only Nrf2-regulated gene to be identified in our MDA-MB-231 proteomics study. L-serine is implicated in regulating redox status in the cell, and it is thought that PHGDH is regulated by Nrf2 as part of this process (DeNicola et al., 2015). CTGF, IGFBP7, and TTN expression have also been previously associated with changes in Nrf2 activity, although their mechanistic connections have not yet been elucidated (Hasselbalch et al., 2014; Kalash et al., 2014). Notably, sequestosome 1 (SQSTM1/ p62), which was one of the most down-regulated markers in our proteomics study, is involved in a mechanistic feedback loop with Nrf2 to regulate its dissociation from KEAP1 to promote transcription of anti-oxidant response

pathway genes (Jain et al., 2010). Given these potential connections between Nrf2 and metformin response genes, I speculate that more investigation is merited into a common Nrf2-dependent signalling axis to control metformin-induced oxidative stress in MDA-MB-231 cells. It would be beneficial to investigate the impact of Nrf2 knock-down on the expression of candidate metformin response genes identified in our study, with special focus on changes in metformin sensitivity or intracellular concentrations of ROS. Additionally, fluorescently-tagged endogenous Nrf2 would allow for visualization of Nrf2 nuclear translocation in response to metformin to help confirm the hypothesis of a mechanistic link.

*4.5.4 Further evaluation of AKR1C3 as a predictive biomarker in breast cancer*

Given this study's findings that AKR1C3 expression modulates metformin sensitivity, it may be worth re-examining AKR1C3 as a predictive biomarker under more controlled experimental conditions. Specifically, all cell lines should be evaluated for both metformin sensitivity and basal AKR1C3 expression while cultured in identical media conditions – these should preferably be low-glucose and low-serum, such as minimal essential media (MEM), to maintain physiological relevance. It would also be highly beneficial to expand the number of cell lines profiled to increase statistical power, as well as to control for known confounding variables that may affect metformin activity in cells. These could include the expression levels of genes like organic cation transporters (OCTs), which are responsible for transporting metformin across the cellular membrane and may impact how much metformin is entering the cell (Dowling et al., 2016; Wang et al., 2002). An alternative method to control for differences in metformin transport is to measure intracellular metformin concentrations with a cell number-

controlled mass spectrometry approach, as outlined in Dowling et al. (2016). This would be a highly favourable study as it is not yet clear exactly which OCTs are involved in shuttling metformin into breast cancer cells, representing an unknown that may confound results.

The same logic would also apply if using patient-derived tumour samples to test the clinical relevance of AKR1C3 as a predictive biomarker. Either of two approaches could be utilized: 1) using samples initially collected from metformin non-users for *ex vivo* culturing or xenotransplantation studies evaluating metformin neoadjuvant therapy without patient participation, or 2) collecting tumour biopsies from patients before undergoing direct metformin neoadjuvant therapy to determine if clinical outcome correlates with basal AKR1C3 expression. The latter allows for more robust findings, as treatment conditions can be better controlled and randomized.

### 4.6 Conclusions

Metformin exerts broad anti-neoplastic activity on *in vitro* models of triple-negative breast cancer in a glucose-dependent manner. The mechanism of action behind these observations is highly complex. This study has identified 11 novel putative modifiers of metformin response in MDA-MB-231 TNBC cells: AKR1C3, AUP1, CTGF, ENPP4, HMGN5, IGFBP7, MAPRE2, PHGDH, SH3BGRL, SPANXB1, and TTN. AKR1C3 has also been identified as a novel metformin response marker in triple-negative breast cancer. AKR1C3 is up-regulated in a dose-dependent manner, which may be due to increased levels of metformin-induced oxidative stress. A trend was also observed that suggests AKR1C3 may have value as a predictive biomarker for metformin

response in breast cancer. Overall, much further investigation is needed to validate these findings *in vivo,* and to help further elucidate the molecular pathways connecting the genes profiled in this study. The findings presented in this thesis contribute to the foundational knowledge of metformin mechanism of action in triple-negative breast cancer, and will hopefully be a first step to help optimize metformin anti-cancer therapy for clinical use.

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## Appendix A: AlamarBlue cell viability assay

AlamarBlue (resazurin) is a cell-permeable compound that functions as a fluorogenic redox indicator for growing cells, thus making it ideal to measure cellular metabolic activity as a proxy for cell viability (Nakayama et al., 1997; Nociari et al., 1998). Resazurin is non-fluorescent blue dye that is converted into the highly fluorogenic pink compound resorufin by metabolic enzymes in viable cells (Figure Appendix A). The assay is optimized for use in high-throughput drug screening in 96-well plates, and can be easily read using a plate reader set to 560 nm excitation and 590 nm emission wavelengths.

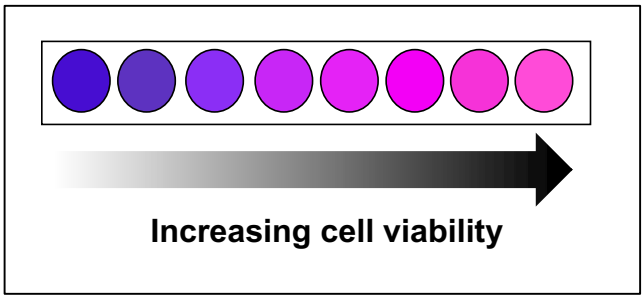


**Figure Appendix A. The conversion of alamarBlue (resazurin) into the highly fluorogenic pink compound resorufin can be used as a reliable measure of cell viability.**

AlamarBlue is considered a favourable alternative to the 3-[4-5-Dimethylthiazol-2-yl]-2-5-diphenyl bromide tetrazolium bromide (MTT) cytotoxicity assay since it is both simpler to use and demonstrates increased sensitivity due to fluorogenic, rather than colorimetric, detection (Hamid et al., 2004; Nociari et al., 1998). Despite metformin's activity on mitochondrial function (possibly impacting metabolic enzymes responsible for converted resazurin to resorufin), alamarBlue assays have been validated for use in

metformin sensitivity screening in previous studies (Klubo-Gwiezdzinska et al., 2012; Zakikhani et al., 2006). The removal of metformin-containing media before addition of alamarBlue reagent further decreases the chance of metabolic interference causing false positive or false negative results.