KAISO'S REGULATION OF MICRORNAS IN TNBC

Kaiso regulates tumor-suppressing *microRNA-31* and *microRNA-200c* in triple negative breast cancer (TNBC) cells

BY

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ABSTRACT

Breast cancer (BC) is a leading cause of death among women worldwide. Despite a recent decline in BC rates due to improved detection and therapeutic options, women diagnosed with the triple negative breast cancer (TNBC) subtype have a poor prognosis relative to other BC subtypes. As TNBCs do not express the estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor-2 (HER2) that are commonly targeted in hormone positive subtypes, they have limited treatment options. While some progress has been made at unraveling the molecular mechanisms behind TNBC, there is still a large knowledge gap regarding how these tumors initiate and progress in such an aggressive manner. Increasingly, the role of microRNA deregulation has been linked to TNBC tumorigenesis. Recently, the transcription factor Kaiso was implicated in the regulation of the pleiotropically acting microRNA-31 (miR-31) and tumor-suppressing microRNA-200c (miR-200c) that promote tumor migration and invasion in prostate cancer. Notably Kaiso is highly expressed in TNBC, relative to other BC subtypes, and high Kaiso expression correlates with poor survival. Herein, we show that Kaiso expression is inversely correlated with miR-31 and miR-200c expression in the TNBC cell lines (MDA-MB-231 and Hs578T). Upon Kaiso depletion, there is an increased expression of these microRNAs and reduced expression of their downstream targets. Misexpression of miR-31 and miR-200c in parental and Kaiso-depleted TNBC cells affects their migratory ability. We also show that Kaiso associates with the promoter regions of both miR-31 and miR-200c. Collectively, these results imply that Kaiso is a regulator of miR-31 and miR-200c, which are important suppressors of epithelial mesenchymal transition (EMT) but are highly downregulated in TNBC. Our findings reveal a novel mechanism for Kaiso in the promotion of TNBC tumorigenesis.

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TABLE OF CONTENTS

LIST OF FIGURES

APPENDIX

LIST OF TABLES

LIST OF ABBREVIATIONS*

***Commonly used scientific abbreviations not included**

AA	**A**frican **A**merican
BC	**B**reast **C**ancer
Bcl-6	**B c**ell **l**ymphoma **6**
BTB	**B**road complex, **T**amtrak, **B**ric a brac
CA	**C**aucasian **A**merican
DAB	**D**i**a**mino**b**enzidine
DMEM	**D**ulbecco's **m**odified **e**agle's **m**edium
DMSO	**D**i**m**ethyl **s**ulf**o**xide
E-cadherin	**E**pithelial **cadherin**
ECL	**E**nhanced **c**hemi**l**uminescence
ECM	**E**xtra**c**ellular **m**atrix
EMSA	**E**lectrophoretic **M**obility **S**hift **A**ssay
EMT	**E**pithelial to **m**esenchymal **t**ransition
FBS	**F**etal **b**ovine **s**erum
FFPE	**F**ormalin-**f**ixed and **p**araffin-**e**mbedded
HDAC	**H**istone **d**e**ac**etylase
HRP	**H**orse **r**adish **p**eroxidase
IHC	**I**mmuno**h**isto**c**hemistry
JMD	**J**uxta-**m**embrane **d**omain
KBS	**K**aiso **b**inding **s**ite
meCpG	**Me**thylated **CpG**
MET	**M**esenchymal to **e**pithelial **t**ransition
miR	**mi**cro**R**NA
mKaiso	**M**urine **Kaiso**
MMP7	**M**atrix **M**etallo**p**roteinase-**7**
MTG	**M**yeloid **t**ranslocation **g**ene
N-CoR	**N**uclear receptor **co-r**epressor-1

p120^{ctn}	**p120 ca**t**eni**n
PBS	**P**hosphate **b**uffered **s**aline
PBS-T	**PBS-T**ween 20
PI3K	**P**hosphat**i**dylinositol-**3**-**k**inase
POZ	**Po**x virus and **z**inc finger
pRS	**pR**etro**S**uper
PUMA	**p**53 **u**pregulated **m**odulator of **a**poptosis
SDS	**S**odium **d**odecyl **s**ulfate
siRNA	**s**hort **i**nterfering **RNA**
sh-K	**sh-K**aiso
shRNA	**s**hort **h**airpin **RNA**
SMRT	**s**ilencing **m**ediator of **r**etinoid and **t**hyroid receptor
TCGA	**T**he **c**ancer **g**enome **a**tlas
TGFβ	**T**ransforming **g**rowth **f**actor β
TMA	**T**issue **m**icro**a**rray
ZBTB33	**Z**inc finger- and **BTB** domain-containing protein **33**
ZF	**Z**inc **f**inger

CHAPTER 1 – INTRODUCTION

1.1 The history of breast cancer

Breast cancer (BC) remains one of the oldest documented forms of cancer–dating back to ancient Egypt.[1] The earliest incidence of BC recorded dates around 1600 B.C.[2] It was first described as "growths" or "ulcers" of the breast, with Hippocrates of Cos being the first to differentiate between benign and malignant tumors.[1] He is also accredited with naming cancer as "karkinoma"–which later became known as carcinoma–because the tumor looked like a crab in that it had a central body and protruding legs or extensions.[1] Interestingly, Hippocrates was also the first to describe an association between menstrual cessation and breast cancer occurrence.[1] During the Roman period, Aulus Cornelius Celsus was responsible for identifying the lymph node enlargement that accompanied many breast cancer cases. He also reported the first instance of metastatic BC, with the swelling of the armpits and subsequent spread to other parts of the body.[2] It was also during this time that the first attempts at breast cancer classification were made, along with the use of a surgical knife in combination with cauterization to remove the cancer.[1] Similar crude removal methods continued into the renaissance era, where the first attempts were made at differentiating between benign and malignant breast tumors.[2] Additionally, it was during this time period that scientists and physicians agreed that BC likely originated in the lymph node, which then spread to the breast and was followed by symptoms of breast swelling and pain.[2]

In the 18th century, surgeons opted to perform mastectomy that involved removing the entire breast, including the *pectoralis major* muscle for all tumors, irrespective of size.[2] This is when theories began to surface surrounding BC predisposition, and the median age of BC presentation for women was calculated to be between 40-60 years. The 19th century sparked a new era of BC surgery with the discovery of nitrous oxide as an anesthetic, as well as tumor histopathological analysis through the use of microscopy.[3] It was during this time that the Halsted radical mastectomy was first implemented – this involved a large tear-drop incision to remove the entire breast including the lymph nodes

and *pectoralis major* muscle.[2] This surgical procedure was viewed as the standard of BC removal surgery for more than 70 years following its initial implementation in 1894.[2] Perhaps the most important discovery during this time was the link between breast cancer and hormone dependency and the subsequent use of chemotherapeutics, which drastically altered the course of BC management.[3] With the 20th century came further refinement of hormone-based therapeutics such as Stilbestrol designed to reduce estrogen production and its effects in advanced BCs.[2] It was also during this time that postoperative radiation in combination with local tumor excision was shown to be just as effective for early stage BC compared to the standard radical mastectomy.[3] By the mid-20th century, many surgeons sought to enhance the standard radical mastectomy, aimed primarily at preserving the *pectoralis major* muscle.[1] This in turn became the "modified" mastectomy, and in 1971 the first randomized controlled trials began to compare conservative surgery combined with radiotherapy versus radical mastectomy.[4] Based on several trials, it was concluded that standard treatment would consist of tumor excision followed by whole breast irradiation for the treatment of early stage BCs.[4] Throughout the 1970s scientists began to experiment with ways of combining treatments to improve patient outcomes, leading to a better understanding of how cancer spreads through the body. During this time, predisposing mutations in the *BRCA1* and *BRCA2* genes were discovered, providing confirmation of a genetic basis of BC as well as a method of identifying a subset of patients at increased risk of developing BC.[2] In 1988, the synthetic estrogen receptor modulator, tamoxifen became the first drug clinically proven to lower BC risk, and with its discovery came the advent of combination treatment regimes, including anthracyclines (fluorouracil, doxorubicin, cyclophosphamide) or FEC (fluorouracil, epirubicin, cyclophosphamide) which showed reduced 5-year recurrence rates along with reduced 15-year mortality rates.[2] In many studies, adjuvant therapy in combination with or following chemotherapy leads to dramatic improvements in disease-free survival. Ultimately, these treatment regimens led to the development of less invasive and more targeted treatments.

This approach to BC treatments and research continues today, with a large focus on the identification of the molecular and genetic factors contributing to BC with the goal of developing individualized BC treatments. To date, BC remains one of the most widely studied diseases, with ongoing research being conducted worldwide. BC management has greatly evolved since its early discovery, and currently involves a complex interplay between surgeons, medical oncologists, radiologists, and the patients themselves.[1] In the last decade, high-throughput technologies have provided valuable information on tumor biology and have led to a better understanding of the gene expression differences amongst various BC tumors, allowing for the classification of breast cancer into six intrinsic subtypes. This has ultimately allowed for the development of new, targeted therapies now being tested in ongoing clinical trials.

1.2 Current breast cancer clinical considerations

Despite being discovered over a hundred years ago, BC remains a complex and challenging disease, and accounts for nearly 30% of cancer diagnoses–at a rate exceeded only by skin cancer.[5] Clinically, breast cancer is broadly categorized into three major subtypes; hormone receptor positive, HER2-overexpressed and triple negative.[5] These subtypes are categorized based on the expression of three main biomarkers–estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2).[6] The presence of these biomarkers is assessed using an approach called immunohistochemistry (IHC).[7] Advances in gene expression profiling by way of gene-based assays, proliferation markers and histologic grade has allowed for further stratification of breast cancer into five distinct subtypes: luminal A, luminal B, normal breast-like, HER2-overexpressing, and basal-like.[6,8] These biological subtypes present a varied spectrum of molecular features, allowing for customized adjuvant treatment for early stage breast cancers.[6] Targeted treatments typically consist of strategies that are directed at specific molecular markers that discriminate malignant from benign cells, and are important for the initiation and progression of breast cancer.[9] Most targeted treatments are aimed at the blocking of a surface or nuclear receptor, whereas chemotherapy is

considered a non-specific treatment because it simply targets proliferating cells.[9] Luminal A breast cancer treatment typically consist of hormonal-based therapies (e.g. Tamoxifen), whereas the luminal B subtype shows improved patient outcomes when therapy consists of chemotherapy in combination with hormonal therapy.[9] Patients with HER2-overexpressing breast cancers have been shown to benefit from targeted therapy with drugs such as Herceptin, which specifically targets the HER2 protein.[10] However, the breast cancer subtype that lacks the expression of all three biomarkers and is thus referred to as Triple Negative Breast Cancer (TNBC) has limited treatment options because it cannot be treated with traditional hormonal or receptor-targeted therapies.[6,8] Consequently, the median patient survival rate for metastatic TNBC is only 13 months.[11] Thus, improved therapeutic strategies are crucial for patients diagnosed with this breast cancer subtype.

1.3 Triple Negative Breast Cancer

TNBCs account for 12-17% of invasive breast cancers.[8,12] Relative to other breast cancer subtypes, TNBCs clinically present as more aggressive, with a poorer overall and disease-free survival, and higher metastatic rate.[11,13] Interestingly, TNBC is more frequently diagnosed in Hispanic and premenopausal women of African ancestry (WAA) compared to Caucasian women, although the cause for this racial disparity remains unknown.[14] Triple negative and basal-like breast cancers (BLBCs) are commonly grouped together, with more than 90% of BLBCs being TNBCs, although only about 50-80% of triple negative tumors fall into the basal-like category.[15,16] Under the current clinical therapeutic guidelines, TNBC is considered a single entity and is uniformly treated with chemotherapy. However, due to high variance within the subtype, the phrase “triple negative” serves as an umbrella term for a subset of breast cancers that can be further differentiated into six subtypes, each displaying unique biological features.[16] Despite progress in further segregating these tumors and developing therapies based on their unique features, none of the intrinsic subtypes differ significantly in terms of pathological complete response (pCR) or survival after neoadjuvant chemotherapy, and all display

similar benefit to platinum therapies.[17] Despite this lack of response, the therapy standard for TNBC treatment remains non-specific radiation and chemotherapy.[17] Although TNBCs elicit a greater initial sensitivity to chemotherapy compared to luminal and HER2-overexpressing breast cancers, over time they become resistant to chemotherapy and display higher rates of distant metastasis and mortality.[12] Therefore, a balanced approach between the simple pragmatism of the current clinical therapeutic guidelines and the molecular complexity of TNBC is required, as it would be the desirable way to manage patients with this disease. Thus, increasing research is currently focused on identifying practical TNBC subtypes that display uniformly targetable molecular features.

1.4 TNBC molecular landscape

To better address the complexity associated with TNBC tumor heterogeneity, Lehmann and colleagues defined six TNBC subtypes through gene expression profiling.[16] These include two basal like (BL1 and BL2), an immune-modulatory (IM), a mesenchymal (M), a mesenchymal stem like (MSL), and a luminal androgen receptor (LAR) subtype.[16] Tumors categorized into the LAR subtype are defined as TNBCs through IHC, but histologically and genetically they resemble luminal-like ER-positive BC. This subtype is characterized by the expression of the androgen receptor (AR), and thus may be a targetable option using agents that target AR, similar to prostate cancer treatment.[16] The BL1 subtype is characterized by elevated cell cycle and DNA damage response gene expression, while the BL2 subtype expresses growth factor signaling and myoepithelial markers.[16] The IM subtype consists of genes encoding immune antigens and cytokine and immune signal transduction pathways.[16] The M and MSL subtypes both display increased expression of genes involved in the epithelial-to-mesenchymal transition and growth factor pathways, differing only in that the MSL shows decreased expression of genes involved in proliferation.[16] Retrospective analysis of the response to neoadjuvant chemotherapy based on TNBC subtype revealed that BL1 tumors demonstrated the highest pCR (52%) and BL2 and LAR tumors exhibited the lowest (0 and 10% respectively).[18] The results from this study suggest that certain TNBC subtypes can be

sensitive or insensitive to neoadjuvant chemotherapy.[18] However, the sample size from this study was small and these findings require additional validation to determine whether TNBC subtypes are in fact able to predict relapse and/or response to therapy.

TNBCs display ~ 1.68 somatic mutations per Mb of coding regions (~60 somatic mutations in each tumor).[19] The most frequently arising mutations (60-70%) are *TP53* mutations and are more commonly found in basal-like tumors than non-basal tumors.[19] The next most frequently occurring mutations in TNBC occur in the *PIK3CA* gene and this mutation occurs most frequently in the LAR TNBC subtype (46.2%) relative to other subtypes (4.5%).[19] Interestingly, the LAR subtype was found to have a significantly higher mutational burden compared to the MSL subtype, which displays a relatively low mutational burden.[19] A recent study examining the mutational profiles across TNBC subtypes using 550 TNBC samples reported that the LAR subtype displayed the most distinct mutational profile, with mutations in *PIK3CA* (55%), *KMT2C* (19%), *CDH1* (13%), *NF1* (13%) and *AKT1* (13%) genes.[19] These results further highlight the significant mutational heterogeneity within TNBC, along with the potential clinical implications. In addition to gene mutations, TNBCs are also known to be associated with unstable genomes and this varies within the different TNBC subtypes. Interestingly, among the distinct subtypes, BL1 and M tumors display significantly higher chromosomal instability (CIN) scores. Delving further into these analyses, *MYC*, *PIK3CA* and *CDK6* were found to be the most frequently amplified genes in TNBC, whereas the *MAP2K4*, *TP53* and *NCOR1* genes were found to be the most frequently deleted.[7,13] Further stratification of the subtypes based on copy number alterations (CNAs) revealed that the BL1 subtype displays the highest gain/amplification levels in genes such as *MYC*, *PIK3CA*, *CDK6*, *AKT2*, and *KRAS*, along with the highest levels of deletions in genes associated with DNA repair such as *BRCA2*, *PTEN*, and *TP53*.[20] In contrast, the LAR subtype is significantly associated with higher amplification levels of *EGFR* and *AKT1*, along with a high frequency of deletions in the *CCND3*, *AKT2*, *ESR1*, *SMAD4*, *TP53*, and *MAP2K4* genes.[20] Lastly, the M subtype is associated with higher amplification levels for

DNMT3A and *TP53*, as well as increased frequency in deletion of *PDGFRA*, *RB1* and *MAP3K1* genes. [16,19,21]

These differences in mutational and copy number profiles characterizing each TNBC subtype may offer novel therapeutics for TNBC patients. For instance, BL1 tumors are characterized by high genomic instability, and high copy number gains for *PPAR1* coupled with high copy number losses for *BRCA1/2* and *TP53*[19], supporting the idea that these tumors may be sensitive to PARP inhibitors. Additionally, LAR and MSL tumors retain *RB1*, while expressing significantly lower *CDK4* and *CDK6* mRNA expression levels, which may be clinically relevant in patients being treated with CDK4/6 inhibitors.[13,16] Similarly, a large proportion of LAR tumors exhibit somatic mutations in the PI3K signaling pathway[7], suggesting a potential benefit to PI3K and AKT inhibitors. EGFR and Notch signaling pathways were found to be enriched in the M TNBC subtype[22], suggesting that targeting these pathways may be a potential therapeutic avenue for patients with these tumors. Lastly, the *MYC* gene was found to be the most frequently amplified gene amongst all TNBC subtypes[22], except for MSL suggesting that targeting *MYC* may be beneficial only to select TNBC subtypes. Interestingly, selective inhibition of *CDK1/2* and spliceosome core component *BUD31* induced synthetic lethal mortality in *MYC* overexpressing TNBC tumors, which indicates that inhibition of *CDK1/2* may have therapeutic benefit specifically for patients with BL1 and M subtypes.[22]

While the exact mechanisms surrounding TNBC initiation and progression remain unknown, it can be assumed that signaling pathways that promote the aberrant gene expression profile responsible for tumor progression are key components of TNBC metastatic tendencies. An emerging field of study is the dysregulation of specific genes such as microRNAs that are capable of negatively influencing gene expression.

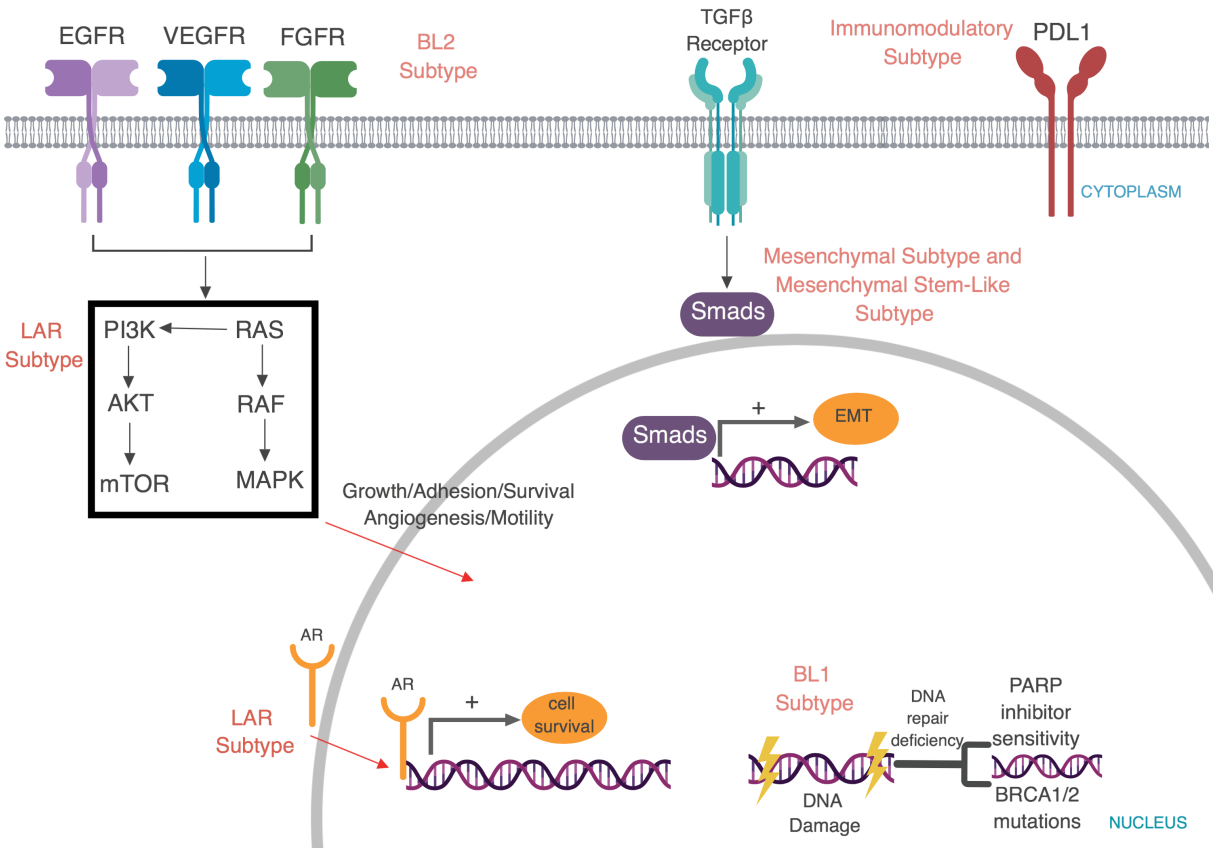

Figure 1.1: Molecular features of TNBC subtypes. In the BL2 and LAR TNBC subtypes, overexpression of EGFR, VEGFR, FGFR lead to the deregulation of downstream PI3K and RAS signaling, resulting in an increase of genes associated with growth, adhesion, survival, angiogenesis and motility. In the BL1 subtype, frequent BRCA1/2 mutations are involved in tumor cell initiation and development and cause homologous recombination deficiencies in response to DNA damage and hypersensitivity to PARP inhibition. The IM subtype is enriched for genes encoding immune antigens and cytokine and immune signal transduction pathways, such as the PDL1 pathway which is a potent mechanism by which tumor cells evade host immune surveillance. The M and MSL TNBC subtypes are enriched for genes involved in EMT and growth factor pathways such as TGFβ signaling.

1.5 MicroRNA regulation

MicroRNAs (miRNAs) are a class of small (18-22 nucleotides), non-coding RNAs that are capable of post-transcriptionally modulating the expression of proteins involved in various cellular processes including cell survival, metastasis, proliferation, senescence, and cell death.[23,24] MiRNAs were first discovered in 1993, when Lee and colleagues described a small non-coding RNA in *C. elegans* that was capable of regulating the expression and function of another protein-coding mRNA.[25]

MiRNAs are transcribed by RNA polymerase II into transcripts called primary-miRNAs (pri-miRNAs) that are characterized by their hairpin structures harbouring the miRNA sequences.[26,27] In the nucleus, these pri-miRNAs are then processed by the Drosha complex, which cleaves the transcript into the shorter (70-100 nts) pre-miRNA (pre-cursor miRNA).[27] These pre-miRNAs are then exported from the nucleus by Exportin-5 and once in the cytoplasm undergo additional cleavage by the RNAse III Dicer complex, forming the 22 nucleotides long miRNA/miRNA* dsRNA.[26] Further processing by this complex yields a mature, single-stranded miRNA product that is then incorporated into the miRNA containing RNA-induced silencing complex (miR-RISC). Once associated with this complex, the mature miRNA regulates gene expression levels post-transcriptionally by binding through partial complementarity to target mRNAs which causes mRNA degradation or silencing. miRNA-RISC-mediated gene silencing has been found to occur in three ways: (1) site-specific cleavage, (2) enhanced mRNA degradation, and (3) translational inhibition.[26] The first process is a very rare event in mammals, requiring perfect complementarity, and is the mechanism of miRNA-mediated gene silencing commonly observed in plants.[27] Most animal miRNAs, however, are thought to use the latter mechanisms, involving mismatched miRNA/target sequences. These miRNAs exert their regulatory effects by binding to imperfect complementary sites within the 3' untranslated regions (UTRs) of their mRNA targets, and subsequently repress gene expression post-transcriptionally. Similar to translational regulation, miRNAs that use this mechanism of mRNA inhibition reduce the protein levels of their targets, but the mRNA levels of their target genes remain relatively unaffected.[26] Given

that this miRNA:target mRNA process occurs without perfect complementarity, it is not surprising that each miRNA is capable of targeting a large number of genes. Similarly, it has been shown that ~ 60% of mRNAs share one or more sequences that are predicted to interact with miRNAs.[26] Consequently, due to their widespread targets across various cell types, miRNA dysregulation has the potential to adversely affect gene expression. Though being referred to as the "fine tuners" of gene expression, miRNAs are now increasingly linked to tumorigenesis.[24]

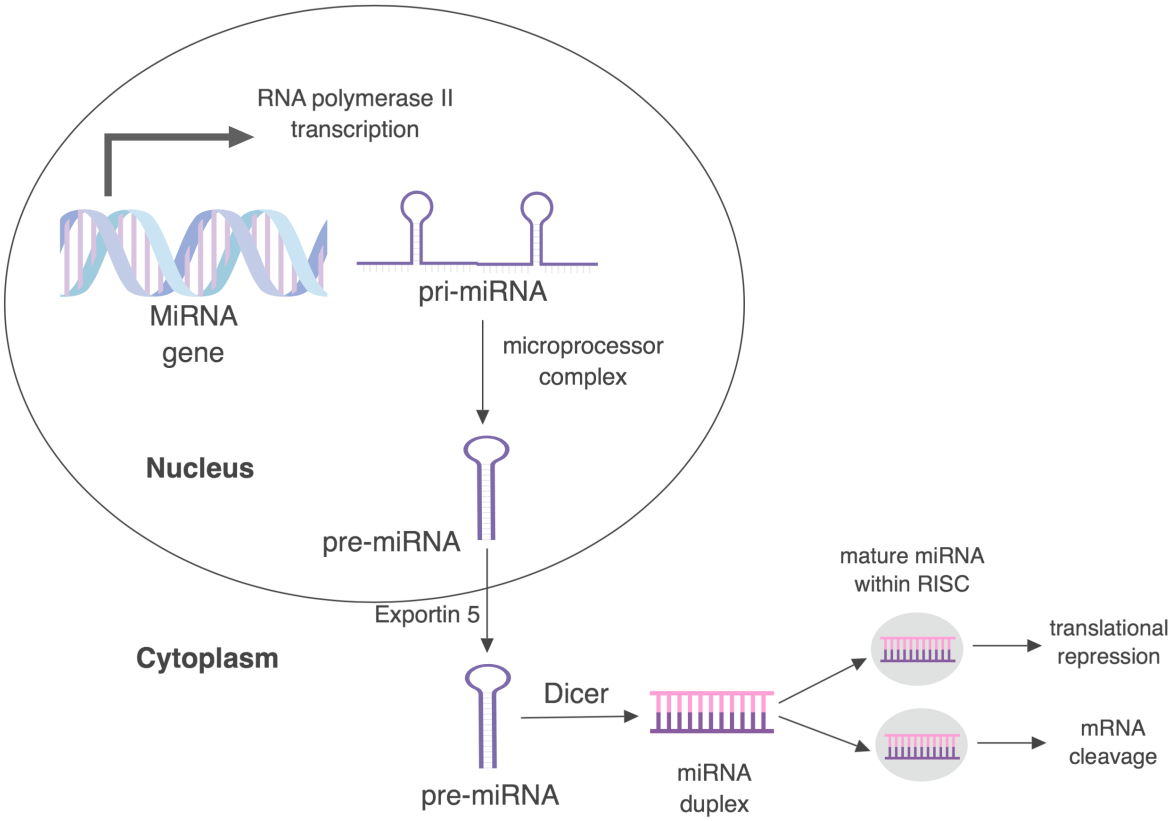

Figure 1.2: MicroRNA synthesis pathway. In the nucleus, RNA polymerase II is responsible for initial transcriptional of the miRNA gene to form transcripts called pri-miRNAs. Pri-miRNAs undergo processing by the Drosha microprocessor complex, cleaving the transcript into a shorter pre-miRNA transcript. Exportin 5 then exports the pre-miRNA from the nucleus and once in the cytoplasm it undergoes further cleavage by the RNAse II Dicer complex, forming a miRNA duplex. Further processing by the Dicer complex yields a mature miRNA product that is incorporated into the RISC complex to regulate gene expression either through translational repression or mRNA cleavage.

1.6 TNBC and microRNA expression

Since their initial discovery, emerging evidence has shown that miRNAs can function as tumor suppressors or oncogenes.[24,26] Tumor suppressive miRNAs exert their effects by taking on an anti-tumor role against cancer cells, whereas oncogenic miRNAs promote tumor initiation and progression.[26] miRNA expression profiling in various diseases has revealed expression signatures of certain miRNAs unique to specific diseases. In particular, in cancer, aberrations in miRNA expression levels are linked to the onset and progression of many cancer types. For instance, in breast cancer it has been shown that several miRNAs are differentially expressed relative to normal breast cells; specifically, the loss of tumor suppressing miR-17-5p, miR-200 or miR-31 lead to the promotion of proliferation, TGF-β signaling or breast tumor metastasis, respectively.[24] Conversely, overexpression of oncogenic miRNAs such as miR-21, miR-10b or miR-373 results in aberrant apoptosis, TGF-β signaling or metastasis, respectively, in these cells.[24] Due to their prognostic potential, microRNAs have been proposed as promising biomarkers of BC because they can be readily detected in tumor biopsies as non-circulating miRNAs, *and* they are easily detected in body fluids such as blood plasma, serum, and saliva as circulating miRNAs.[28] Recently, miRNA expression profiling has been used to differentiate patients with BC to determine whether or not they will respond to certain therapies, with promising results.[28]

In TNBC specifically, it has been repeatedly shown that there is a significant correlation between a number of miRNAs and TNBC progression.[23] For example, TNBC patients uniquely express higher levels of the miR-17-92 family, which is comprised of seven miRNA genes that act cooperatively with the oncogene, *c-MYC* to escape programmed cell death, ultimately leading to tumorigenesis.[29] Similarly, TNBCs frequently express increased miR-146a and miR-146b-5p levels, which function to negatively regulate BRCA1 expression, leading to increased cell proliferation and a reduced homologous recombination rate in TNBC.[23] Alternately, the expression of miR-205–a novel transcriptional target of p53–that functions to reduce cell proliferation, cell

cycle progression and tumor growth inhibition, is highly downregulated in TNBC patients relative to other breast cancer subtypes.[23]

One of the greatest obstacles for effective therapies against TNBC is the lack of highly sensitive and specific prognostic markers pertaining to early metastasis, which is a leading cause of TNBC-associated death.[30] Encouragingly, several studies have identified miRNAs as prognostic and therapeutic markers in TNBC.[23,28,31] Several oncogenic miRNAs have prognostic potential for TNBC patients, including miR-27b-3p, which when overexpressed is an independent predictor for distant metastasis in TNBC patients.[23] Similarly, high expression of miR-21 and miR-210 revealed a positive correlation with poor prognosis for TNBC patients.[32] In contrast, several tumor-suppressing miRNAs are negatively correlated with TNBC prognosis. For example, the reduced expression of miR-17 and miR-19a, common in TNBC tumors are associated with the leukocyte trans-endothelial migration pathway–a crucial regulator of the inflammatory response–and may represent an area of therapeutic targeting.[28,29] In addition to the potential prognostic miRNAs described, there are other miRNAs that show involvement in the therapeutic aspect of TNBC. For instance, several miRNAs have been implicated in the chemo-resistant nature of TNBC such as miR-130a-3p and miR-451a whose expression in TNBC cells is significantly lower compared to normal breast epithelial cells.[32] When overexpressed, these miRNAs increased TNBC cells sensitivity to doxorubicin, a chemotherapeutic agent.[32] Similarly, overexpression of miR-155 in TNBC cells increased their chemo-resistance relative to control cells, which suggests that it could be potentially therapeutically targeted in patients expressing high levels of this miRNA.[32] Collectively, this evidence indicates that miRNAs can be used as therapeutic targets for TNBC patients, however there is a large knowledge gap pertaining to the exact mechanisms by which microRNAs regulate their target mRNAs and how miRNAs are regulated themselves. Therefore, understanding the mechanisms through which miRNA regulation occurs, in both normal and tumor environment, is crucial for their development as therapeutic targets.

While there are a number of studies focused on elucidating miRNA targets and their downstream effects, there is little known about the mechanisms responsible for promoting or inhibiting miRNA expression. Intriguingly, the protein Kaiso has been implicated in cancer-associated miRNA regulation[33,34] and serves as an attractive target to explore in the context of TNBC.

1.7 The POZ-ZF transcription factor Kaiso

Kaiso is a member of the BTB/POZ (Broad Complex, Tramtrak, Bric à brac/ pox virus and zinc finger) family of transcription factors.[35,36] Members of this family are characterized structurally by an N-terminal POZ domain that mediates protein-protein interactions and a C-terminal domain comprised of three Kruppel-like C_2H_2 zinc fingers that mediate its DNA-binding ability.[37] Subsequent analyses revealed Kaiso's zinc fingers 2 and 3 specifically are both required for sequence-specific DNA binding.[37] Kaiso was first identified as a binding partner of the E-cadherin catenin cofactor p120-catenin ($p120^{ctn}$), using a yeast two-hybrid screen for unique $p120^{ctn}$ binding partners, with full length $p120^{ctn}$ used as bait.[37,38] Further analyses identified Kaiso as the first member of a subfamily of zinc finger methyl-CpG binding proteins that include the Kaiso-like ZBTB38 and ZBTB4 proteins.[37,39] Surprisingly, although Kaiso, ZBTB38 and ZBTB4 share very similar structures, ZBTB38 and ZBTB4 do not interact with $p120^{ctn}$, and highlight the uniqueness of the Kaiso-$p120^{ctn}$ interaction.

Investigations into Kaiso's regulatory function revealed three different ways in which Kaiso regulates its targets, and led to Kaiso's subsequent classification as a dual-specificity DNA-binding transcription factor. The first was revealed during Kaiso's initial characterization where the consensus DNA binding site, TC**CTGC**N**A** (core sequence bolded) was termed the Kaiso Binding Site (KBS).[37] This sequence was shown to be specific to Kaiso through an interaction between the Kaiso protein and KBS's in the *matrilysin* promoter using EMSA.[38] Additional assays revealed that Kaiso also regulates its target DNA via binding to the unmethylated palindromic TCT**CGCG**AGA sequence (core sequence **bolded**) or at methylated CpG dinucleotides.[40] The relative affinity of

Kaiso for the KBS versus methylated CpG dinucleotides continues to be debated, although this variability may be due to cell-type specific differences across studies, and further highlights Kaiso's regulation complexity.[40,41] While Kaiso's preference for methylated versus unmethylated regions warrants further investigation, Kaiso's mechanism of regulating gene silencing has been fairly well established. This mechanism involves recruitment of macromolecular corepressor complexes, consisting of N-CoR, silencing mediator of retinoid and thyroid receptor (SMRT), histone deacetylases (HDACs) and myeloid translocation gene 16 (MTG) proteins.[40–42] A study by Barrett and colleagues demonstrated Kaiso's recruitment of the MTG16 corepressor to the MMP7 promoter in a sequence-specific manner, which was shown to be required for Kaiso-mediated repression of *MMP7*.[42] Although the majority of studies surrounding Kaiso's regulatory roles have shown that Kaiso appears to function primarily as a transcriptional repressor, Kaiso also functions as a transcriptional activator like the MIZ1 POZ-ZF.[43] This was first shown in a 2004 study by Rodova and colleagues, where Kaiso was found to activate the neuromuscular gene, *rapsyn*.[43] Since then, additional studies have supported a role for Kaiso in gene activation[44], and further highlights Kaiso's diverse transcriptional capabilities. While these transcriptional functions have been intensely studied, there is still little known regarding Kaiso's exact roles in mammals, and it's increasing association with carcinogenesis.

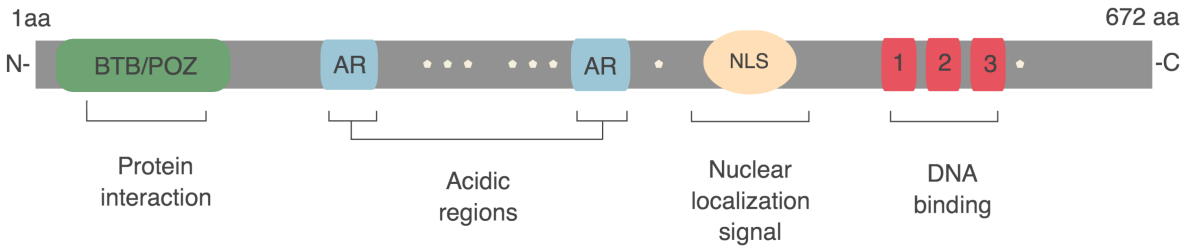

Figure 1.3: Schematic diagram of Kaiso and its functional domains. The transcription factor Kaiso possesses the highly conserved BTB/POZ protein interaction domain at its amino-terminus, while its carboxyl-terminus contains three zinc finger (ZF) DNA binding domains. Kaiso also possesses two acidic regions (AR) which are involved in transcriptional activation, as well as a nuclear localization signal (NLS). Kaiso also contains multiple putative serine/threonine phosphorylation sites (asterisks).

1.8 Kaiso and cancer

Since its discovery, Kaiso has been unveiled as a regulator of the epithelial and cell adhesion marker, E-cadherin, and similarly the epithelial-to-mesenchymal transition (EMT)–a process that confers normal, immobile epithelial cells with traits that allow their increased motility and invasiveness.[45]

Upon E-cadherin downregulation, $p120^{ctn}$ was found to translocate to the nucleus where it bound to Kaiso and subsequently inhibited Kaiso's transcriptional activities.[37,46] This data demonstrates the importance of Kaiso's subcellular localization on it's regulatory functioning. In fact, studies investigating Kaiso and its biological role found Kaiso expression and subcellular localization correlated with several cancers, including breast, prostate, lung, ovarian and colorectal.[33,47–49] Early studies examining the effect of Kaiso depletion in *Xenopus* embryos led to abnormal gastrulation, premature gene activation, and embryonic lethality.[49,50] These studies suggested a role for Kaiso in the developmental gene regulatory pathway that moderates vertebrate morphogenesis.[50] Interestingly, a study by Prokhortchouk and colleagues revealed alternative physiological roles for Kaiso in mouse tissues by generating Kaiso knockout mice. In examining the loss of Kaiso and its effects in these mice, they found that unlike the *Xenopus* models, Kaiso-null mice showed no overt phenotype, and the tissues and organs from these mice did not demonstrate any visible deformities or developmental abnormalities.[49] Collectively, these studies demonstrated distinct physiological roles for Kaiso that was dependent on the vertebrate species and context. Interestingly, continued monitoring of the effect of Kaiso loss in the 3rd generation of the Kaiso-null progeny revealed continuous loss of Kaiso led to abnormal spleen enlargement, accompanied by increased cell proliferation resulting from increased *Bcl-6* and *c-Myc* expression, along with decreased expression of the cell cycle arrest genes *p27*, *p21*, and *Gadd45a*.[51] Thus, these studies demonstrate Kaiso's multiple roles even within the same species. Despite exhibiting no obvious phenotype when compared to control mice, further analysis of the Kaiso knockout mouse model also revealed that Kaiso-null mice exhibited delayed onset of intestinal tumorigenesis when bred with the $APC^{Min/+}$ mouse model of colorectal

cancer (CRC).[49] The APC$^{Min/+}$ mouse, used to model human familial adenomatous polyposis is characterized by mutations in the APC gene, hyperactive Wnt/β-catenin signaling and multiple intestinal tumors.[51] The results from this study led Prokhortchouk and colleagues to suggest that Kaiso expression positively regulated the Wnt signaling cascade, and they were the first to imply a pro-tumorigenic role for Kaiso in intestinal tumorigenesis. Indeed, studies performed in our lab showed that Kaiso overexpression in murine intestines (hereafter referred to as Kaiso transgenic mice, *Kaiso*^{Tg}), led to increased expression of Wnt target genes, along with increased inflammation.[52] Additional studies performed by our lab found that *Kaiso*^{Tg} exhibited secretory cell and crypt hyperplasia, which has been shown to increase the risk of CRC.[53] Although Kaiso overexpression on its own was not enough to induce tumorigenesis in these mice, increased Kaiso expression has been observed in advanced-grade colon and tumors compared to lower-grade tumors and suggests a potential link between Kaiso and CRC.[53]

Recent studies by Yates and colleagues examined the link between Kaiso in prostate cancer development and progression and revealed a role for Kaiso in the regulation of the pleiotropically acting *miR-31*, and *miR-200c*, a member of the tumor-suppressing miR-200 family; both were upregulated upon Kaiso depletion.[33,34] Further analyses revealed a direct, methylation-dependent association between Kaiso and the promoter regions of these miRNAs, and *in vivo* mouse models using Kaiso-depleted PC3 prostate cancer cells revealed that xenografted mice exhibited reduced tumor formation and metastasis.[34,54] Collectively, these studies suggest a mechanism through which Kaiso promotes prostate cancer cell migration, invasiveness and metastasis via miRNA regulation. **In Chapter 3 of thesis, we show that Kaiso similarly regulates *miR-31* and *miR-200c* in a methylation-dependent manner in TNBC cells. These findings implicate Kaiso in TNBC aggressiveness, in part, via miRNA regulation.**

1.9 Kaiso subcellular localization and tumor tissue type

In addition to Kaiso's high or low expression demonstrating diagnostic significance for certain cancers, Kaiso's subcellular localization in different tumor types correlates with disease prognosis. For instance, in lung cancer tissues, Kaiso is expressed mainly in the cytoplasm, and higher cytoplasmic Kaiso expression correlates with advanced grade lung tumors compared to lower grade tumors as well as lymph node metastasis.[47] Similarly, in prostate cancer tissues, high nuclear Kaiso correlates with higher grade tumors compared to low grade tumors.[55] Interestingly, in BC tissues, Kaiso subcellular localization is dependent on tumor type, with nuclear Kaiso expression being linked to invasive ductal carcinoma, and cytoplasmic Kaiso expression is more commonly observed in invasive lobular carcinoma.[56] Interestingly, in the same study it was also shown that nuclear Kaiso positively correlated with tumor grade and ER, PR and HER2 negativity.[56] The exact mechanisms through which Kaiso regulates tumorigenesis still remains to be elucidated, however emerging studies have linked Kaiso with several tumor processes including tumor cell growth, proliferation, progression, metastasis and apoptosis,[33,52,57] and will aid us in our understanding of Kaiso-mediated tumorigenesis.

1.10 Kaiso as a regulator of BC metastasis, apoptosis and proliferation

Relative to other BC subtypes, TNBCs are characterized by an increased propensity to metastasize to vital organs, along with high rates of proliferation and quick growth rates.[17] Mounting evidence from our lab and others suggest that Kaiso is a crucial promoter of aggressive and metastatic breast cancers, such as TNBC.[48] In support of previous findings from a study by Vermuelen and colleagues, our lab found that Kaiso is highly expressed in TNBC relative to other BC subtypes, and that higher Kaiso expression is associated with increased aggressiveness, metastasis and lower survival outcomes.[48] **In Chapter 5, we show through mechanistic *in vitro* studies that Kaiso-depleted TNBC cells display reduced proliferation and increased apoptosis.**[57] **Specifically, Kaiso-depleted MDA-MB-231 TNBC cells displayed delayed tumor onset and reduced expression of several notable cell proliferation markers such as**

Ki67 in mouse xenografts.[57] In addition, Kaiso-depleted cells expressing mutant p53 also exhibited increased apoptosis and increased expression of pro-apoptotic genes such as *PUMA* and *Bax*.[57]

In addition to Kaiso's role in proliferation and apoptosis, previous studies have also implicated Kaiso in EMT,[34,48,58] another crucial oncogenic process that is regulated by various signal transduction pathways, one of which is the transforming growth factor beta (TGFβ) signaling pathway.

1.11 Roles of TGFβ signaling in non-transformed and tumorigenic cells

During normal cell homeostasis, TGFβ signaling is initiated upon ligand-induced oligomerization of serine/threonine receptor kinases (type I and type II), with the TGFβ receptor type I (TGFβ-R1) mediating most gene expression responses.[59,60] Heterodimerization of the receptors (R1 and R2) leads to subsequent phosphorylation of the cytoplasmic signaling molecules Smad2 and Smad3 for the TGFβ/activin pathway.[61] Phosphorylation of Smads by activated receptors results in their interaction with Smad4 – a signaling transducer that translocates to the nucleus and interacts with other transcription factors to regulate gene expression.[62] In non-malignant breast cells, TGFβ signaling primarily functions to inhibit cell cycle progression, specifically the transition from growth (G1) to synthesis (S) phase, and ultimately cell proliferation.[63] However in addition to cell proliferation, TGFβ signaling also exerts a diverse range of biological effects on a variety of cell types, including regulation of cell growth, differentiation, matrix production and apoptosis.[62] Specifically, during embryogenesis, many TGFβ family members have crucial roles in pattern formation and tissue specification, and in adults TGFβ proteins are involved in cellular events such as tissue repair and immune system modulation.[62] However, in the context of tumorigenesis, TGFβ signaling plays a multifunctional role; during the initial stages, when cells are still responsive to its anti-mitogenic effects, TGFβ acts as a tumor suppressor, through repression of *c-Myc, CDK4,* and *CDC25A*.[64] During malignant progression, the cells become desensitized to the growth inhibitory effects of TGFβ, and instead TGFβ promotes a pro-metastatic

phenotype.[64] One way that this is achieved is through an increase in *c-Myc* expression concomitant with a loss of the growth-inhibitory response to TGFβ.[65,66] Consequently, TGFβ then functions to stimulate processes required for malignant progression such as angiogenesis, EMT and suppression of the immune system, collectively providing an optimal microenvironment for tumor growth, proliferation and metastasis.[65,66]

In BC, TGFβ roles in the promotion of breast tumors have been extensively investigated. Notably, TNBC cells tend to be the most frequently utilized to study TGFβ-induced metastasis, which highlights the crucial role TGFβ plays in tumor metastasis. In 2013, Taylor and colleagues demonstrated a role for TGFβ in the promotion of EMT and subsequent metastasis of TNBC cells through an upregulation of WAVE3.[67] WAVE3, an actin-binding protein and member of the WASP/WAVE family of proteins, is crucial for regulating cell morphology, actin polymerization and cytoskeleton remodeling, events that lead to increased cell motility and invasion.[67] Indeed, depletion of WAVE3 in TNBC cells prevented TGFβ−induced initiation of EMT and the subsequent proliferation, migration and lamellipodia formation in these cells.[67] WAVE3 thus appears to be required for EMT initiation upon TGFβ stimulation, which suggests it is an essential component of BC tumor progression.[68]

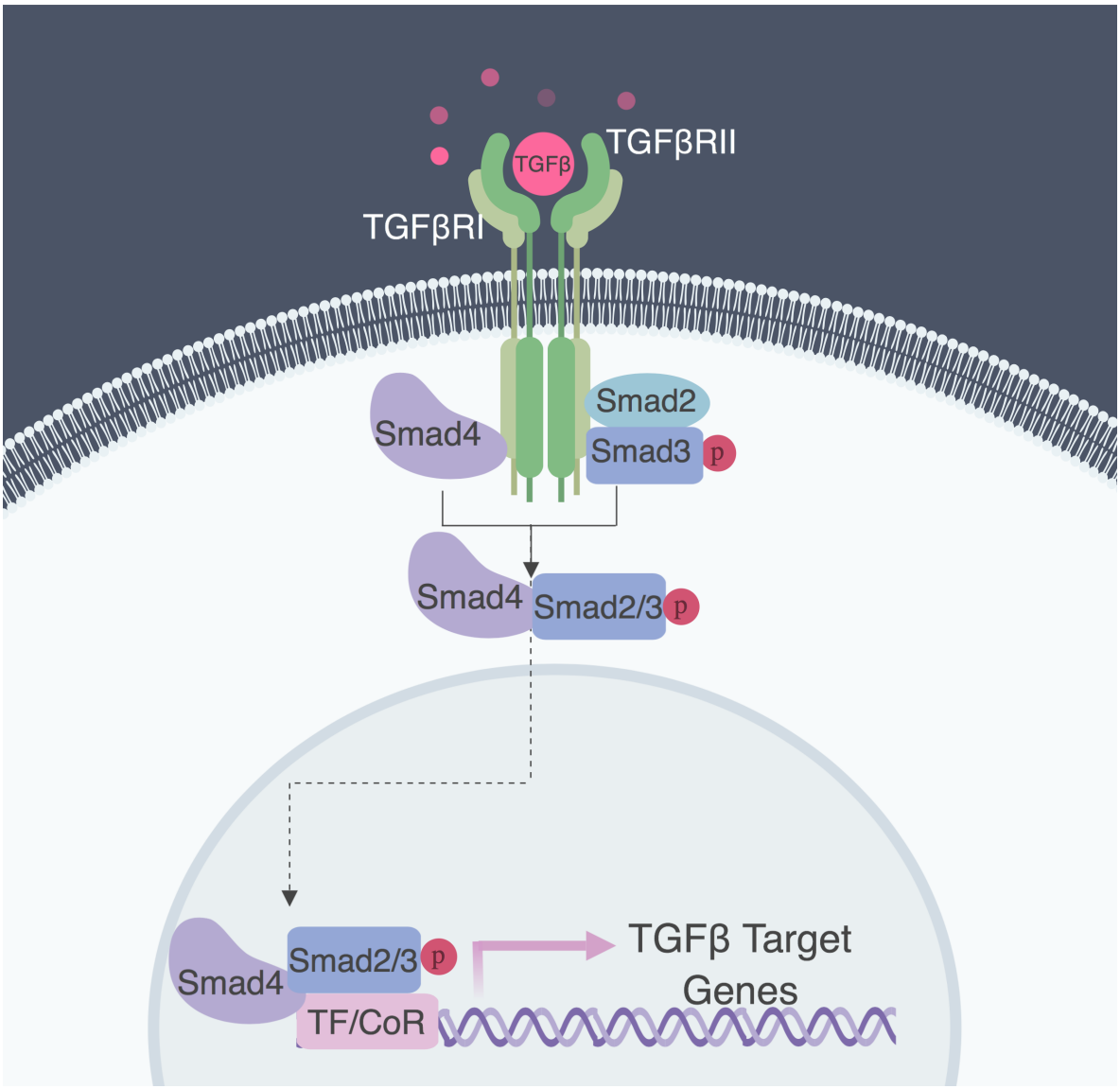

Figure 1.4: The canonical TGFβ signaling pathway. Upon active TGFβ signaling, the TGFβ ligand binds to TGFβRII, initiating the recruitment and subsequent phosphorylation of TGFβRI. Once activated, TGFβRI then phosphorylates Smad2/3, resulting in their interaction with the signaling transducer, Smad4 and subsequent translocation to the nucleus where the complex interacts with various transcription factors (TF) and nuclear coactivators or corepressors to regulate the expression of TGFβ target genes.

1.12 Kaiso and TGFβ signaling

We have shown that Kaiso depletion attenuates the TGFβ signaling pathway, which resulted in reduced cell motility and invasiveness *in vitro* and reduced cell metastasis to the liver and lungs of mice injected with Kaiso-depleted TNBC cells.[48] This attenuation in metastasis is due to the altered expression of genes regulated by TGFβ signaling, such as the ZEB1 transcription factor, whose expression is reduced upon Kaiso depletion.[48] Kaiso depletion also resulted in increased expression of the TGFβ receptors, TGFβR1 and TGFβR2.[48] Interestingly, Kaiso bound to the promoter regions of both receptors, suggesting a direct role for Kaiso in regulating TGFβ signaling.[48] Remarkably, TGFβ signaling resulted in increased Kaiso expression in BC cells, thus hinting at a potential positive feedback loop between Kaiso and TGFβ.[48] Together these data suggest that Kaiso may be a pertinent driver of metastasis in these cells. **In Chapter 3, we demonstrate that Kaiso-depletion attenuates TNBC cell motility, in part via the reduction of WAVE expression through various signaling events associated with an attenuation of TGFβ signaling.**

1.13 Kaiso and TNBC racial disparity

Mounting evidence supports the idea of genetically-based racial disparities across various human diseases, including cancer. For instance, in prostate cancer it was observed that high Kaiso expression in African American men led to poorer survival outcomes compared with Caucasian men.[55] Similarly, increasing evidence from our lab and others have shown that Kaiso is also highly expressed in TNBC tissues from women of African ancestry (WAA) relative to Caucasian TNBC tissues.[69] We also found a positive correlation between the "percentage" of African Ancestry and Kaiso expression in TNBC tissues.[69] Collectively, this evidence suggests that high Kaiso expression in people of African ancestry may be linked and/or contribute to the racial disparity that is observed within various cancer types. Our lab reported the correlation between high Kaiso expression and WAA with TNBC through immunohistochemistry analyses of a pilot human tissue microarray (TMA) comprised of TNBC tissues from West African (Nigeria)

and Caribbean (Barbados) women that represented more homogenous populations of African ancestry than African American women.[69] Interestingly, the highest Kaiso expression was observed in the Nigerian TNBC tumor tissues, followed by Barbadian TNBC tumor tissues, with the lowest expression observed in the Caucasian TNBC tumor tissues.[69] Upon further examination of the tissue staining, we found increased nuclear Kaiso levels in the patients with higher African ancestry compared to Caucasian women.[49] Collectively, these data support a potential role for Kaiso in the racial disparity associated with TNBC prevalence and outcomes, and may reveal a role for Kaiso as a TNBC diagnostic biomarker. However, there is still much to be uncovered about the mechanisms that Kaiso uses to exacerbate this BC subtype in this select population. **In Chapter 3 of this thesis, we show that Kaiso negatively regulates *miR-31* and that pre-miR-31 expression is inversely correlated with degree of African heritage in TNBC tumor tissues from WAA.**

RATIONALE & RESEARCH GOALS

TNBC is a highly aggressive and metastatic BC subtype, that is most prevalent in WAA.[11,19,70] As TNBCs do not express the biomarkers used to classify and treat other BC subtypes, they lack targeted therapies and thus have poor treatment outcomes. Recent studies have implicated Kaiso in promoting TNBC aggressiveness and metastasis, although the exact mechanisms through which Kaiso achieves this outcome remain unknown.[48,57] Recently, it was shown that Kaiso regulates the tumor suppressing miR-31 and miR-200c in prostate cancer cells, and hinted at a novel role for Kaiso in promoting the migration and invasion of these cells.[33,34] The tumor suppressing miR-200c functions to suppress TNBC invasion and metastasis through the targeting and subsequent inhibition of the metastasis-inducing transcription factor ZEB1.[71,72] Previously, our lab has shown that Kaiso depletion leads to an attenuation of TGFβ signaling, through the decreased expression of this pathway's regulatory genes–including ZEB1, supporting a potential role for Kaiso in miRNA regulation in TNBC.[48] Thus, **we hypothesized that Kaiso functions as a transcriptional repressor to inhibit *miR-31* and *miR-200c* expression, contributing to TNBC tumorigenesis.**

The research objectives of this thesis were to:

(i) Determine the effect of Kaiso depletion on miR-31 and miR-200c expression

(ii) Determine if Kaiso regulates *miR-31* and/or *miR-200c* in TNBC cells

(iii) Determine the effect of Kaiso depletion on the expression of downstream miRNA targets *in vitro* and *in vivo*

(iv) Determine the clinical relevance of the Kaiso/miRNA relationship in a cohort of WAA TNBC tissues

To address these objectives, Kaiso-depleted MDA-MB-231 and Hs578T TNBC cell lines were generated and further utilized to examine the expression of miR-31 and miR-200c in these cells relative to scrambled controls. Parental MDA-MB-231 and Hs578T cells were then treated with and without a demethylation agent and utilized to conduct chromatin immunoprecipitation experiments to determine if Kaiso associates with the promoter regions of these miRNAs in a methylation-dependent manner. Next, I examined the effect of Kaiso depletion on the downstream targets of miR-31 and miR-200c, i.e. WAVE3 and ZEB1. Functional wound-healing assays were then performed to determine how mis-expression of miR-31 and miR-200c would affect TNBC cell motility in parental and Kaiso-depleted TNBC cells. Lastly, I examined the expression of pre-miRNA-31 in a cohort of TNBC tissues from WAA (Nigeria and Barbados) and compared this to pre-miRNA-31 expression in an African American and Caucasian cohort.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Cell Culture

The triple negative breast cancer cell lines used in this study were MDA-MB-231 (hereafter MDA-231) and Hs578T obtained from ATCC (Manassas, VA). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone/Mediatech) and supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone), and 1% penicillin and streptomycin (Invitrogen). Cells were passaged when they reached 95% confluency or culture medium was changed every three (3) days. All cells were grown in a 5% CO_2 humidified incubator at 37°C. For 5-aza-2'-deoxycytidine (5-aza-dC) treatment, cells were treated with 10 μM 5-aza-dC (Sigma, MO, USA) daily for 72 hours before proceeding to chromatin immunoprecipitation experiments.

2.2 Generation of Stable Kaiso-depleted MDA-MB-231 and Hs578T Cells

Kaiso knockdown was attained using the pRetroSuper (pRS) Kaiso vector and its control pRS-empty vector. MDA-231 and Hs578T cells were seeded $6.30x10^5$ cells/ml in 60 mm dishes and allowed to grow for at least 24 hours until the cells were ~50– 60% confluent. Each plate was subsequently transfected with 6 μg of either the pRS-Kaiso plasmid vector or its pRS-Empty control vector by diluting the DNA in serum-free media and vortexing gently before the addition 20 μL of Turbofect transfection reagent (ThermoScientific, USA) according to the manufacturer's protocol and optimizations. The plasmid DNA-Turbofect mixture was vortexed for ~10 seconds and subsequently incubated at room temperature (RT) for 20 minutes. Approximately 15 minutes later, the media from the 60-mm plates of cells was poured off and replaced with 3 mL of fresh serum-supplemented DMEM media. When the 20-minute incubation had commenced, the transfection solution was added to each respective plate in a drop-wise manner. Cells were grown in a 5% CO_2 supplemented humidified incubator at 37°C for 48 hours after which the media/transfection reagent solution was aspirated and replaced with 5 mL of media supplemented with 0.8 μg/mL Puromycin (Invitrogen) for selection. Selection media was replaced every 2-3 days for approximately three weeks until individual colonies formed.

Cells were washed in 1X phosphate buffered saline (PBS) and colonies were separated using cloning caps (Corning Incorporated, NY) and high temperature silicone grease (Dow Corning Incorporated, Midland USA). Once in the cloning caps, colonies were trypsinized using 0.5% Trypsin EDTA diluted to a 1X solution and transferred to 48-well culture plates. Cells were grown until 100% confluency in selection media and expanded out until cells were confluent enough to grow in 100 mm plates. Each colony was then assayed for optimal Kaiso-depletion using immunoblot analysis.

2.3 Western Blot Analysis

Cells were cultured in 100 mm plates until they reached ~90% confluency. Cells were then washed with 5 mL of 1X PBS at pH 7.4 and lysed on ice for 10 minutes with 200 µL Nonidet P40 (NP40) lysis buffer containing 0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM sodium orthovanadate and a complete mini protease inhibitor tablet without EDTA (Roche). Cells were collected using a cell scraper, and lysates were transferred to a 1.5 mL Eppendorf tube and centrifuged at 4°C for 10 minutes at 13,200 rpm to pellet cell debris. The lysate supernatant was quantified by Bradford Assay (Bio-Rad, CA). Protein was run on a 10% SDS-PAGE gel in equal concentrations for 4 hours at 32 mA. The resolved proteins were then transferred onto a nitrocellulose membrane (Bio-Rad, CA) for 2 hours at 140 mA utilizing a semi-dry transfer apparatus (Amersham/ Pharmacia). After transfer, the membrane was blocked in 4% milk in 1X TBS for 30 mins and incubated with the appropriate primary antibody at 4°C overnight. The antibodies used were: anti-Kaiso rabbit polyclonal antibody (1:10,000), anti-WAVE3 polyclonal antibody (1:1000; Cell Signaling Technology), anti-GAPDH polyclonal antibody as loading control (1:10,000; Cell Signaling Technology). The next day, primary antibodies were removed and membranes were washed in 1X TBS pH 7.4 five times for 5 minutes each. Membranes were then incubated at RT for two hours with either horse radish peroxidase (HRP)-conjugated donkey anti-mouse or goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) diluted at 1:10,000 in 4% milk in 1X TBS pH 7.4. After incubation, membranes underwent additional washes (five times for 5 minutes each) and

subsequently imaged using Western Lightning Enhanced Chemiluminescence reagent (PerkinElmer LAS, Inc) according to the manufacturer's protocol. Blots were visualized on Kodak XAR film (Amersham) at various time points.

2.4 qRT-PCR

Small RNAs were isolated from control and Kaiso-depleted MDA-231, Hs578T, and control and Kaiso-overexpressing MCF7 cells at ~95% confluence using the Total RNA Purification Kit (Norgen Biotek) according to the manufacturer's instructions. 10 ng of small RNA from each sample were reverse transcribed using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Thermo Fisher) according to the manufacturer's instructions. The resulting cDNA was used to perform the qRT-PCR reaction using TaqMan® Advanced miRNA Assay primers for miRNA31, miRNA200c and miRNA423 (endogenous control) (Thermo Fisher). Each reaction was performed in triplicate using Applied BioSystems Prism 7900Ht sequence detection system. The quantity of each target was determined by the Applied Biosystems Software powered by the Thermo Fisher Cloud and the data were normalized to miRNA423 expression levels.

2.5 Chromatin Immunoprecipitation (ChIP)

MDA-231 and Hs578T cells were cultured to achieve ~80% confluency and cross-linked with 1% formaldehyde in DMEM medium. The cells were placed on a belly dancer and gently shaken for 10 minutes at room temperature. Formaldehyde fixation was stopped by adding 1M glycine to a final concentration of 125 mM and the cells were rocked for 5 minutes at RT. The cells were then washed twice with 5 mL of ice cold 1X PBS pH7.4, and cells were collected using a cell scraper in 1 mL PBS containing complete Mini Protease Inhibitor Cocktail Tablet (Roche, Mannheim, Germany). The cells were placed in 15 mL falcon tubes and pelleted by centrifugation at 4°C for 5 minutes at 2,000 rpm and the supernatants were aspirated. Cells were re-suspended in 2 mL of ice cold ChIP lysis buffer containing 5 mM PIPES pH 8.0, 85 mM KCL, 0.5% NP-40, and protease inhibitors, and subsequently dounced 10 times with a homogenizer before incubating on

ice for 15 minutes. After incubation, cells were centrifuged at 5,000 rpm for 5 minutes at 4° C and the nuclear pellet was resuspended in 250 µL of Nuclear Lysis Buffer containing 50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS and protease inhibitors prior to incubating on ice for 10 minutes. After incubation, the nuclear pellets were sonicated at 90% duty, 5% power for 5 rounds of 15 second pulses in order to shear chromatin to lengths of ~100-1000 bps. The lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4° C and the supernatant was transferred to a new microfuge tube. 10 µg of chromatin was pre-cleared by incubating end-over-end for 1 hour at 4° C with 5 µL of rabbit IgG (Abcam, Cambridge, MA) in a 500 µL reaction. Fifty µL of salmon sperm-blocked Protein A beads was added to the pre-cleared lysate and rotated end-over-end before centrifuging at 5,000 rpm for 3 minutes. The supernatant was subjected to immunoprecipitation with 4 µg Kaiso 6F monoclonal antibody, 2 µg Histone H3 polyclonal antibody (Abcam) or negative control mouse IgG antibody (Active Motif, Carlsbad, CA) at 4°C and rotated end-over-end overnight. The immunoprecipitated samples were centrifuged at 13,000 rpm for 2 minutes at 4° C before 50 µL of Protein-A rabbit-anti-mouse bridge or Protein-A beads (depending on antibody isotype used for IP) was added to each immunoprecipitated supernatant sample. Samples were rotated end-over-end at 4° C for 1 hour and the precipitated samples were washed as follows at 4° C: 1X with 1 mL RIPA buffer for 10 minutes, 1X with high salt buffer for 10 minutes, 1X with LiCl buffer for 5 minutes and 2X with TE buffer for 10 minutes. After removing the supernatant, 300 µL of 1X TE buffer and 1.5 µL of RNase A (10 mg/mL) was added to the immunoprecipitate and 10% input samples before incubating for 30 minutes at 37°C. 15 µL of 10% SDS and 3.75 µL of proteinase K (20 mg/mL) were added and the samples were incubated at 37°C for a minimum of 4 hours. The samples were then reverse cross-linked overnight at 65°C and DNA was purified using standard phenol-chloroform extraction and ethanol precipitation. The DNA was resuspended in 50 µL of sterile dH_2O and used for PCR amplification.

2.6 ChIP-PCR

One microliter of recovered DNA from each chromatin immunoprecipitated sample was used in a PCR reaction with the following primer pairs:

Table 1: List of primers used for ChIP-PCR with their annealing temperatures and product size

Target	Annealing Temp (ºC)	Product Size (bp)	Sequence (5' – 3')
miR-31	62.0	177	FWD: **AAG GGG TCA CTT TTG CCT AA** REV: **TTG CCC TGT ACA GCT GAG AA**
miR-200c	62.0	229	FWD*:* **AGT GTC TGG GGA AAG ACG TG** REV: **GGC CCA TCT CAT CTT CCT CT**
WAVE3	62.3	277	FWD: **TTGCCAGTCTCTCCAGTGTG** REV: **AAGTGGTGAAAGGTGCCATC**
ZEB1	64.0	413	FWD: **GGGACTGATGGTAGCCCTGC** REV: **GTTTACGACACTCCCGGCTT**

2.7 MiRNA Mimic/AntagomiR Transfection and Wound Healing Assay

MDA-231 and Hs578T cells were seeded in 60 mm cultures dishes to achieve 40-50% confluency on the day of transfection. Each plate was then transfected with either miR-31, miR-200c or control mimic/antagomiR and RNAiMAX Lipofectamine reagent (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. Cells were grown in a 5% CO_2 supplemented humidified incubator at 37°C for 24 hours or until they reached ~90-100% confluency. The transfection media was then aspirated into a 15 mL falcon tube and 2 mL of fresh serum supplemented media was added to the dish. A scratch was then created in the confluent layer of cells using the fine end of a 200 µL pipette tip. The media was then poured off and cells were rinsed with 2 mL of 1X PBS pH 7.4 and an additional 2 mL of fresh media was added to the dish. A perpendicular scratch was made on the bottom of the plate using a razor blade to use as a guide when imaging. The dishes were then placed on a 37°C heated insert and imaged using phase

contrast microscopy. After imaging, the appropriate transfection media was poured back on the cells and cells were put back in the incubator. Subsequent images were taken of the same region of the monolayer at the 24 and 48-hour time interval. The images were analyzed using ImageJ and statistical analysis was performed using GraphPad software. The experiment was performed independently in triplicate.

2.8 Immunohistochemistry

Tissues harvested from the mice xenograft metastatic studies were fixed in 10% formalin and were embedded in paraffin (John Mayberry Histology Facility, McMaster University, ON, CAN) prior to mounting 5 μm thick tissues sections onto slides. Slide sections containing primary tumor MDA-231 or Hs578 cells (parental controls) and Kaiso-depleted were first de-waxed by warming on a slide warmer at 60°C for 20 minutes, followed by de-paraffinization in xylenes 3 times for 5 minutes each and rehydration in a decreasing ethanol gradient (100%, 95%, 70%). Antigen retrieval was performed by boiling slides at low power (10%) for 15-20 minutes in a low pH buffer (6.0) solution (DAKO, Glostrup, Denmark). Endogenous peroxidase activity was quenched through treatment with 3% hydrogen peroxide in 1X PBS. Tissues were blocked for non-specific staining with a solution containing 5% normal donkey serum in 1X PBS for 1 hour at room temperature. Endogenous biotin and avidin binding sites on tissues were blocked using the Avidin/Biotin blocking kit (Vector Labs, CA, USA) prior to and during primary antibody incubations. Tissues were then incubated overnight at 4°C with the anti-WASF3 rabbit antibody (Sigma) at a 1:75 dilution and the anti-ZEB1 rabbit antibody (Santa Cruz) at a 1:200 dilution. The next day, tissues were washed twice with a 1X PBS solution containing 0.05% Tween-20 (PBS-T) and once for 10 minutes each in 1X PBS. Secondary antibody incubations were performed at room temperature for 2 hours with biotinylated, donkey anti-rabbit antibody at a dilution of 1:1000. Subsequent washes were as described above. Tissues were then incubated in Vectastain (Vector Labs) for 30 minutes followed by diaminobenzidine (DAB) incubation (Vector Labs) for 8 minutes in order to visualize immunostaining. Tissues were counterstained with Harris hematoxylin

(Sigma), differentiated in acid ethanol (0.3% HCl in 70% ethanol) and blued in Scott's tap water substitute. Tissues were dehydrated by incubating slides in an ascending ethanol gradient (70%, 95% and 100%). Tissues were then dried in xylenes two times for 5 minutes each, and mounted using Polymount (Polysciences Inc., Warrington, PA, USA).

2.9 BaseScope™ Manual Assay

BaseScope assays were performed in accordance with guidelines provided by the supplier (Advanced Cell Diagnostics - ACD, Newark, CA). 5 μm thick sections were mounted onto Superfrost plus slides (Fisher Scientific, Loughborough, UK) and were then baked at 60°C for 1 hr before deparaffinizing in xylene (2 × 5 min) and ethanol (2 × 2 min), followed by baking at 60 °C for 2 min. Pretreatment condition 1 (hydrogen peroxide) was applied for 10 min at RT, Pretreatment condition 2 (target retrieval) for 15 min at 100°C and Pretreatment condition 3 (protease) for 30 minutes at 40°C, with two rinses in distilled water between pretreatments. BaseScope probes (against pre-miRNA-31) were then applied for 2 hr at 40°C in a HybEZ oven (ACD) before incubation with reagents AMP0 (30 min at 40°C), AMP1 (15 min at 40°C), AMP2 (30 min at 40°C), AMP3 (30 min at 40°C), AMP4 (15 min at 40°C), AMP5 (30 min at RT) and AMP6 (15 min at RT). Slides were rinsed with wash buffer (2 × 2 min) between each AMP incubation. Finally, slides were incubated with Fast Red for 10 min at RT in the dark. Slides were counterstained with 50% Gill's hematoxylin before drying for 15 min at 60°C and mounting in VectaMount permanent mounting medium (Vector labs, Burlingame, CA).

2.10 Statistics

All statistical analyses were performed using GraphPad software. Results are presented at mean ± SD of three experiments. P-values were determined by unpaired students t-tests and one-way ANOVA, Turkey comparison's test. A p value of <0.05 is considered to be statistically significant.

CHAPTER 3 – RESULTS

3.1 Expression of tumor-suppressing microRNAs 31 and 200c inversely correlate with Kaiso expression in TNBC cells

Previous studies by our lab and others have revealed a positive correlation between Kaiso and the metastatic nature of multiple human cancers, supporting a role for Kaiso's involvement in the key processes required for metastasis, such as EMT.[34,45,48] Recent studies by Yates and colleagues found that Kaiso inhibits two miRNAs involved in EMT in prostate cancer; they both suppress downstream targets such as ZEB1/2, SNAIL, SLUG and TWIST that are involved in the EMT process.[33,34] Recently, we found that Kaiso is involved in the TGFβ signaling pathway, with its depletion leading to an attenuation of downstream targets of the pathway, notably the transcription factor ZEB1.[48] Interestingly, both miR-31 and miR-200c are downregulated in highly metastatic and aggressive breast cancers, such as TNBC.[28,73] MiR-200c is a prominent inhibitor of EMT through suppression of TGFβ signaling pathway components such as ZEB1/2.[71,74] In contrast, miR-31 exerts its tumor suppressive effects by inhibiting targets such as WAVE3, GNA13 or RhoA that are involved in cell proliferation and invasion.[73,75] Based on this evidence, we sought to determine whether Kaiso depletion in TNBC cells would affect miR-31 and/or miR-200c expression as was seen in prostate cancer cells. The TNBC cell lines MDA- 231 and Hs578T were used as they both exhibit high Kaiso expression and display basal characteristics similar to most TNBC cases. These cell lines were also chosen as they have previously been reported to express low levels of miR-31 and miR-200c.[23,73]

To ascertain whether Kaiso is involved in the expression of these miRNAs, we used shRNA technology to generate stable Kaiso-depleted (sh-K) MDA-231 and Hs578T cells (**Appendix Figure 1A)**. Next, we performed qRT-PCR analysis using parental control and Kaiso-depleted TNBC cells. Upon efficient Kaiso silencing, there appears to be increased miR-31 and miR-200c expression (**Figure 3.1A and B**).

3.1.1 Kaiso overexpression in a non-TNBC cell line leads to decreased miRNA expression similar to that observed in high Kaiso expressing TNBC cells

To determine if this increase in miR-31 and miR-200 expression was specific to Kaiso depletion, we expressed a sh-resistant murine Kaiso cDNA (mKaiso) in the low Kaiso expressing MCF7 Luminal A BC cell line and subjected these cells to qRT-PCR analysis. As observed in Figure 4C, Kaiso overexpression results in decreased miR-31 and miR-200c expression, compared to MCF7 parental cells transfected with an empty (E) control (Ctrl) vector. Similar to that seen in the parental MDA-231 and Hs578T cells, high Kaiso appears to inhibit the expression of tumor-suppressing miRNAs, perhaps to promote the cell motility and invasion required for BC metastasis.

3.1.2 Kaiso associates with miR-31 and miR-200 promoter regions in a methylation-dependent manner

Previous studies have shown that Kaiso is a potent transcriptional regulator that can act as an activator or repressor.[45,76] Furthermore, Kaiso is a unique dual-specific transcription factor that recognizes and binds sequence-specific Kaiso binding sites (KBS) or methyl-CpG dinucleotides.[37] In TNBC tumor tissues Kaiso is highly expressed in the nucleus.[69] This nuclear localization results in Kaiso's methylation-dependent silencing of various genes, importantly E-cadherin; which in turn promotes cell migration and subsequent metastasis.[50] Interestingly, in TNBC as well as other human cancers, promoter hypermethylation has been found among various miRNAs, including *miR-31* and the *miR-200* family, leading to their subsequent transcriptional deregulation or inhibition.[77] Because Kaiso is highly expressed in TNBC, and participates in methylation-dependent silencing of target genes, we sought to examine Kaiso's function in the regulation of *miR-31* and *miR-200c* in TNBC cells. We first determined whether Kaiso associates with the promoters of *miR-31* and *miR-200c*, and if so, whether Kaiso associated with these miRNAs in a methylation-dependent manner. To this end, we conducted chromatin immunoprecipitation (ChIP) and ChIP-PCR assays on parental control MDA-231 and Hs578T cells that were either untreated (promoter methylation intact) or treated with 5'-

aza to induce DNA demethylation. These assays revealed an enrichment of Kaiso at the proximal promoter regions of both *miR-31* and *miR-200c* in the untreated cells **(Figure 3.2A and B).** In contrast, there was no Kaiso binding observed in cells treated with 5'-aza, suggesting that Kaiso's association with and regulation of the *miR-31* and *miR-200c* promoters was methylation-dependent (**Figure 3.2C and D)**.

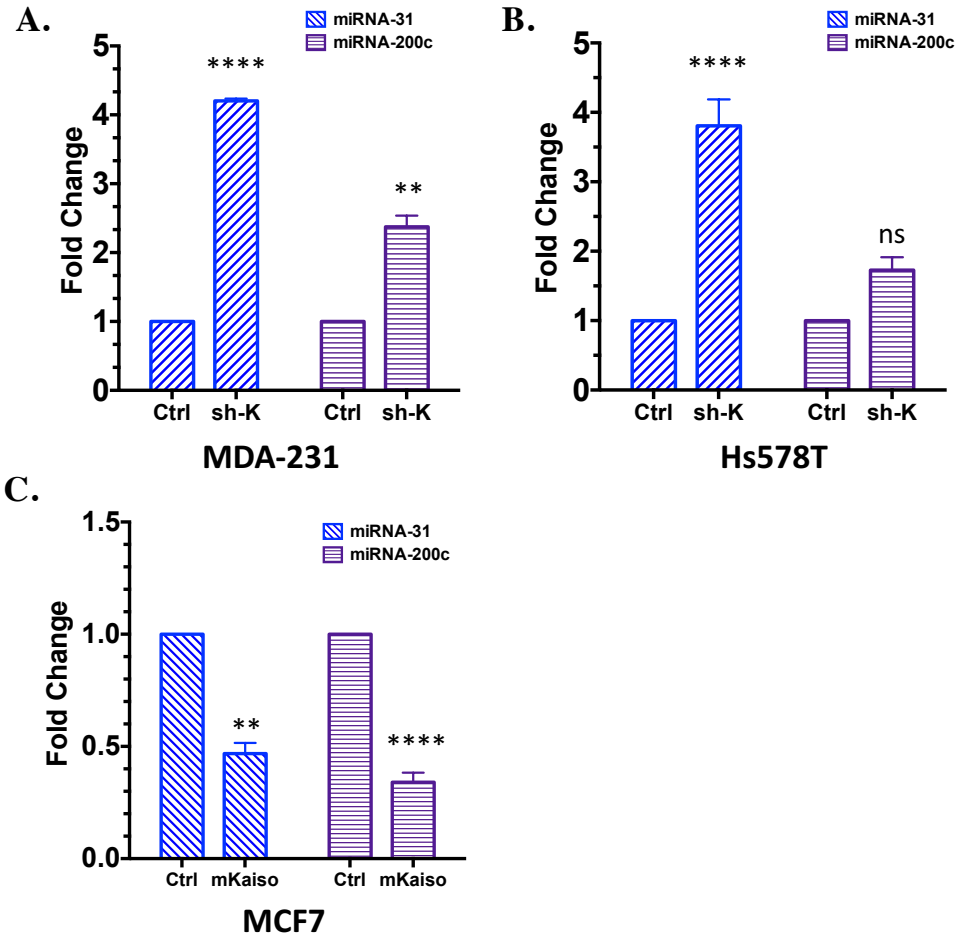

Figure 3.1: miR-31 and miR-200c expression are inversely correlated with Kaiso expression in TNBC cells. (**A**) Kaiso-depleted MDA-231 and **(B)** Hs578T cells exhibit higher mRNA levels of miR-31 and miR-200c relative to control cells. (**C**) MCF7 cells with Kaiso overexpression results in decreased miR-31 and miR-200c expression. ****P<0.0001, **P<0.001, P<0.05 is considered significant.

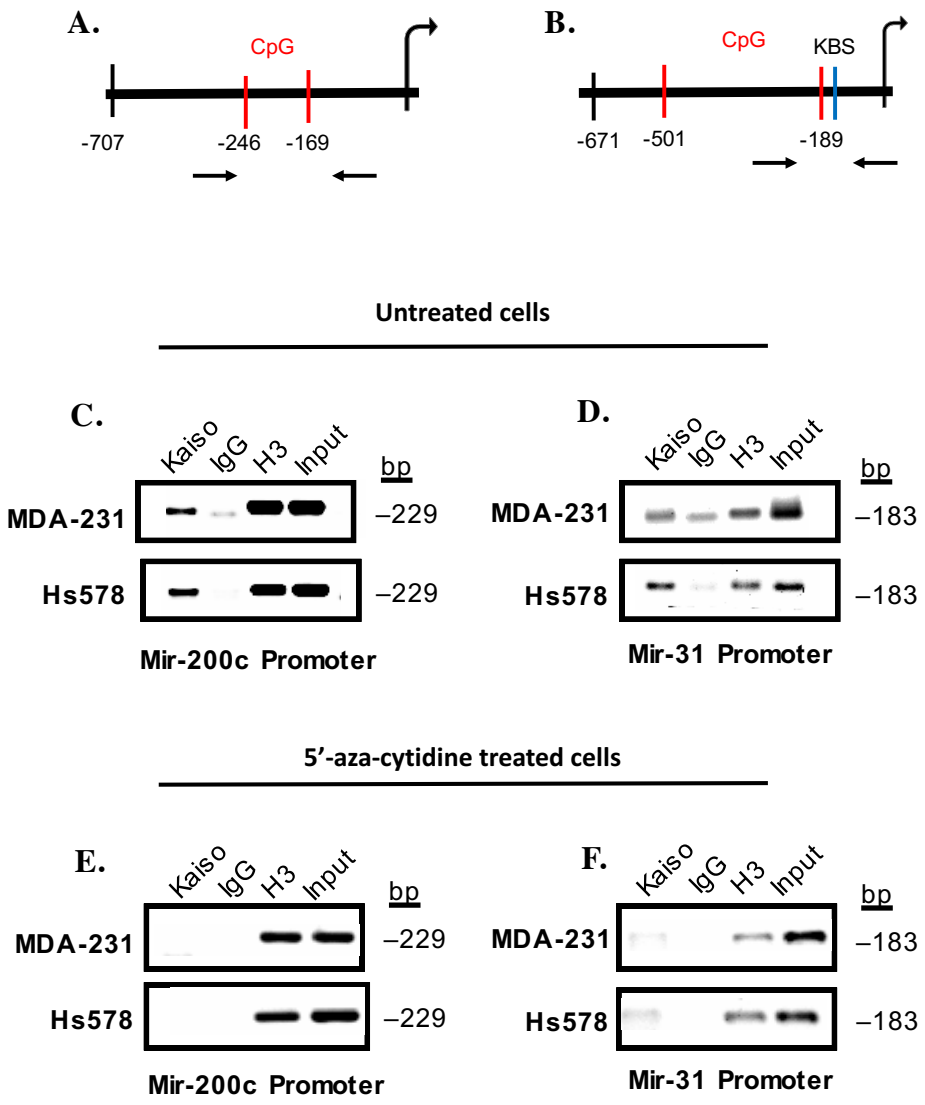

Figure 3.2: Kaiso interacts with the miR-31 and miR-200c promoter regions in a methylation dependent manner. *In silico* analysis of (**A**) miR-200c and (**B**) miR-31 promoter regions. Primer pairs were generated for miR-200c to amplify the methylated CpG region (-246 to -169) and for miR-31 to amplify the methylated CpG region and encompass the core KBS (-189 to -26). ChIP-PCR analysis reveals an enrichment of Kaiso at the miR-200c and miR-31 promotor regions in **(C)** MDA-231 and **(D)** Hs578T TNBC cells. **(E)** Upon 5'-aza treatment, Kaiso binding at the miR-200c and **(F)** miR-31 promoter is abolished.

3.2 Kaiso-depletion attenuates downstream targets of miR-200c and miR-31 in TNBC cells

Multiple studies have found that miR-200c and miR-31 exert their anti-metastatic effects through repression of various genes involved in the metastatic process.[72,74,75] Thus, not surprisingly, in highly metastatic BCs such as TNBC, miR-31 and miR-200c are both downregulated.[23] Subsequently, this leads to an increase of several genes that have been identified as common targets of both these miRNAs, followed by the eventual invasion and metastasis of the cancer cells.

The actin cytoskeleton remodelling protein and member of the WASP/WAVE family, WAVE3, is a common target of both miR-31 and miR-200c.[67] Upon WAVE3 activation, WAVE3 associates with the Arp2/3 complex that regulates the actin cytoskeleton.[68] This then leads to the rapid polymerization of actin filaments necessary for cell motility and ultimately invasion and metastasis.[68] Interestingly, WAVE3 is highly expressed in aggressive breast cancers, including TNBC.[68] Due to its involvement in metastasis and invasion, as well as being a common target between miR-200c and miR-31, we sought to determine whether Kaiso depletion affected WAVE3 expression. Relative to parental control TNBC cells, there appears to be an ~1.5-fold reduction in WAVE3 expression upon Kaiso depletion in MDA-231 and Hs578T cells (**Figure 3.3A and B)**. Similar to the previously conducted qRT-PCR assays, we transiently inhibited and overexpressed murine Kaiso (pRS Kaiso and mKaiso, respectively) in the low Kaiso expressing MCF7 cell line and subjected these cells to western blot analysis. As observed in Figure 3C, Kaiso inhibition results in decreased WAVE3 expression, compared to MCF7 parental cells transfected with an empty (E) vector. Conversely, when Kaiso is overexpressed in MCF7 cells (mKaiso), we see a marked increase in WAVE3 expression (**Figure 3.3D)**, similar to the levels observed in the less invasive Hs578T cells. Interestingly, ChIP-PCR assays revealed an enrichment of Kaiso at the WAVE3 proximal promoter region, suggesting that Kaiso may be directly regulating WAVE3 expression in TNBC cells (**Figure 3.3E**).

3.2.1 Kaiso-depletion attenuates downstream targets of miR-200c and miR-31 in TNBC cells in vivo

Previous studies found that ZEB1 and the miR-200 family participate in an autoregulatory feedback loop whereby miR-200 represses ZEB1 in order to promote an epithelial state.[78] However at the same time ZEB1 can also repress the miR-200 family members (miR-200a/b/c and miR-141) to promote EMT and subsequent cell invasion and metastasis.[74] Since recent studies from our lab found Kaiso-depletion attenuates ZEB1 expression, this may in part be due to an attenuation of the miR-200-ZEB feedback loop.[48]

To ascertain whether the *in vitro* effects of Kaiso depletion on the expression of downstream targets of miR-31 and miR-200c would be sustained *in vivo,* we examined MDA-231 and Hs578T TNBC cells xenografted into the mammary fat pads of immuno-compromised mice.[48,57] Consistent with our *in vitro* findings, IHC analysis of the Kaiso-depleted MDA-231 xenografts revealed reduced cytoplasmic and nuclear WAVE3 and nuclear ZEB1 expression (**Figure 3.4C and D)**. Similarly, the Kaiso-depleted Hs578T xenografts, exhibited decreased cytoplasmic and nuclear WAVE3 expression (**Figure 3.4A)** as well as reduced nuclear ZEB1 expression (**Figure 3.4B**).

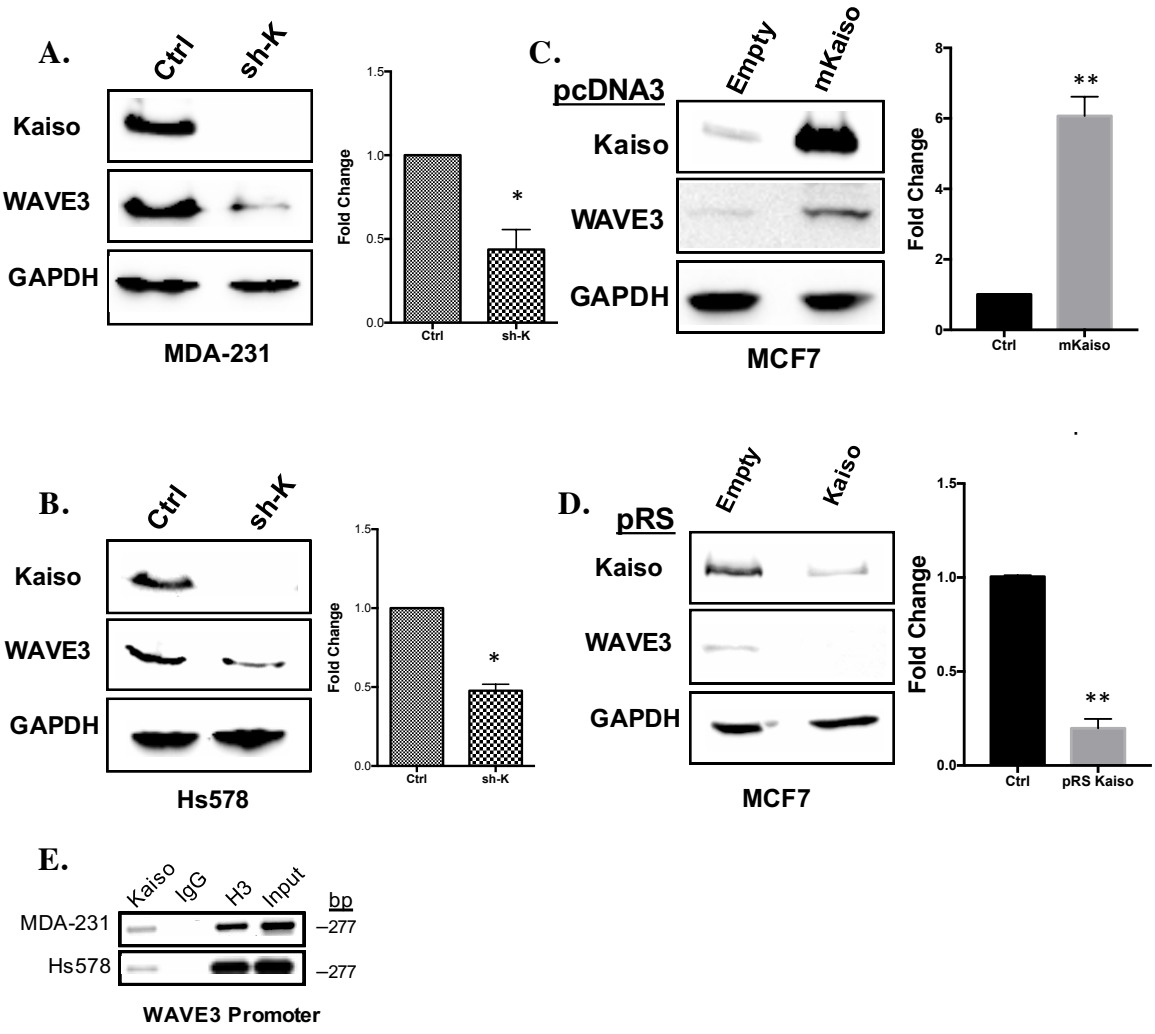

Figure 3.3: Inverse correlation between Kaiso and miR-31 and miR-200c downstream target WAVE3. 50μg of protein extracts from (**A**) Kaiso-depleted (sh-K), parental control (ctrl) MDA-231 and (**B**) Hs578T cells were used to perform WB analysis using antibodies against Kaiso, WAVE3 and GADPH as loading control. Upon Kaiso depletion, there appears to be 1.5-fold reduction in WAVE3 expression relative to control. (**C**) Control MCF7 cells underwent Kaiso depletion (pRS Kaiso) and (**D**) Kaiso overexpression (mKaiso) and the subsequent WAVE3 protein expression was observed. **(E)** ChIP-PCR analysis reveals faint enrichment of Kaiso at the WAVE3 promoter.

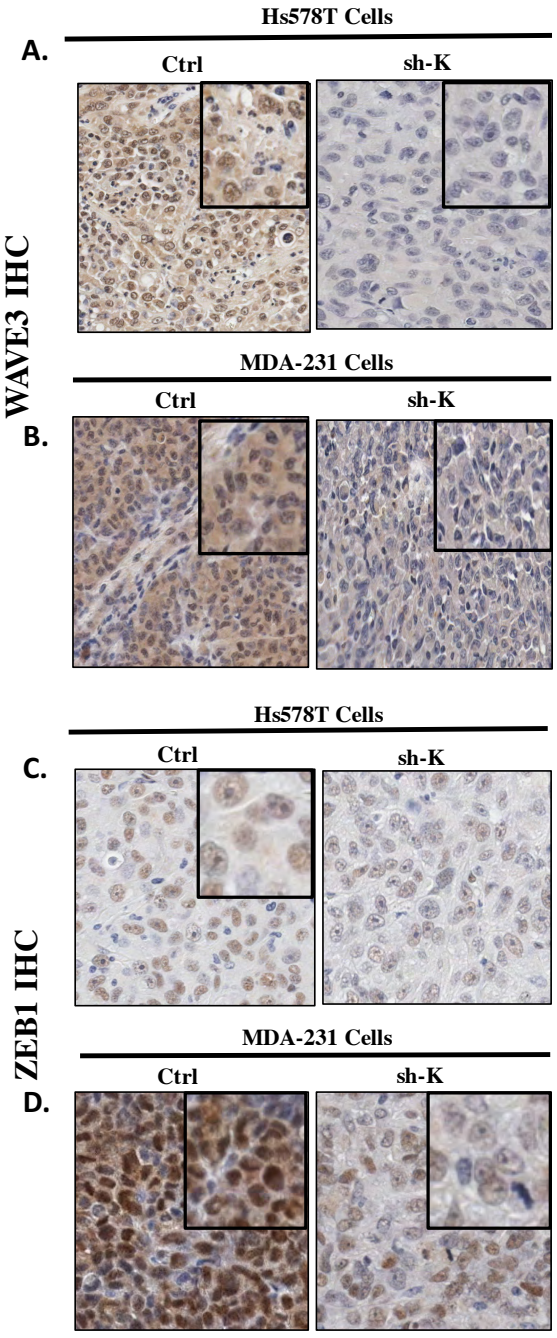

Figure 3.4: Kaiso depletion attenuates WAVE3 and ZEB1 expression in TNBC cells *in vivo*. (**A**) Kaiso-depleted Hs578T and (**B**) MDA-231 xenografts exhibit decreased WAVE3 expression levels relative to control. (**C**) Kaiso-depleted Hs578T and (**D**) MDA-231 xenografts exhibit decreased ZEB1 expression levels relative to control.

3.3 miR-31 and miR-200c overexpression decreases WAVE3 and ZEB1 expression in parental control TNBC cells

To further investigate the impact of miR-31 and miR-200c regulation via Kaiso, we used commercially purchased synthetically engineered oligonucleotides to either mimic or antagonize miR-31 and miR-200c expression in parental and Kaiso-depleted TNBC cells and examined the subsequent expression of their downstream targets WAVE3 and ZEB1.

As expected, when parental (ctrl) MDA-231 and Hs578T cells were transiently transfected with miR-31 and miR-200c mimics, we observed reduced WAVE3 expression relative to parental control cells (**Figure 3.5A and C)**. These results were obtained in two independent trials and using densitometry analysis, we found that miR-31 and miR-200c overexpression resulted in an ~ 2.5-fold reduction in both the MDA-231 and Hs578T cells (**Figure 3.5B and D)**. Unpaired sample t-test results showed that these finding were statistically significant for the MDA-231 cells for miR-31 and miR-200c ($p=0.008$ and $p=0.010$) as well as for the Hs578T cells for miR-31 and miR-200c ($p=0.001$ and $p=0.004$). Conversely, when antagomiRs (antag) were transiently transfected into the Kaiso-depleted TNBC cells, we observed increased WAVE3 expression relative to parental control cells (**Figure 3.5E and G)**. Densitometry analysis revealed an ~ 1.5-fold increase in WAVE3 expression in both the MDA-231 and Hs578T cells for both miR-31 and miR-200c antagomiR conditions (**Figure 3.5F and H)**. These results were statistically significant for the MDA-231 sh-K cells for miR-31 and miR-200c ($p=0.016$ and $p=0.022$) as well as the Hs578T sh-K cells ($p=0.032$ and $p=0.003$).

Consistent with previous studies, when control MDA-231 and Hs578T cells are transfected with miR-31 and miR-200c mimics, we observed decreased ZEB1 expression in cells treated with miR-200c, but not miR-31 (**Figure 3.6A and C)**. Using densitometry analysis, we observed an ~ 1.5-fold reduction in ZEB1 expression in the MDA-231 and Hs578T cells upon treatment with miR-200c (**Figure 3.6B and D)**. Unpaired t-test results showed this reduction was statistically significant for MDA-231 cells ($p=0.002$) and the

Hs578T cells (p=0.011). To further tease apart this Kaiso-ZEB1-miR-200 relationship, Kaiso-depleted cells were transiently treated with antagomiRs against both miR-31 and miR-200c. Under these conditions (Kaiso depletion coupled with miR-200c inhibition) we observed a statistically significant increase (~1.5-fold) in ZEB1 expression relative to parental control cells in MDA-231 cells (p=0.026) (**Figure 3.6E).** Although treatment with miR-200c antagomiRs did not elicit a statistically significant increase in ZEB1 expression in Hs578T cells, there was a subtle increase observed relative to control cells **(Figure 3.6G)**. Notably, when we performed ChIP-PCR using primers designed against a core KBS site in the ZEB1 promoter region, no Kaiso binding was observed (**Appendix, Figure A2).** This suggested that Kaiso regulates ZEB1 expression indirectly through miR-200c. Collectively, these data support a role for Kaiso in the miR-200-ZEB1 feedback loop, and suggests that Kaiso and ZEB1 may function in partnership to inhibit miR-200c expression during tumorigenesis.

Further misexpression experiments were performed to unravel the nature of the Kaiso/miRNA relationship. Interestingly, when parental MDA-231 and Hs578T cells were transfected with antagomiRs against both miRNAs, there did not appear to be any subsequent increase in ZEB1 or WAVE3 expression in both conditions **(Appendix Figure 4A)**. However, when the Kaiso-depleted cells were treated with mimics of miR-31 and miR-200c, there was a slight decrease in ZEB1 expression observed **(Appendix Figure 5A)**. Collectively, these results suggest that in the parental control TNBC cells, miR-31 and miR-200c expression levels are likely already too low for further suppression with the antagomiRs, which would explain why we did not see any changes in WAVE3 or ZEB1 protein expression. This theory is supported by previous studies using qRT-PCR analysis which found that miR-31 and miR-200c levels in these cell lines are very low, with one study specifically showing that miR-31 expression was undetectable in MDA-231 cells.[32,73–75,79]

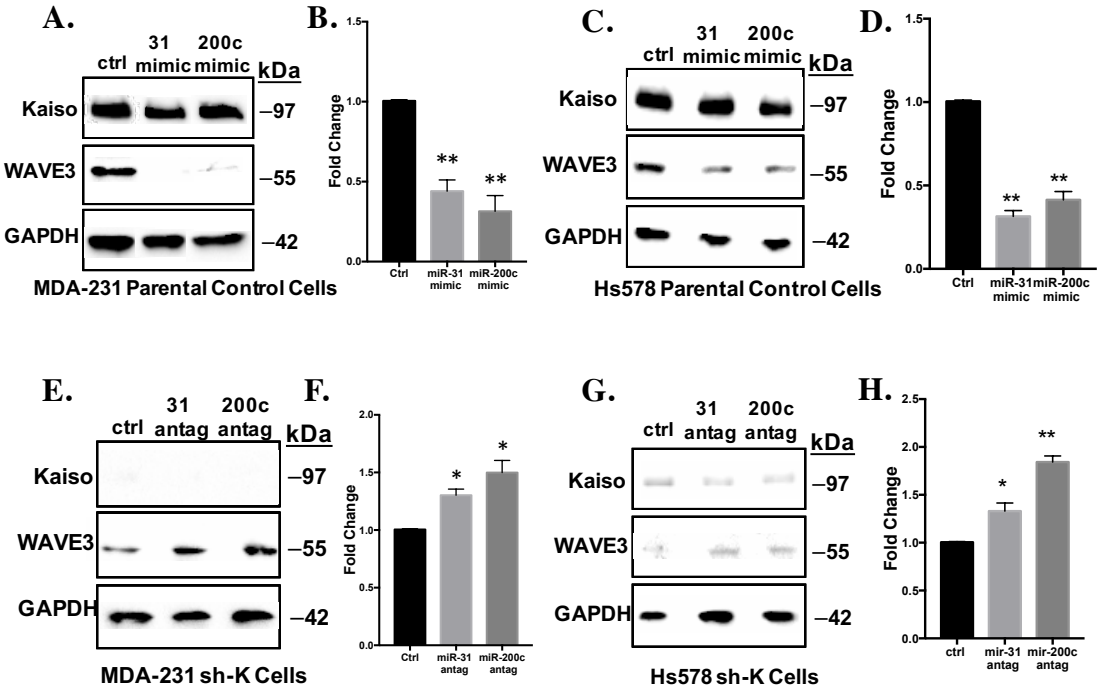

Figure 3.5: miR-31 and miR-200c overexpression decreases WAVE3 expression and inhibition increases WAVE3 expression in MDA-231 and Hs578T TNBC cells. (**A and C**) Parental control MDA-231 and Hs578T cells were transfected with mimics against miR-31 and miR-200c and subjected to western blot analysis. (**B and D**) Densitometry analysis of control cells treated with mimics and reveal miR-31 and miR-200c overexpression reduces WAVE3 expression in these cells. (**E and G**) Kaiso-depleted (sh-K) MDA-231 and Hs578T cells were transfected with inhibitors against miR-31 and miR-200c. (**F and H**) Densitometry analysis of Kaiso-depleted cells treated with antagomiRs and reveal miR-31 and miR-200c inhibition increases WAVE3 expression in these cells. 35 μg of protein was isolated and used to perform WB analysis using antibodies against Kaiso, WAVE3 and GADPH as loading control.

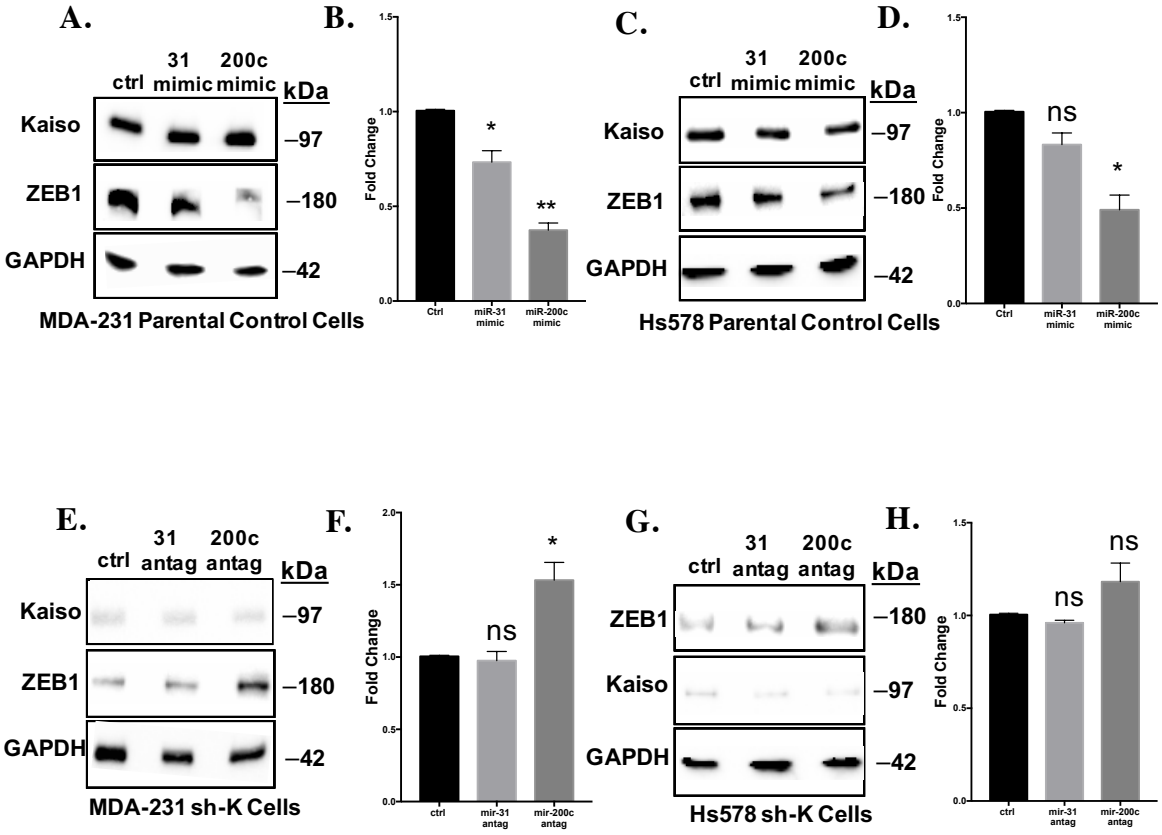

Figure 3.6: miR-200c but not miR-31 overexpression decreases ZEB1 expression and miR-200c inhibition increases ZEB1 expression in MDA-231 and Hs578T TNBC cells. (**A and C**) Parental control MDA-231 and Hs578T cells were transfected with mimics against miR-31 and miR-200c and subjected to western blot analysis. (**B and D**) Densitometry analysis of control cells treated with mimics revealed that miR-200c overexpression significantly reduced ZEB1 expression (**E and G**) Kaiso-depleted (sh-K) MDA-231 and Hs578T cells were transfected with antagomiRs against miR-31 and miR-200c. (**F and H**) Densitometry analysis of Kaiso-depleted cells treated with antagomiRs revealed that miR-200c inhibition significantly increased ZEB1 expression. 35 μg of protein was isolated and used to perform WB analysis using antibodies against Kaiso, ZEB1 and GADPH as loading control.

3.4 miR-31 and miR-200c overexpression reduces cell motility in parental TNBC cells, and their inhibition increases cell motility in Kaiso-depleted TNBC cells

Cell motility and invasion are tumor progression processes that require dynamic actin cytoskeleton remodelling events to allow for cell movement from the primary tumor site.[68] As TNBCs are well known for their increased invasiveness and metastatic potential relative to other BC subtypes, identifying the key contributors/players to these metastatic events is critical. To gain insight into the effect of miR-31 and miR-200c misexpression on cell motility and invasion in control and Kaiso-depleted MDA-231 and Hs578T cells, wound-healing assays were performed. This assay was performed over a 24-hour period with images obtained at the 0-hour and 24-hour time points after transfection with miRNA mimics for miR-31 and miR-200c in parental MDA-231 and Hs578T cells and antagomiRs against miR-31 and miR-200c in Kaiso-depleted MDA-231 and Hs578T cells. Overexpression of miR-31 and miR-200c reduces the migratory capacity of both the MDA-231 and Hs578T TNBC cell lines compared to parental control, and this was statistically significant for cells transfected with miR-31 ($p=0.043$ and $p=0.022$) and miR-200c ($p=0.047$ and 0.0012) **(Figures 3.7A-B and 3.8A-B)**. To further tease apart the Kaiso/miRNA relationship in the context of cell motility, we next transfected Kaiso-depleted MDA-231 and Hs578T cells with inhibitors against miR-31 and miR-200c and repeated the wound-healing assay. In the MDA-231 Kaiso-depleted cells transfected with inhibitors against miR-31 and miR-200c, there was a significant increase in cell motility ($p=0.004$ and $p=0.013$). This trend was also observed in the Hs578T Kaiso-depleted cells, with miR-200c inhibited cells displaying a significant increase ($p=0.022$) in cell motility relative to parental Kaiso-expressing cells (**Figure 3.8C-D).** Together this data suggests that the Kaiso/miR-31/miR-200c interplay may be driving cell invasion and migration that is frequently observed in TNBC.[17,30]

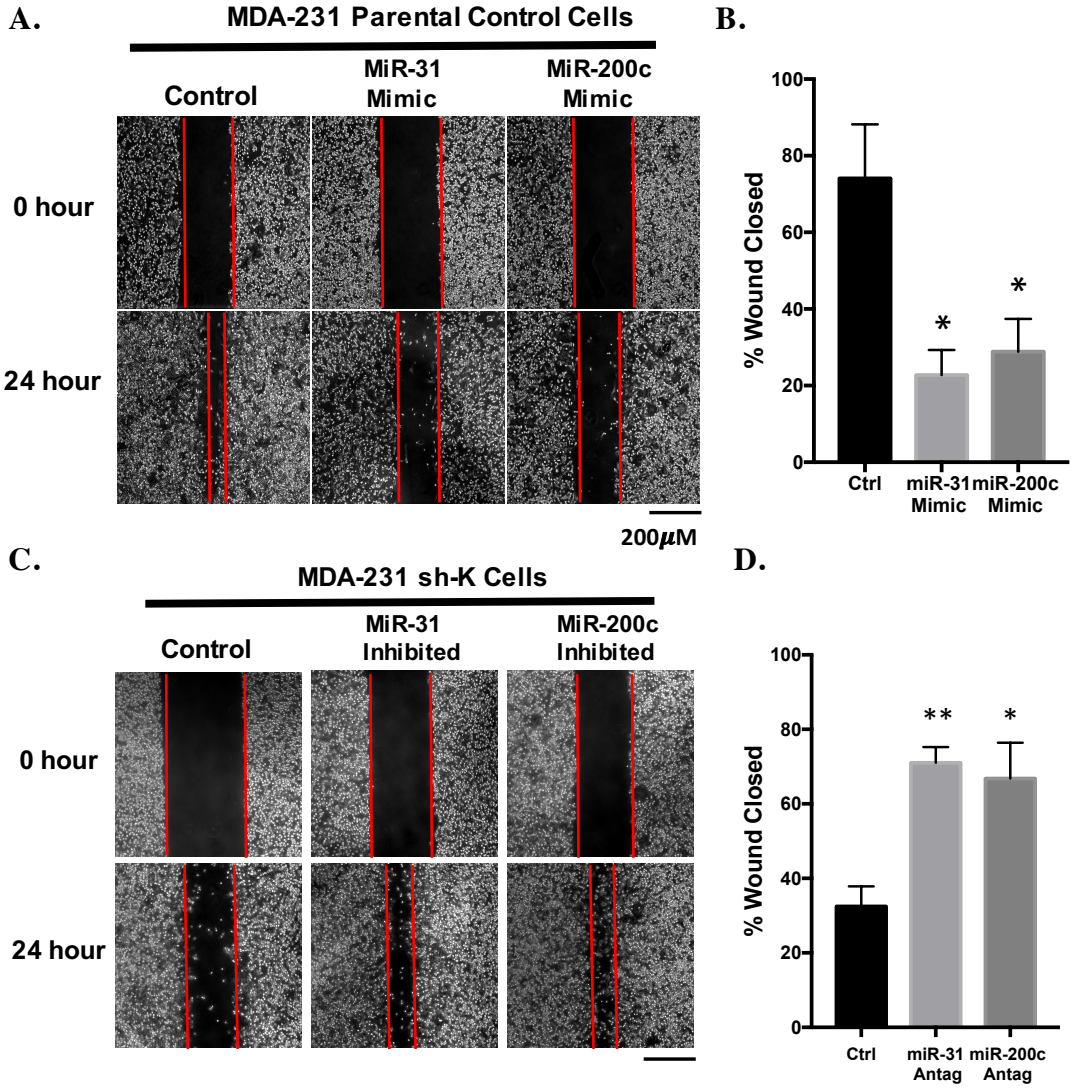

Figure 3.7: miR-31 and miR-200c overexpression reduces cell motility and inhibition increases cell motility in parental and Kaiso-depleted MDA-231 TNBC cells. (**A**) Parental control MDA-231 cells were transfected with mimics for miR-31 and miR-200c and (**C**) Kaiso-depleted MDA-231 cells were transfected with antagomiRs against miR-31 and miR-200c. Cells underwent wound healing assays to examine the effects of these miRNAs on cell motility was measured and showed miR-31 and miR-200c overexpression decreased cell motility **(B)** and **(D)** miR-31 and miR-200c inhibition increased cell motility in sh-K cells**.**

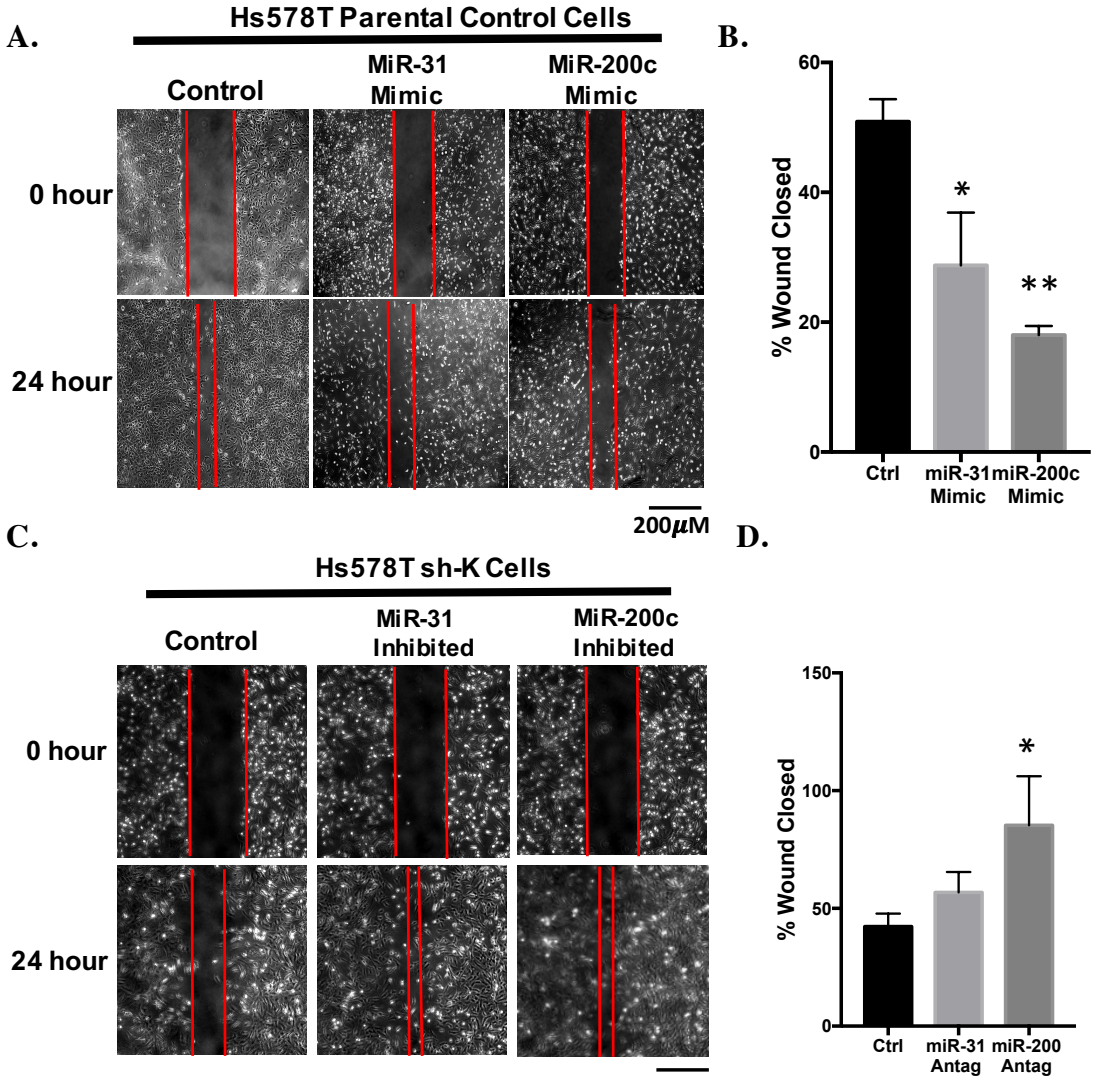

Figure 3.8: miR-31 and miR-200c overexpression reduces cell motility and inhibition increases cell motility in parental and Kaiso-depleted Hs578T TNBC cells. (**A**) Parental control Hs578T cells were transfected with mimics for miR-31 and miR-200c and (**C**) Kaiso-depleted Hs578T cells were transfected with antagomiRs against miR-31 and miR-200c. Cells underwent wound healing assays to examine the effects of these miRNAs on cell motility was measured **(B and D).**

3.5 Kaiso, miR-31 and miR-200c expression are linked to overall survival and TNBC racial disparity

3.5.1 High miR-31 and miR-200c expression correlate with improved prognosis in BCa patients

Previously, we found that Kaiso is more highly expressed in TNBC relative to other BC subtypes, and nuclear Kaiso has been linked to EMT and aggressiveness in TNBC.[69] Specifically, Kaplan-Meier survival curves revealed that patients with higher Kaiso transcript expression had worse survival compared to patients with lower Kaiso expression.[34] Interestingly, there appears to be an inverse trend between miR-31 and miR-200c expression and survival in invasive breast cancers. Using the PROGmiRV2 Pan Cancer miRNA Prognostics Database[62], we found that high expression of miR-31 and miR-200c individually, correlates with better overall survival, (**Figure 3.9A and B)** and this was statistically significant for miR-31 expression ($p=0.002$) but not miR-200c expression ($p=0.093$).

3.5.2 Pre-miR-31 expression correlates with degree of African Ancestry in WAA and Caucasian TNBC tissues

To further explore the role of Kaiso in TNBC racial disparity, we examined the miRNA expression differences in TNBC tumor tissues among a mixed ethnic cohort. Based on our earlier findings that miR-31 expression levels correlated significantly with overall survival, we examine its expression in subsequent analyses. Using a tissue microarray (TMA) consisting of African American and Caucasian TNBC tumor tissues, pre-miRNA-31 expression was assessed using BaseScope™ technology. We also assessed pre-miRNA-31 expression in our previously constructed TMA comprised of Nigerian and Barbadian TNBC tissues that represented cohorts with a higher degree of African heritage, and compared the pre-miRNA-31 expression levels to the Caucasian and African American cohorts. pre-miRNA-31 expression levels were quantified using the manufacturer's suggested scoring methodology. Although there were no significant differences observed between the various ethnicities, there did appear to be a trend

towards an inverse relationship between pre-miR-31 expression and degree of African Ancestry (**Figure 3.9C**).

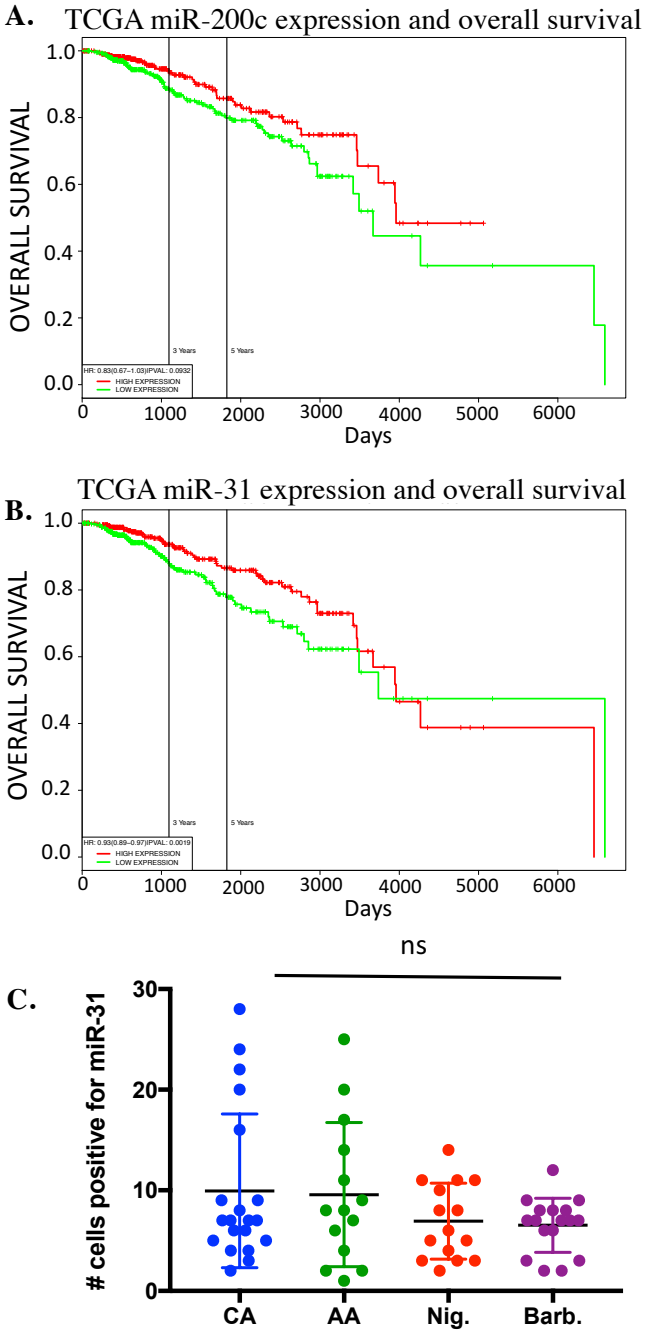

Figure 3.9: miR-31 and miR-200c expression correlate with BC survival in WAA TNBC tissues of various ethnicities. (**A**) Using the progmiR online tool, extracted TCGA Breast invasive carcinoma data show high miR-31 and (**B**) miR-200c expression are associated with improved overall survival. Cohort divided at median of miRNA expression. (**C**) Comparative analysis of pre-miR-31 expression in a cohort of Caucasian, African American, Nigerian and Barbadian TNBC tumor tissues using the BaseScope assay reveal an inverse trend between degree of African Ancestry and pre-miR-31 expression.

CHAPTER 4 – DISCUSSION

TNBCs currently represent a clinical challenge due to their highly metastatic nature and lack of targeted therapies.[11,70] Thus, there is a great need to better understand the underlying molecular genetic mechanisms underlying TNBC so that targeted and more effective therapeutic options may be developed. Emerging evidence indicates that microRNA deregulation may be an essential factor in TNBC tumorigenesis.[23] Indeed, several aberrantly expressed miRNAs have been linked with TNBC growth and progression including miR-31 and miR-200c that functions to suppress the metastatic potential of tumor cells via the silencing of pro-metastatic genes like WAVE3 and ZEB1/2. [23,34,71,73,75] Specifically, both miR-31 and miR-200c are downregulated in triple negative tumors.[23,31] While the ZEB transcription factors have been shown to repress miR-200 expression in a reciprocal feedback loop that modulates epithelial cell plasticity in TNBC and other cell types, the exact mechanisms via which miR-31 is repressed in TNBC cells remains relatively unknown.[24]

Recently, Jones and colleagues found that the transcription factor Kaiso attenuates miR-31 and miR-200c expression in prostate cancer cells, which resulted in the increased migration and invasion of these cells.[33,34] Interestingly, we and others have shown that Kaiso is highly expressed in TNBC.[56,57,69] We therefore wondered whether Kaiso may transcriptionally regulate miR-31 and miR-200c in TNBC cells, similar to what was previously found in prostate cancer cells. Since we have also shown that Kaiso depletion results in the reduced expression of the TGF-β target gene ZEB1 that represses miR-200 (Appendix, Figure 3A), we also wondered whether Kaiso might modulate miR-200c expression in part via promotion of ZEB1 expression in TNBC cells. The current study was conducted to address these questions and unveil the potential relationship between Kaiso, miR-31 and miR-200c in TNBC progression.

4.1 Kaiso depletion attenuates miR-31 and miR-200c expression in TNBC cells

As a first step towards examining Kaiso's role in miR-31 and miR-200c regulation, we established stable Kaiso depletion in two aggressive and invasive TNBC cell lines – MDA-231 and Hs578T. Similar to what was reported by Yates and colleagues in prostate cancer cells, we observed a significant increase in miR-31 and miR-200c expression upon stable Kaiso depletion in MDA-231 and Hs578T cells.[34,54] Complementary studies via Kaiso overexpression in the low Kaiso expressing MCF7 breast tumor cell line, showed a drastic reduction in both miR-31 and miR-200c levels upon Kaiso overexpression in MCF7 cells. Importantly, in *in vitro* studies, Kaiso depletion resulted in a decrease in the expression of the miR-200c target, ZEB1 and the actin cytoskeleton remodeling protein WAVE3, a downstream target shared by both miR-31 and miR-200c.[67] The same trend was found *in vivo*, where reduced ZEB1 and WAVE3 expression was observed in Kaiso-depleted MDA-231 xenograft tissues compared to control MDA-231 xenograft tissues **(Figure 3.4)**. These findings suggest the plausibility of miR-31 and miR-200c as *bona fide* Kaiso target genes.

4.1.1 Kaiso directly modulates miR-31 and miR-200c expression in TNBC cells

To support this theory, additional *in vitro* studies revealed that Kaiso associates with the proximal promoter regions of miR-31 and miR-200c **(Figure 3.1)**. This binding was methylation-dependent as a loss of Kaiso binding to the proximal promoter regions of miR-31 and miR-200c was seen when control TNBC cells were treated with the demethylating agent – 5'-aza **(Figure 3.2)**. Similar results were also observed in prostate cancer cells[34,54] and indicates that Kaiso may regulate miR-31 and miR-200c expression across multiple cell types in a methylation-dependent manner. Increasing studies suggest that DNA methylation is crucial in the abnormal gene expression changes associated with tumorigenesis.[80] Although the mechanisms responsible for inducing these methylation-dependent changes still remain largely unclear, it appears that Kaiso may exact some of its tumorigenic roles in TNBC and other cancers in a methylation dependent manner.[58]

4.2 Kaiso's effects on TNBC cell migration in TNBC cells is miR-200c dependent

Previous studies in our lab and others indicates a role for Kaiso in TNBC cell migration.[57,81] However, the mechanism(s) via which Kaiso influences migration in TNBC cells is still unclear although Kaiso has been implicated in the regulation of the EMT process that is increasingly linked to the motility and invasion of several cancer types including breast cancers.[34,82] EMT is associated with a with major re-organization of the actin cytoskeleton, which is perpetrated either directly or indirectly by various proteins such as WAVE3 and ZEB1 that are increased at the molecular level during the process of EMT.[63,67] Interestingly, we show that both WAVE3 and ZEB1 are downregulated by miR-200c but not miR-31 in TNBC cells (that is miR-31 only downregulates WAVE3 consistently but not ZEB1 across all TNBC cells examined) **(Figures 3.5 and 3.6)**. Expression of miR-31 and miR-200c mimics in parental high Kaiso-expressing MDA-231 and Hs578T cells resulted in reduced cell motility in these cells **(Figure 3.7A and 3.8A)**. However, when Kaiso-depleted cells were treated with antagomiRs against miR-31 and miR-200c respectively, only miR-200c antagomiRs resulted in an increase in cell migration **(Figure 3.7C-D and 3.8C-D)** in both Kaiso-depleted MDA-231 and Hs578T cells. Inhibition of miR-31 expression only resulted in increased migration in Kaiso-depleted MDA-231 **(Figure 3.7C-D)** but not Kaiso-depleted Hs578T cells **(Figures 3.8C-D)**. The sustained effect of miR-200c on migration across multiple TNBC cell types may likely be due to its inhibitory effects on both WAVE3 and ZEB1 expression in both cell types **(Figures 3.5 and 3.6; See Model in Figure 4.1)**. Functionally, this suggests that Kaiso may consistently regulate TNBC cell migration in part via miR-200c repression.

To further extend these findings, *in vivo* studies should be conducted using miR-200c overexpressing high-Kaiso TNBC cells (MDA-231 and Hs578) to see if miR-200c expression will attenuate the migration and metastatic abilities of these cells. In the event that miR-200c expression attenuates TNBC cell invasion and metastasis, subsequent *in vivo* studies using the miR-200c antagomiRs in Kaiso-depleted TNBC cells could be performed to further characterize and validate the role of miR-200c in Kaiso's mechanism of action in TNBC cell metastasis.

4.3 Kaiso, miR-31 and miR-200c expression and TNBC racial disparity: is there a link?

While it is now well known that there is a racial disparity in TNBC prevalence and outcomes, there is little research currently aimed at deciphering its possible genetic origin. Recent studies in our lab have reported high levels of nuclear Kaiso in TNBC patients with higher levels of African heritage compared to Caucasian women, which suggests a potential role for Kaiso in TNBC racial disparity.[69] Remarkably, although both miR-200c and miR-31 expression were found to positively correlate with survival in a subset of invasive BCs **(Figure 3.9A and B)**, there was no ethnicity information available for further stratification of the data to establish a relationship between these miRNAs and African ancestry. Nonetheless, a recent study has revealed a differential miRNA expression profile among African American and non-Hispanic white women with TNBC.[31] Among the miRNAs analyzed in the study, miR-200c was shown to be differentially expressed among African American and Caucasian TNBC tumors, supporting a role for miRNA misexpression in TNBC racial disparity.[31] Specifically, low miR-200c expression was observed in African American TNBC tumors compared with their Caucasian counterparts.[31]

Given the fact that there are currently no studies that have reported on ethnic differences in miR-31 expression, our preliminary finding that miR-31 expression inversely correlates with TNBC in women of higher degree of African ancestry relative to African American cohorts **(Figure 3.9C)** suggests that miR-31 like miR-200c may play a role in

the racial disparities in TNBC outcomes. The lack of statistical significance between miR-31 expression and TNBC in WAA compared to their Caucasian counterparts may be likely due to the small tissue sample size (~N=15) for each cohort that could be scored due to multiple TNBC tumor tissues failing initial quality control. However, our preliminary findings are intriguing and warrant further investigation of both mIR-31 and miR-200c in a larger cohort of TNBC tissues to achieve adequate statistical power.

Conclusions and Significance

Studies published in the past several years have revealed numerous roles for Kaiso in human tumorigenesis. However, there is still a large knowledge gap regarding the biological role of Kaiso in triple negative breast cancers especially in the context of miRNA deregulation. While there has been significant progress made in elucidating miRNA targets and their downstream effects, there is still very little known about the upstream mechanisms responsible for promoting or inhibiting miRNA expression during oncogenesis. This study is the first to examine a role for the transcription factor Kaiso in the regulation of the tumor-suppressive miR-31 and -200c in TNBC. Herein, we reveal a novel role for Kaiso in the inhibition of miR-31 and miR-200c, and provide further insight into the diverse molecular functions of Kaiso in breast tumor cells. Importantly, we show that Kaiso depletion increases the expression of these miRNAs, which are known negative regulators of TNBC cell invasion, migration and EMT. Collectively, our findings have led us to generate a model of Kaiso's potential biological role in TNBC migration in the context of miR-31 and miR-200c where inhibition of tumor-suppressing miRNAs in high-Kaiso expressing TNBC cells promote cell migration **(Figure 4.1)**. While more research is needed to further elucidate Kaiso's role in TNBC metastasis in the context of miR-31 and miR-200c deregulation, our findings build on previous reports that Kaiso functions in an oncogenic manner to induce tumorigenesis in triple-negative breast tumors.

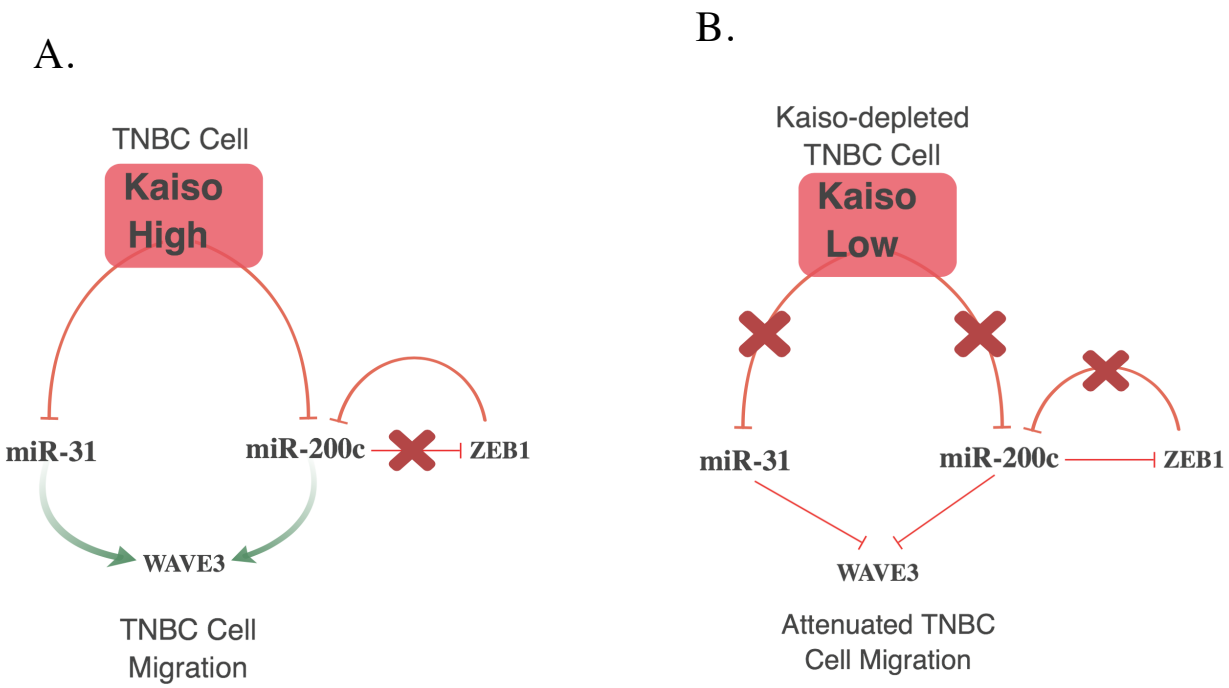

Figure 4.1: A potential model for Kaiso's regulation of miR-31 and miR-200c in TNBC cells. (A) High Kaiso expression in TNBC cells undergo inhibition of mir-31 and 200c, which alleviates their suppression of WAVE3 and miR-200c's suppression of ZEB1, which in turn function to promote TNBC cell migration. **(B)** Upon Kaiso depletion, we propose a mechanism whereby Kaiso is unable to suppress miR-31 and 200c, which then function to silence their targets WAVE3, and ZEB1, leading to an attenuation of TNBC cell migration.

CHAPTER 5 – KAISO'S ROLE IN TNBC CELL GROWTH AND SURVIVAL

Kaiso depletion attenuates the growth and survival of triple negative breast cancer cells. Bassey-Archibong BI, Rayner LG, Hercules SM, Aarts CW, Dvorkin-Gheva A, Bramson, JL, Hassell JA & Daniel JM. (Cell Death Dis. 2017; 8(3) e2689 Doi.1038/cddis.2017.92)

Preface

This chapter contains the published journal article entitled: "Kaiso depletion attenuates the growth and survival of triple negative breast cancer cells." **by Bassey-Archibong *et al.* This is an open-access article, which permits unrestricted use, reproduction and distribution in any format, provided necessary credit is given to the author and attribution parties, and properly cited.**

This manuscript reports the effects of Kaiso depletion on TNBC cell proliferation and survival. We show that upon Kaiso depletion, TNBC cells show reduced proliferation and exhibit delayed tumor growth, along with increased apoptosis *in vivo* and *in vitro.* More importantly, we also report a positive correlation between high Kaiso and BRCA1 mRNA expression and poor overall survival in TNBC patients. These findings suggest an important role for Kaiso in TNBC promotion & progression.

Contributions

BI Bassey-Archibong wrote the manuscript and conducted the experiments for the data found in Figures 1A-C, 1E, Figure 2A, C-D, Figure 3B, Figures 4A-E, Figures 5A-B, Figures 6A-C and the model for Figure 8A-B. BI Bassey-Archibong, SM Hercules and C Aarts performed experiments for data found in Figure 3C-D. **LG Rayner** conducted the experiments for Figure 3A and **LG Rayner** and BI Bassey-Archibong conducted the experiments for the data found in Figures 4E and 6D. A Dvorkin-Gheva generated the data for Figures 4F, and 7A-C. Dr. JM Daniel and BI Bassey-Archibong conceived the study and co-wrote the manuscript. All authors assisted with reagents/resources, optimization of experimental procedures, and edited the manuscript text.

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Kaiso depletion attenuates the growth and survival of triple negative breast cancer cells

Blessing I Bassey-Archibong[1], Lyndsay GA Rayner[1], Shawn M Hercules[1], Craig W Aarts[2], Anna Dvorkin-Gheva[2,3], Jonathan L Bramson[2], John A Hassell[3] and Juliet M Daniel*[,1]

Triple negative breast cancers (TNBC) are highly aggressive and lack specific targeted therapies. Recent studies have reported high expression of the transcription factor Kaiso in triple negative tumors, and this correlates with their increased aggressiveness. However, little is known about the clinical relevance of Kaiso in the growth and survival of TNBCs. Herein, we report that Kaiso depletion attenuates TNBC cell proliferation, and delays tumor onset in mice xenografted with the aggressive MDA-231 breast tumor cells. We further demonstrate that Kaiso depletion attenuates the survival of TNBC cells and increases their propensity for apoptotic-mediated cell death. Notably, Kaiso depletion downregulates BRCA1 expression in TNBC cells expressing mutant-p53 and we found that high Kaiso and BRCA1 expression correlates with a poor overall survival in breast cancer patients. Collectively, our findings reveal a role for Kaiso in the proliferation and survival of TNBC cells, and suggest a relevant role for Kaiso in the prognosis and treatment of TNBCs.

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Triple negative breast cancers (TNBC) represent a heterogeneous subtype of breast tumors that generally lack expression of estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2.[1] TNBCs are highly proliferative and have a high rate of recurrence compared to other breast cancer (BCa) subtypes.[2] Currently, there are no specific targeted therapies for the management of TNBC, hence treatment is limited to radio- and chemotherapy. Although TNBCs initially respond to chemotherapy, many patients relapse and this contributes to a shortened overall survival for affected patients.[3]

Various proteins have been implicated in the survival and chemo-resistant nature of TNBC. Two of the most understood are the tumor suppressors BRCA1 and p53.[4–6] BRCA1 is mutated in ~45% of familial BCa[7] and a high proportion of sporadic BCa, especially of the TNBC subtype.[8,9] However, some TNBCs retain the expression of wild-type (wt) BRCA1 (which plays a role in DNA repair) and this has been associated with their resistance to chemotherapeutic drugs such as Cisplatin.[10] Similarly, p53 is mutated in ~30% of BCa[11] with a higher frequency observed in TNBCs, reviewed in Walerych *et al.*[12] The inability of mutant p53 to bind and activate the expression of canonical p53 target genes such as the pro-apoptotic genes Noxa, Bax and Puma is believed to contribute to the chemo-resistance and survival of BCa.[13–18]

Several recent studies have implicated increased nuclear expression of the transcription factor Kaiso in the aggressiveness of certain tumors including basal/triple negative tumors.[19,20] Kaiso is a POZ-ZF transcription factor that was first identified as a binding partner of the E-cadherin catenin cofactor p120-catenin ($p120^{ctn}$).[21] In the absence or downregulation of E-cadherin, $p120^{ctn}$ is able to translocate to the nucleus[22,23] where it can bind and inhibit Kaiso's transcriptional activities.[23–25] Although high Kaiso expression is associated with TNBC aggressiveness,[19,26] Kaiso's specific role in the growth and survival of TNBCs remains unknown. Interestingly, roles for Kaiso in cell growth (proliferation) and survival (decreased apoptosis) have been demonstrated in several cell types. For instance, Kaiso depletion results in decreased Cyclin D1, reduced proliferation and increased apoptosis of cervical cancer (HeLa) cells, but decreased apoptosis of human embryonic kidney (HEK293) cells.[27] Similarly, loss of Kaiso decreased prostate tumor cell proliferation[20] and delayed the onset of intestinal polyp formation in Apc^{Min+} mice.[28] Finally, loss of Kaiso-mediated transcriptional repression is associated with increased anchorage-independent cell growth of mouse lobular BCa cells.[23] Collectively, these studies suggest context-dependent roles for Kaiso in cell proliferation and apoptosis.

Herein, we report that Kaiso depletion attenuates the proliferative ability of TNBC cells, reduces the anchorage-independent growth of MDA-231 cells and delays the tumor onset of MDA-231 xenografts. We also show that Kaiso depletion increases the apoptosis of TNBC cells. More importantly, we report for the first time that silencing Kaiso results in the downregulation of BRCA1 in mutant-p53-expressing TNBC cells. Together, our findings suggest that high Kaiso expression promotes the growth and survival of TNBCs and raise the possibility that Kaiso may be a useful biomarker for the prognosis and treatment of a subset of TNBCs.

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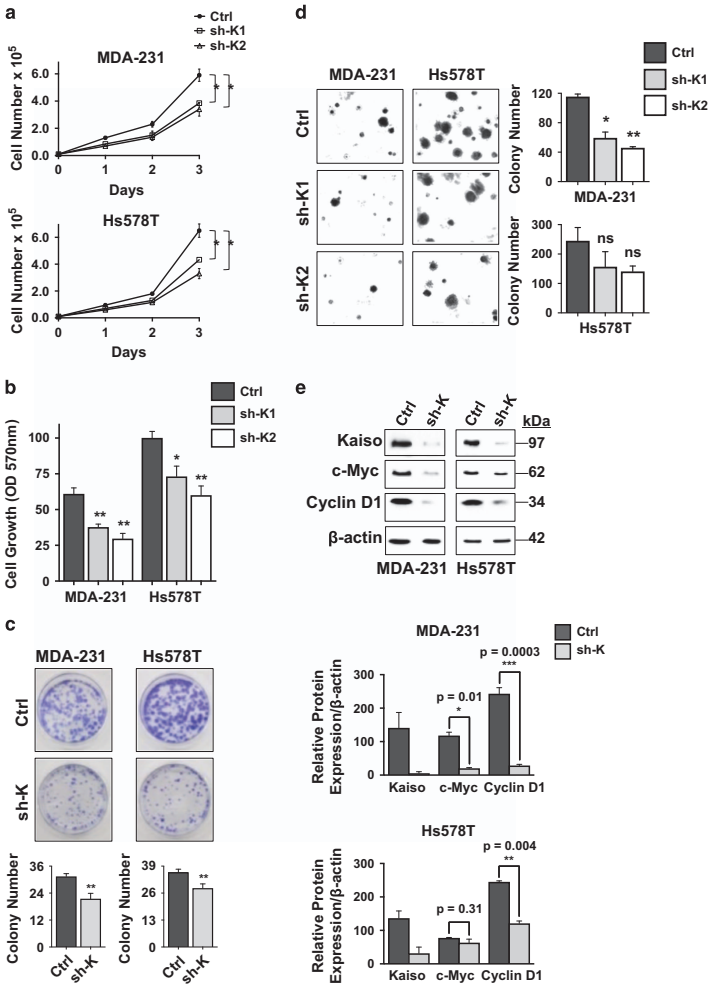

Figure 1 Kaiso depletion attenuates the proliferation of TNBC cells. Growth curve (**a**), MTT assays (**b**) and colony formation assays (**c**) were conducted on Ctrl and Kaiso-depleted (sh-K1 and sh-K2, hereafter referred to as sh-K) cells, and reveal that Kaiso depletion inhibits proliferation of MDA-231 and Hs578T cells. (**d**) Soft agar assays were also conducted on Ctrl, sh-K1 and sh-K2 MDA-231 and Hs578T cells and revealed that Kaiso-depletion diminished anchorage-independent growth of MDA-231 but not Hs578T cells. (**e**) Kaiso depletion resulted in decreased c-Myc and cyclin D1 expression in MDA-231 and Hs578T cells as detected by IB analysis and densitometry analysis. The reduction in c-Myc levels in response to Kaiso depletion was more significant in MDA-231 compared to Hs578T cells. GraphPad Prism software was used to generate graphs and for all statistical calculations. Data shown are representative of three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$

Results

Kaiso depletion inhibits TNBC cell proliferation. Recently, we reported that Kaiso is highly expressed in triple negative tumors and correlates with the metastatic propensity of TNBC cells.[19,26] To ascertain whether Kaiso is also involved in TNBC cell growth and survival, we performed cell viability assays (direct cell counts, MTT and colony formation assays) on control (Ctrl) and Kaiso-depleted (sh-K1 and sh-K2, hereafter referred to as sh-K) TNBC cell lines (MDA-231 and Hs578T), see Bassey-Archibong *et al.*[26] Notably, silencing Kaiso significantly inhibited the proliferation

of MDA-231 and Hs578T cells in all assays performed (Figures 1a–c). We also conducted soft agar assays on Ctrl and Kaiso-depleted MDA-231 and Hs578T cells, and found that Kaiso-depletion mitigates the anchorage independence of MDA-231 but not Hs578T cells (Figure 1d). As previous studies had implicated Kaiso in the regulation of the pro-proliferation genes *c-myc* and *cyclin D1*,[24,29,30] we examined the expression of these proteins in Kaiso-depleted TNBC cells. We found that Kaiso-depleted MDA-231 and Hs578T cells expressed less c-Myc and Cyclin D1 than control cells (Figure 1e and Supplementary Figure 1A), which further supported a role for Kaiso in cell proliferation.

Kaiso depletion results in delayed tumor onset of MDA-231 xenografts. To ascertain whether the *in vitro* effect of Kaiso depletion on TNBC cell proliferation would be sustained *in vivo*, we performed mouse xenograft studies with the well-characterized TNBC cell line–MDA-231. Equal numbers (4.5×10^6) of Ctrl and Kaiso-depleted (sh-K) MDA-231 cells were injected into the mammary fat pad of immunocompromised mice ($n = 5$ for each experimental condition) and allowed to form tumors as previously described.[26] Interestingly, we observed a significant delay (~8 weeks) in tumor formation in the Kaiso-depleted xenografts compared to controls which took ~5 weeks to form visible tumors (Figure 2a). Furthermore, upon tumor formation, the Kaiso-depleted tumors took ~4 weeks to reach the endpoint size of 3300 mm^3 compared to the control tumors which took ~3 weeks to reach 3300 mm^3 (Figure 2a). To determine if the delayed tumor onset observed in the Kaiso-depleted xenografts was due to Kaiso-depletion effects on proliferation, size-matched (~3300 mm^3) Ctrl and sh-K MDA-231 tumor tissues were harvested and examined for the expression of the well-established proliferation markers (Ki-67 and PCNA). Immunohistochemical (IHC) analyses revealed less proliferating cells in the Kaiso-depleted tumor tissues compared to control tissues (Figure 2b and see Bassey-Archibong *et al*.[26] for IHC analysis of Kaiso expression in the Ctrl and sh-K tumor tissues). This suggests that the delayed tumorigenesis of the Kaiso-depleted MDA--231 cells may be due to their reduced proliferative capacity. However, the delayed tumor onset may also be due to the reduced colonization of Kaiso-depleted MDA-231 cells, since these cells displayed decreased anchorage-independence *in vitro* (Figure 1d). Nonetheless, consistent with our *in vitro* proliferation studies, IHC analysis revealed reduced c-Myc and Cyclin D1 expression in Kaiso-depleted MDA-231 tumors compared to control MDA-231 tumor tissues (Figures 2c and d). Collectively, these findings further support a role for Kaiso in TNBC cell proliferation.

Kaiso depletion induces apoptosis in TNBC cells. As the delay in MDA-231 tumor onset could also have been due to increased apoptosis in Kaiso-depleted cells, we investigated the effect of Kaiso depletion on the expression of the apoptotic/cell-death marker–cleaved Caspase 3 (c-Caspase 3) in MDA-231 tumor tissues. Remarkably, we observed an increased number of c-Caspase 3 stained cells in Kaiso-depleted MDA-231 tumors compared to control MDA-231 tumors (Figure 3a). Quantification of the Caspase 3 activity of control and Kaiso-depleted (sh-K1 & sh-K2) MDA-231 cells *in vitro,* using the Caspase 3 colorimetric assay, also revealed increased Caspase 3 activity in the Kaiso-depleted (sh-K1 & sh-K2) MDA-231 cells compared to control cells (Figure 3b). Similar results were also observed in Kaiso-depleted (sh-K1 & sh-K2) Hs578T cells compared to their control counterparts (Figure 3b). Further verification of Kaiso depletion effects on apoptosis with the Annexin V-fluorescein isothiocyanate (FITC) staining assay also confirmed that Kaiso depletion resulted in increased apoptosis of MDA-231 and Hs578T cells as evidenced by the elevated number of Annexin V-FITC stained cells in Kaiso-depleted (sh-K) cells compared to controls (Figure 3c). Similar results were also obtained in an additional TNBC cell line–MDA-157 (Supplementary Figure 2A). To determine if the increased apoptosis in the TNBC cells was specific to Kaiso depletion, we expressed a sh-resistant murine Kaiso cDNA (mKaiso) in the MDA-231 and Hs578T sh-K cells, and subjected these cells to Annexin V-FITC staining. As observed in Figure 3d, Kaiso re-expression rescued the apoptotic phenotype observed in the Kaiso-depleted (sh-K) MDA-231 and Hs578T cells, as seen by the reduced number of Annexin V-FITC stained cells in the MDA-231 and Hs578T sh-K (mK) cells compared to Kaiso-depleted MDA-231 and Hs578T cells transfected with an empty (E) vector. Together these findings suggest that silencing Kaiso enhances the apoptosis of TNBC cells.

Our observation that Kaiso depletion caused increased apoptosis in TNBC cells was intriguing but contradictory to recent findings in other cell types (MEF and HEK293) where Kaiso was implicated as a pro-apoptotic protein, and promoter of p53-mediated apoptosis.[31] Since the TNBC cells utilized in this study (MDA-231, Hs578T, MDA-157) contain a mutant (mut)-p53 gene[32–34] compared to MEF and HEK293 cells that express wt-p53,[31] we postulated that Kaiso's role in apoptosis was contingent on the status of p53 rather than cell type per se. To test this hypothesis, we performed Annexin V-FITC staining of MCF-7 cells transiently overexpressing Kaiso. The MCF-7 BCa cell line was selected for these studies as it expresses low levels of Kaiso and wt-p53. As seen in Supplementary Figure 2B, transient overexpression of Kaiso in MCF-7 cells enhanced the apoptosis/death of these cells, as evidenced by more Annexin V-FITC stained cells in the Kaiso-overexpressing (mKaiso) MCF-7 cells compared to their parental (empty) counterparts, consistent with the findings of Koh et *al*.[31]

Pro-apoptotic proteins are up-regulated in Kaiso-depleted TNBC cells. As Kaiso expression promotes survival in TNBC cells expressing mut-p53 (Figure 3c), we hypothesized that the pro-survival role of Kaiso in TNBC cells is due to its interaction with mut-p53. To test this hypothesis, we performed co-immunoprecipitation experiments and found that Kaiso associated with mut-p53 in MDA-231 and Hs578T cells, although a stronger interaction was observed between Kaiso and mut-p53 in MDA-231 compared to Hs578T cells (Figure 4a). Wt-p53-expressing MCF-7 cells were also examined as a positive control to confirm Kaiso's interaction with wt-p53 (Supplementary Figure 3A) as previously reported.[31]

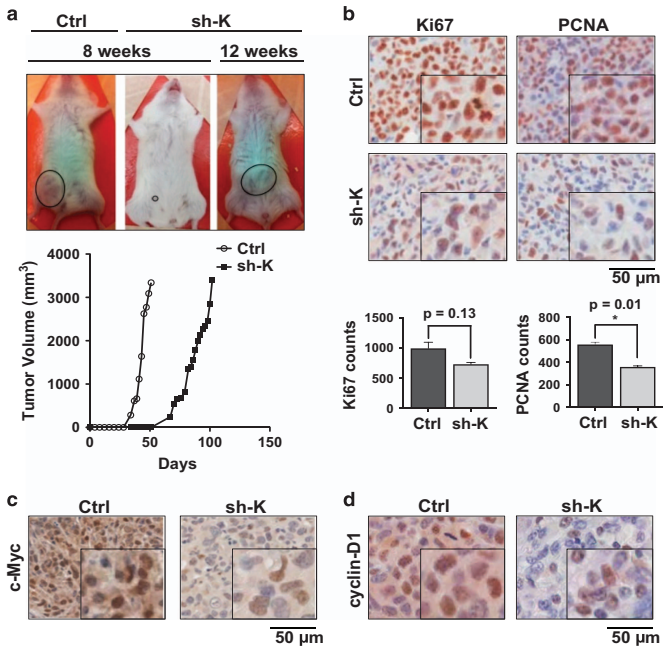

Figure 2 Kaiso-depleted MDA-231 cells exhibit delayed tumor onset in mouse xenografts. (**a**) Kaiso-depleted MDA-231 xenografts (sh-K) are delayed ~ 3 weeks in tumor onset and development compared to control (Ctrl) MDA-231 xenografted tumors as seen by time-course analysis of the tumor volume of Ctrl and sh-K MDA-231 xenografted cells. (**b**) IHC-stained images of MDA-231 xenograft tissues with Ki-67 and PCNA antibodies show a marked decrease in proliferating cells in MDA-231 Kaiso-depleted tumor tissues as indicated by the reduced expression of the proliferation markers Ki-67 and PCNA. (**c** and **d**) IHC-stained images of MDA-231 xenograft tissues with c-Myc and Cyclin D1 antibodies show that Kaiso-depletion results in reduced numbers of c-Myc and cyclin-D1 stained cells and reduced staining intensity. Representative images shown from 3 or more independent experiments

To gain more mechanistic insight into Kaiso's role in TNBC cell survival and apoptosis, we assessed the effect of Kaiso depletion on the expression of the pro-apoptotic proteins PUMA and Bax. As Kaiso augments the expression of PUMA and Bax in wt-p53-expressing cells[31] and (Supplementary Figure 3B), we postulated that in mut-p53-expressing TNBC cells, Kaiso would inhibit PUMA and Bax expression. Indeed, low levels of PUMA were detected in control MDA-231 and Hs578T cells that express high levels of Kaiso and mut-p53 (Figure 4b). PUMA was also detected at low levels in the high Kaiso and mut-p53 MDA-157 cells (Supplementary Figure 4). Similarly, low Bax levels were detected in control MDA-231 and MDA-157 cells (Figure 4b and Supplementary Figure 4) but not control Hs578T cells (Figure 4b). Remarkably, Kaiso depletion resulted in a striking upregulation of PUMA in all three cell lines (MDA-231, Hs578T and MDA-157 cells; Figure 4b and Supplementary Figure 4). While Bax expression was increased ~ 2-fold in MDA-231 and MDA-157 cells, there was only a slight increase in Bax expression in Hs578T cells (Figure 4b and Supplementary Figure 4), which may be due to the fact that Bax was expressed at higher levels in parental Hs578T cells compared to parental MDA-231 and MDA-157 cells (Figure 4b and Supplementary Figure 4). This suggests that Kaiso may not exhibit a repressive function on Bax expression in parental (Ctrl) Hs578T cells, probably due to the increased expression of $p120^{ctn}$ observed in these cells (Figure 4b), which co-localized with Kaiso in the nucleus of some but not all Ctrl Hs578T cells (Supplementary Figure 5). Notably, there was no change in mut-p53 levels upon Kaiso depletion. The specificity of Kaiso depletion effects on Bax and PUMA protein levels was confirmed by the expression of a sh-resistant Kaiso (mKaiso) cDNA in the MDA-231 sh-K cells; this resulted in reduced Bax and PUMA protein expression in the MDA-231-sh-K (mKaiso) cells (Figure 4c).

Additional analyses using qRT-PCR revealed significantly increased PUMA transcripts but no significant changes in Bax transcript levels in Kaiso-depleted MDA-231 and Hs578T cells compared to controls (Figure 4d). Consistent with this observation, chromatin immunoprecipitation (ChIP)-PCR experiments showed an enrichment of Kaiso at a minimal PUMA promoter region rich in Kaiso binding sequences (KBS) but not at a minimal Bax promoter region containing a core KBS (see schematic, Supplementary Figure 6A) in MDA-231 and Hs578T cells (Figure 4e, and data not shown). Similar

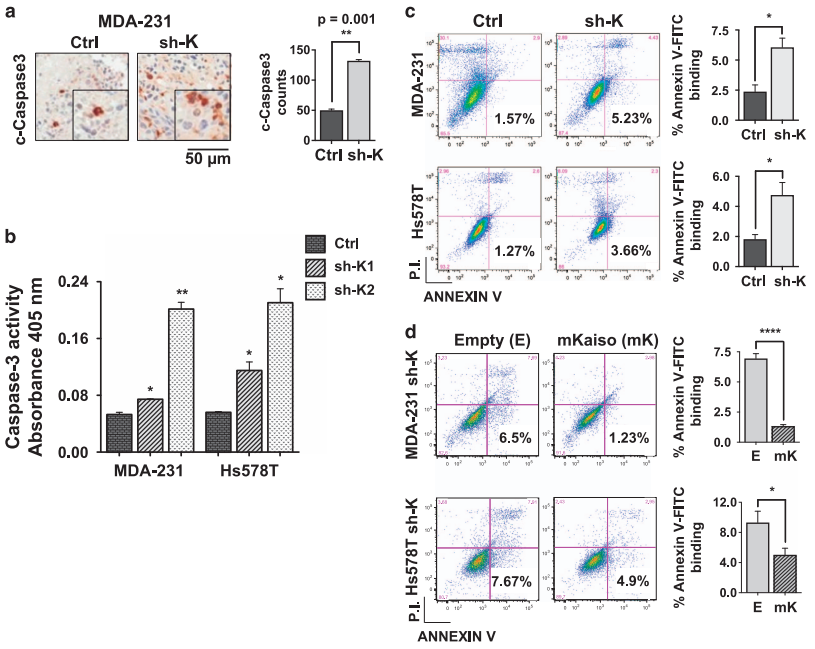

Figure 3 Kaiso depletion results in increased apoptosis of TNBC cells. (**a**) IHC-stained images of control (Ctrl) and Kaiso-depleted (sh-K) MDA-231 tumor tissues with cleaved (c)-Caspase 3 antibody show that Kaiso-depletion results in increased c-Caspase 3 expression in MDA-231 tumor tissues. (**b**) Caspase 3 assay conducted on Ctrl, sh-K1 and sh-K2 MDA-231 and Hs578T cells show that Kaiso depletion resulted in increased Caspase 3 activity in MDA-231 and Hs578T sh-K1 and sh-K2 cells compared to their control counterparts. (**c**) Kaiso-depleted TNBC cells (sh-K MDA-231 & Hs578T) expressing mut-p53 exhibit increased apoptosis as revealed by Annexin V-FITC staining. (**d**) Expression of a sh-resistant Kaiso cDNA in Kaiso-depleted MDA-231 and Hs578T cells mitigates the apoptosis induced by Kaiso depletion as assessed by Annexin V-FITC staining. Data shown are representative of three independent experiments. *$P<0.05$, **$P<0.01$, ****$P<0.0001$

results were also obtained with chromatin isolated from MDA-157 cells (Supplementary Figure 6B). Interestingly, an enrichment of mut-p53 was also observed at the minimal PUMA promoter region rich in KBS but not at the minimal Bax promoter region containing a core KBS (Figure 4e, and data not shown). Nonetheless, Kaiso's interaction with the PUMA promoter was independent of p53 as evidenced by its association with the PUMA promoter in MDA-157 cells, which lack detectable p53 protein expression (Supplementary Figure 4). Collectively, these data imply that Kaiso may directly or indirectly inhibit Bax and PUMA expression in TNBC cells that lack wt-p53.

High Kaiso and low PUMA expression does not correlate with poor survival in BCa patients. Considering the consistent effect of Kaiso depletion on PUMA expression in all TNBC cell lines (MDA-231, Hs578T and MDA-157) studied, we explored whether the inverse correlation of Kaiso and PUMA expression could account for Kaiso's role in the survival of breast tumors. We thus utilized The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) BCa data sets and examined the effect of high Kaiso and low PUMA expression on the overall survival of either TNBC patients specifically (data not shown), or all BCa cases. Kaplan–Meier survival curves revealed that patients bearing tumors with high Kaiso and low PUMA expression exhibited a decreased but non-significant overall survival trend compared to patients with tumors that had a low Kaiso and high PUMA expression (log-rank test, *P*-value = 0.16; Figure 4f). This suggests that while Kaiso's effect on PUMA expression does have some effect on BCa survival, the clinical relevance does not appear to be statistically significant. Thus, Kaiso may cooperate with other protein(s) to influence BCa survival.

Kaiso depletion enhances the sensitivity of TNBC cells to Cisplatin. Most metastatic BCas such as TNBCs are resistant to chemotherapeutic agents,[3] a phenomenon which may be due to reduced apoptosis and increased DNA repair.[35] As Kaiso depletion stimulated the apoptosis of TNBC cells, we investigated whether silencing Kaiso would sensitize these cells to chemotherapeutic drugs. Control and Kaiso-depleted TNBC cells were treated with the chemotherapy drug Cisplatin and then subjected to immunoblot (IB)

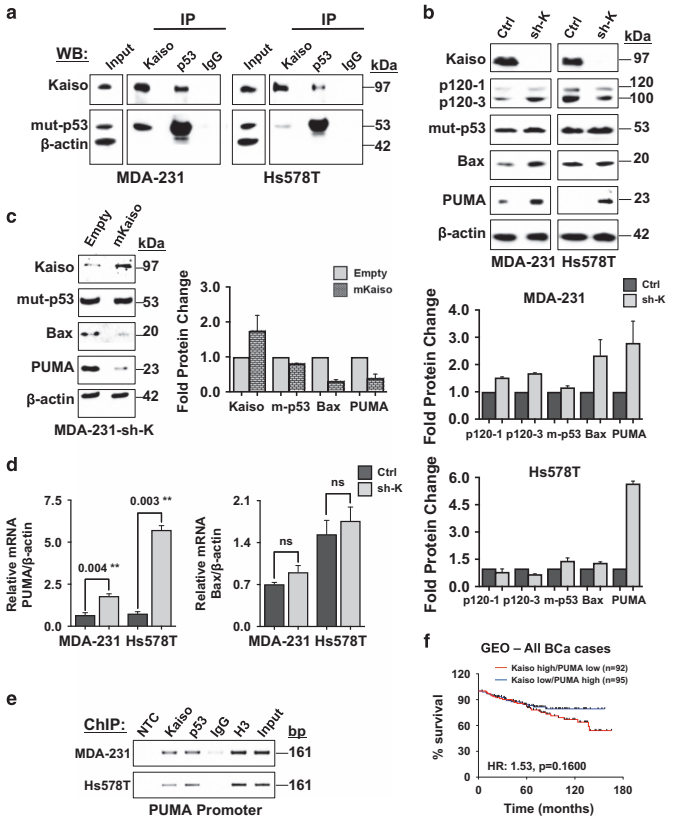

Figure 4 Kaiso depletion increases expression of pro-apoptotic proteins in TNBC cells lacking wt p53. (**a**) Kaiso co-precipitates with mutant p53 in MDA-231 and Hs578T cell lysates. Parental MDA-231 and Hs578T cells were subjected to immunoprecipitation with anti-Kaiso, anti-p53 and anti-IgG antibodies, and immunoblotted with the indicated antibodies. Kaiso-depleted MDA-231 and Hs578T cells express increased amounts of Bax and PUMA protein compared to control cells (**b**), that is decreased upon expression of a sh-resistant Kaiso cDNA in the MDA-231 and Hs578T sh-K cells (**c**). Graphical representation of the quantitated protein values is shown. (**d**) Kaiso-depleted MDA-231 and Hs578T cells (that express mut-p53) exhibit a statistically significant increase in PUMA mRNA levels as measured by qRT-PCR. Although Bax mRNA levels were also slightly increased, it was not significant. (**e**) ChIP-PCR analysis of MDA-231 and Hs578T chromatin revealed that Kaiso and mut-p53 associate endogenously with the PUMA promoter in TNBC cells. (**f**) Transcript profiles of patients from the GEO (GSE20685, GSE21653, GSE16446, GSE19615 and GSE9195) BCa data sets were pooled and segregated into Kaiso high/PUMA low, and Kaiso low/PUMA high groups. Kaplan–Meier survival curves revealed a correlation between high Kaiso and low PUMA expression with poor overall survival in all BCa cases. However, it was not statistically significant. Data representative of three independent experiments. $^{**}P < 0.01$ and NS, not significant. For Kaplan–Meier survival curves, log-rank test was performed to determine statistical significance. $P < 0.05$ is considered statistically significant

analysis for the expression of the apoptotic marker, cleaved-PARP. Intriguingly, loss of Kaiso enhanced the sensitivity of MDA-231, Hs578T and MDA-157 cells to Cisplatin as evidenced by the increased expression of cleaved-PARP in the treated Kaiso-depleted cells compared to the control-treated cells (Figures 5a and b).

High Kaiso and BRCA1 expression correlates with poor survival in BCa patients. As BRCA1 expression has been linked to the resistance of TNBC cells to Cisplatin,[10,36] we examined the effect of Kaiso depletion on BRCA1 expression. We observed that Kaiso depletion led to downregulation of BRCA1 in MDA-231 and Hs578T cells at both the transcript and protein level (Figures 6a and b). This was partially rescued (~1.6-fold increase) by the expression of a sh-resistant Kaiso cDNA (mKaiso) in the Kaiso-depleted MDA-231 and Hs578T cells (Figure 6c). More importantly, we found an enrichment of Kaiso at a minimal BRCA1 promoter region containing several core KBS (Figure 6d), which suggests that BRCA1 may be a Kaiso target gene.

In light of these findings, we utilized the TCGA and GEO BCa data sets and correlated the expression levels of Kaiso and BRCA1 with BCa survival. Kaplan–Meier survival curves revealed that TNBC patients bearing tumors with high Kaiso and BRCA1 expression, exhibit a significantly worse overall survival compared to TNBC patients with low Kaiso and low BRCA1 expression (log-rank test, $P=0.017$; Figure 7a). A similar trend was also observed in all BCa cases with high Kaiso and high BRCA1 expression (log-rank test, $P=0.0003$) compared to cases with low Kaiso and low BRCA1 expression, and high or low BRCA1 expression alone (log-rank test, $P=0.13$), Figures 7b and c. This finding suggests that Kaiso and BRCA1 function together to promote the survival of BCa cells.

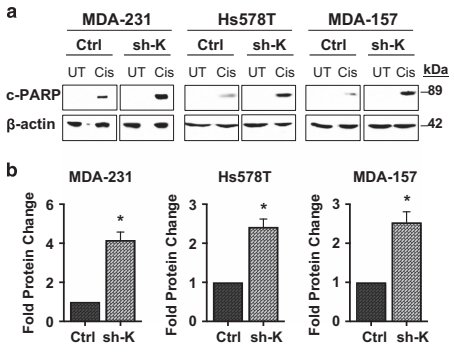

Figure 5 Kaiso depletion enhances the sensitivity of TNBC cells to Cisplatin. (**a**) Kaiso-depletion sensitizes TNBC cells to Cisplatin treatment, as demonstrated by the increased cleaved-PARP expression observed in Kaiso-depleted MDA-231, Hs578T and MDA-157 cells treated with Cisplatin (Cis) for 48 h compared to similarly treated control cells. UT, untreated cells. (**b**) Graphical representation of the fold change in protein expression is shown. All experiments were conducted independently at least three times. Representative images shown. $^*P<0.05$

Discussion

TNBCs remain a clinical challenge due to their highly aggressive nature, lack of specific targeted therapies and resistance to routine chemotherapeutic regimens including anthracyclines and taxanes.[37] Consequently, there is an urgent need to understand the molecular mechanisms underlying TNBC growth, aggressiveness and chemo-resistance. Herein, we report that depletion of the transcription factor Kaiso attenuates the proliferation of, and increases apoptosis in, the TNBC cell lines MDA-231 and Hs578T. These findings suggest that in addition to Kaiso's potential role in TNBC metastasis,[26] Kaiso may also be a key regulator of triple negative tumor cell growth and survival.

In the past decade, several independent studies have implicated Kaiso in various cancers; while some studies suggest a pro-oncogenic role for Kaiso,[19,20,26,28,38–42] others associate Kaiso with a tumor suppressive role.[23,25,31,43] Together, these diverse studies highlight context-dependent roles for Kaiso in human cancer, which might be due to the fact

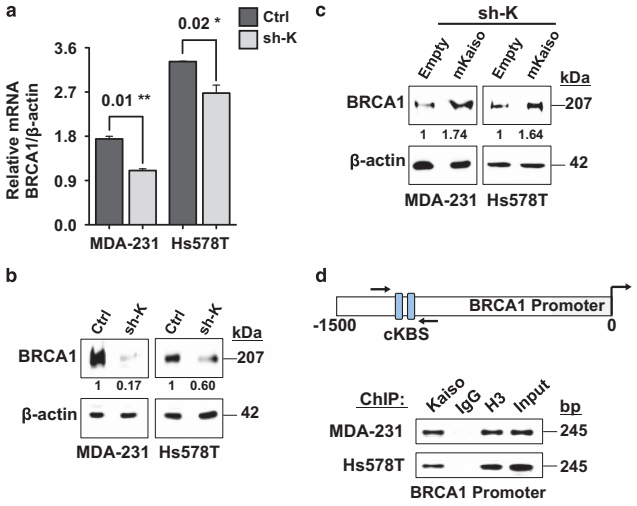

Figure 6 Kaiso depletion attenuates BRCA1 expression in sporadic TNBC cells. (**a**) BRCA1 mRNA expression was significantly reduced in Kaiso-depleted MDA-231 and Hs578T cells compared to controls as measured by qRT-PCR. (**b**) WB analysis with a BRCA1-specific antibody shows decreased BRCA1 protein expression in Kaiso-depleted MDA-231 and Hs578T cells, which is partially rescued by expression of a sh-resistant Kaiso cDNA in the MDA-231 and Hs578T sh-K cells (**c**). (**d**) Schematic illustration of the minimal BRCA1 promoter region showing the location of a core KBS (cKBS) that was amplified by ChIP-PCR. Kaiso was enriched at the BRCA1 promoter region indicated in MDA-231 and Hs578T cells. Data representative of three independent experiments. $^*P<0.05$, $^{**}P<0.01$

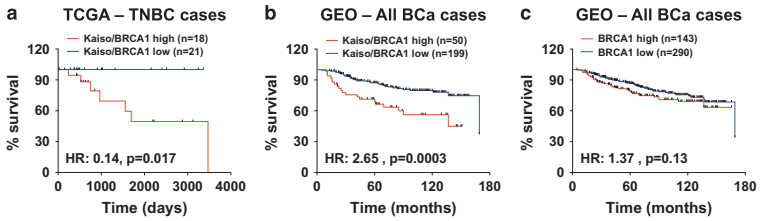

Figure 7 High Kaiso and BRCA1 expression correlates with poor prognosis in BCa patients. Transcript profiles of patients from the TCGA and GEO (GSE20685, GSE21653, GSE16446, GSE19615 and GSE9195) BCa data sets were pooled and segregated into Kaiso/BRCA1 high or Kaiso/BRCA1 low, and BRCA1 high or BRCA1 low groups. Kaplan–Meier survival curves revealed that high Kaiso and BRCA1 expression significantly correlates with poor overall survival in TNBC patients specifically (**a**) or all BCa cases (**b**) whereas increased BRCA1 expression did not correlate with poor overall survival in BCa patients (**c**). Log-rank test was performed to determine statistical significance. $P<0.05$ is considered statistically significant

that Kaiso acts as both a transcriptional repressor and an activator.[24,29,30,44,45] In addition, as Kaiso also possesses dual-specificity DNA-binding properties,[29,46,47] there may be a large repertoire of tumorigenic target genes that may be differentially regulated by Kaiso. To date, only a few *bona fide* Kaiso target genes—*c-Myc*, *Wnt 11*, *Cyclin D1*, *Siamois*, *Matrilysin* and *Rapysn* have been characterized.[24,25,29,45,48] Two of these genes (*c-Myc and Cyclin D1*) are well-established pro-proliferation oncogenes[49,50] that were found to be repressed by Kaiso in *Xenopus laevis* embryos and colon cancer cells.[24,29] Therefore, it was surprising to find that loss of Kaiso in TNBC cells led to their decreased, rather than increased, expression (Figure 1e). Our findings thus indicate context-dependent roles for Kaiso in the regulation of *c-Myc* and *Cyclin D1* expression, an idea that is supported by a recent study which demonstrates differential regulation of *Cyclin D1* by Kaiso.[27]

Kaiso's role in specific cancers may also be dictated or modulated by its interaction with other transcriptional cofactors or proteins that may be uniquely expressed in these cancers. For example, Kaiso was shown to interact with nuclear p120^{ctn} in mouse invasive lobular BCa cells, which inhibited Kaiso's repression of *Wnt11*, and fostered anoikis resistance in these cells.[23] In another study, Kaiso was shown to interact with wt-p53, and promote apoptosis through increased p53-mediated expression of the pro-apoptotic *Bax* and *PUMA* genes.[31] Our findings in this study also support distinct roles of Kaiso that may be based on its interaction with p53, as we found that Kaiso differentially regulates apoptosis in BCa cells that express different forms of p53 (Figure 3c, Supplementary Figures 2A and B). Specifically, Kaiso exhibits an anti-apoptotic role in TNBC (MDA-231, Hs578T and MDA-157) cells that express mut-p53 as its depletion promotes apoptosis in these cells (Figure 3c and Supplementary Figure 2A). As mut-p53 expression is implicated in the survival of MDA-231 and Hs578T cells,[51,52] it was interesting to note that loss of Kaiso attenuated the survival of these cells, despite having no significant effects on mut-p53 expression in these cells.

Conversely, in non-TNBC cells that express wt-p53, Kaiso exhibits a pro-apoptotic role (Supplementary Figure 2B), which is consistent with reports in other cell types that demonstrated a pro-apoptotic role for Kaiso in a wt-p53-dependent

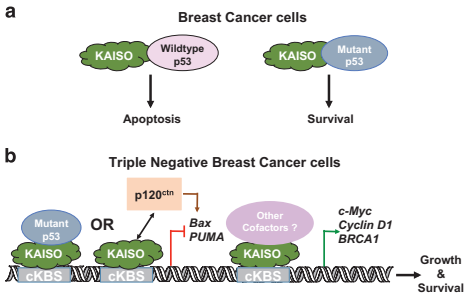

Figure 8 Schematic diagram of proposed model for Kaiso's role in TNBC. (**a**) Kaiso interacts with both wt p53 and mutant p53 in BCa cells and this differential interaction may modulate Kaiso's function in apoptosis. (**b**) In TNBC cells lacking wt p53 (but expressing mutant p53), Kaiso might directly or indirectly inhibit the activation of the pro-apoptotic genes Bax and PUMA, which leads to tumor survival. However, Kaiso's inhibitory effect on Bax protein expression may be attenuated by Kaiso interaction with other proteins like p120^{ctn}. Kaiso may also activate c-Myc, Cyclin D1 and BRCA1 expression independently or in collaboration with other cofactors in TNBC cells, which would also promote TNBC cell growth and survival

manner.[31] Based on these findings, we surmise that the distinct roles of Kaiso in apoptosis may be due to its ability to interact with both wt-p53 and mut-p53 as shown in Figure 4a and Supplementary Figure 3A (see model indicated in Figure 8a). Indeed, several recent studies have reported differential activities of transcription factors that interact with both wt-p53 and mut-p53, reviewed in Kim *et al*.[53] As Kaiso behaved in an anti-apoptotic manner in TNBC cells lacking wt-p53, we postulate that Kaiso may only function in a pro-apoptotic manner in BCa cells expressing wt-p53.

An unexpected finding of this study was that Kaiso depletion reduced BRCA1 expression at both the transcript and protein levels in TNBC cells, suggesting that BRCA1 may be a Kaiso target gene. Indeed, we observed that Kaiso associates with the BRCA1 promoter in both MDA-231 and Hs578T cells (Figure 6d) but more importantly, we also found that high Kaiso and BRCA1 expression correlates with poor overall survival in TNBC patients, as well as all BCa cases in general (Figures 7a and b). Collectively, our findings suggest that Kaiso may

augment the survival and aggressiveness of TNBC cells by promoting BRCA1 expression (see model, Figure 8b). Hence, our demonstration that Kaiso-depletion enhanced the sensitivity of TNBC cells to the chemotherapy drug Cisplatin raise the exciting possibility that Kaiso may be a target for TN tumors with BRCA1 expression.

Together, this study reveals an essential role for Kaiso in the growth and survival of TNBC cells and suggests that Kaiso could be targeted for the treatment of a subset of triple negative tumors especially those expressing BRCA1. Future experiments (e.g. ChIP-sequencing and RNA-sequencing of control and Kaiso-depleted TNBC cells) are needed to fully delineate and understand the molecular mechanisms and signaling pathways that Kaiso participates in to contribute to the pathogenesis and survival of triple negative tumors.

Materials and Methods

Cell culture. The human triple negative breast tumor cell lines MDA-MB-231 (hereafter MDA-231) and Hs578T, and their stable Kaiso-depleted (sh-K1 and sh-K2) derivatives were cultured as previously described.[26] The non-TNBC cell line MCF-7 was purchased from ATCC (Manassas, VA, USA), while the triple negative breast tumor cell line MDA-MB-157 (hereafter MDA-157) and the non-TNBC cell line ZR75.1 were a kind gift from Dr. John Hassell (McMaster University, Hamilton, Canada). These cells were cultured as previously described.[54] All cells were grown in 5% CO_2 at 37 °C.

Generation of stable Kaiso-depleted MDA-157 cell lines. Depletion of Kaiso in MDA-157 cells was achieved by stably transfecting cells with a pRetroSuper (pRS) vector containing shRNAs that targeted the Kaiso mRNA sequences; 5′-AAAAGATCATTGTTACCGATT-3′ and 5′-TTTTAACATTCATTCTTG GGAGAAG-3′ termed sh-K1 and sh-K2 as previously described.[26] Stable control (transfected with a pRS-Kaiso scrambled shRNA)[26] and Kaiso-depleted MDA-157 cells were maintained in media containing 1.0 μg/ml of Puromycin (Invitrogen, Carlsbad, CA, USA). Only the most efficient Kaiso-depleted cells were selected for further analysis (Supplementary Figure 1b).

Cell proliferation assay. Equal numbers (1×10^4) of control and Kaiso-depleted (sh-K1 and sh-K2) MDA-231 and Hs578T cells were grown in 24-well plates for 3 days. Direct cell counts were obtained each day using the BioRAD TC10 automated cell counter and averaged using Microsoft Excel. Graphical representation of counts was achieved using GraphPad Prism software (La Jolla, CA, USA).

MTT assay. Equal numbers (1×10^4) of control and Kaiso-depleted (sh-K1 and sh-K2) MDA-231 and Hs578T cells were grown in duplicate in 96-well plates for 22 h. Cells were then immediately treated with MTT (3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2, 5-diphenyl-**t**etrazolium bromide; Sigma Aldrich, USA) for 2 h. The precipitated formazan crystals were subsequently dissolved with 100 μl of dimethyl sulfoxide and the optical density of the resulting reaction solution measured at 570 nm using the SpectraMax Plus 384 Microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Colony formation and soft agar assay. 5×10^2 control and Kaiso-depleted MDA-231 and Hs578T cells were cultured in 60 mm dishes in duplicate and allowed to grow and form colonies for 10–14 days. For the soft agar assays, 5×10^4 control and Kaiso-depleted MDA-231 and Hs578T cells were cultured in 0.3% Agarose in 60 mm dishes, and allowed to grow and form colonies for 10 days. After the incubation period, colonies were stained with 0.5 and 0.05% Gentian Violet diluted in methanol for the colony formation and soft agar assays respectively. Images of colonies from the colony formation assay were obtained by using a Canon digital camera and then colonies were counted manually. For the soft agar assay, 10× images of colonies were obtained using the Zeiss Axiovert 200 microscope (Carl Zeiss Canada Ltd., ON, Canada), and then counted using the ImageJ software. Graphical representation of counts (colony numbers) was achieved using GraphPad Prism software.

Xenograft studies. All mice studies were approved by the Animal Research Ethics Board, McMaster University (AUP# 14–05–14) and performed in accordance with the guidelines of the Animal Research Ethics Board as previously described.[26] In brief, equal numbers (4.5×10^6) of control and Kaiso-depleted MDA-231 cells were injected subcutaneously into the mammary fat pad of ~5–8-week-old female NOD SCID gamma mice ($n = 5$ each) and allowed to form tumors. Tumor growth was monitored using vernier calipers and tumor volume measurements calculated as previously described.[26] Mice were euthanized at endpoint (tumor size 3300 mm^3) as previously described[26] and tumor tissues harvested for histological examination and IHC analyses.

Immunohistochemistry. 5 μM sections of harvested MDA-231 xenografted tumor tissues were stained with mouse monoclonal antibody against Ki-67 (BD Biosciences; 1:50), rabbit monoclonal antibody against PCNA (**C**ell Signaling **T**echnology (CST)-D3H8P; 1:30 000), rabbit monoclonal antibody against c-Myc (Abcam; 1:100), rabbit monoclonal antibody against Cyclin D1 (CST-2978; 1:100) or rabbit monoclonal antibody against cleaved Caspase 3 (CST-9661; 1:50) overnight at 4 °C as previously described.[26] Images were captured using the Aperio Slide scanner (Leica Biosystems, ON, Canada). Ki-67, PCNA and cleaved Caspase 3 counts were obtained from 5 different fields that represented staining observed in whole-tissue sections. The stained cells in these fields were counted blindly and independently by 2 students. Bar graphs representing counts were generated using GraphPad Prism software. Statistically analyses were also conducted using GraphPad Prism statistical software.

Transient transfection assay and rescue experiments. MCF-7 and ZR75.1 parental cells were transfected with either a pCDNA3-empty vector (empty), or a pCDNA3 vector containing the sequence that encodes a sh-resistant mKaiso cDNA using the Turbofect transfection reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. 48 or 72 h post transfection, cells were either subjected to IB analysis, or treated with Geneticin (Invitrogen) at 250 μg/ml for MCF-7 cells and 750 μg/ml for ZR75.1 cells to select for efficient Kaiso overexpression. Three to four weeks post transfection, whole-cell lysates were obtained from the pCDNA3-empty and mKaiso transfected cells and subjected to IB analysis of interested proteins.

For rescue of Kaiso overexpression, pCDNA3 vector expressing the mKaiso cDNA coding sequence that is not targeted by the Kaiso-specific shRNA was transfected into MDA-231 and Hs578T sh-K2 (or sh-K) cells using the Turbofect transfection reagent (Thermo Scientific) as per the manufacturer's protocol. 24 or 48 h post transfection, cells were treated with Puromycin (Invitrogen) at 0.8 μg/ml and Geneticin (Invitrogen) at 1000 μg/ml for MDA-231 sh-K cells and Puromycin (Invitrogen) at 1.5 μg/ml and Geneticin (Invitrogen) at 1000 μg/ml for Hs578T sh-K cells to select for efficient Kaiso overexpression. Three to four weeks post transfection, MDA-231 and Hs578T sh-K (empty and mKaiso) cells were subjected to Annexin V-FITC staining. Whole-cell lysates were also obtained from MDA-231 and Hs578T sh-K (empty and mKaiso) cells and subjected to IB analysis of interested proteins.

Caspase 3 assay. The Caspase 3 assay (colorimetric) kit was purchased from Abcam (Boston, MA, USA), and the assay performed according to the manufacturer's instructions. In brief, 1×10^6 control and Kaiso-depleted (sh-K1 and sh-K2) MDA-231 and Hs578T cells were re-suspended in 50 μl of chilled cell lysis buffer, incubated on ice for 10 min and pelleted by centrifugation at 13 000 r.p.m. for 1 min. The resulting supernatant (cytosolic extract) was then transferred to a new tube, quantified and then ~200 μg of protein per 50 μl cell lysis buffer transferred into 96-well plates in duplicate per cell condition. 50 μl cell lysis buffer without protein samples was also aliquoted into 96-well plates to provide background readings. 50 μl reaction buffer (2×) containing 10 mM DTT was added to each well containing experimental samples (in duplicate) and cell lysis buffer (without samples) followed by 5 μl of 4 mM DEVD-p-NA substrate (200 μM final concentration). The resultant mixture was incubated at 37 °C for 2 h, and then the optical density of the solution was measured at 405 nm using the SpectraMax Plus 384 Microplate reader (Molecular Devices).

ANNEXIN V-FITC staining assay. The FITC–conjugated Annexin V apoptosis detection kit was purchased from Abcam, and staining performed according to the manufacturer's instructions. In brief, equal numbers (1×10^5) of Ctrl and sh-K MDA-231, Hs578T and MDA-157 cells, MDA-231 and Hs578T sh-K (empty and mKaiso) cells, as well as MCF-7 (empty and mKaiso) cells were

re-suspended in 1 × binding buffer (Abcam) after being washed with 1 × PBS and trypsinized with 1 × Trypsin (Invitrogen). These cells were subsequently stained with Annexin V-FITC and propidium iodide (PI) and incubated for 10 min prior to analysis by Flow cytometry. Data were acquired using the LSRFortessa flow cytometer (BD Biosciences, Mississauga, Canada) and analyzed with FlowJo version 9 software.

Quantitative reverse transcription-PCR (qRT-PCR). qRT-PCR experiments were conducted as previously described[26] using the following primers: Bax forward: 5′-GCCCTTTTGCTTCAGGGTTT-3′ and reverse: 5′-GCAATCATCCTCT GCAGCTC-3′ at 60 °C, PUMA forward: 5′-AGCAGGGCAGGAAGTAACAA-3′ and reverse: 5′-CCCTGGGGCCACAAATCT-3′ at 55 °C, BRCA1 forward: 5′-CTCGCTG AGACTTCCTGGAC-3′ and reverse: 5′-TCAACTCCAGACAGATGGGAC-3′ at 62 °C. The SensiFAST cDNA synthesis kit and the SensiFAST SYBR Hi-ROX kit (FroggaBio Scientific Solutions, Toronto, ON, Canada) were used in place of the qScript cDNA SuperMix and Perfecta SYBR Green SuperMix ROX (Quanta BioSciences, Gaithersburg, MD, USA) as previously described.[26]

ChIP and ChIP-PCR. ChIP and ChIP-PCR were performed as previously described.[26] The following primers were used to amplify a minimal Bax, PUMA and BRCA1 promoter region, respectively, containing one or more KBS: Bax KBS forward: 5′-CTAATTCCTTCTGCGCTGGG-3′, and reverse: 5′-GTCCAATCGCAGCT CTAATGC-3′ at 64 °C; PUMA KBS forward: 5′-GATCGAGACCATCCTGGCTA-3′ and reverse: 5′-CGATCTCAGCAAACTGCAAG-3′ at 64 °C; and BRCA1 KBS forward: 5′-AGGGCTCTCTCATCCTGTCA-3′ and reverse: 5′-TGTCCGCCATGT TAGATTCA-3′ at 64 °C.

Immunoprecipitation. Whole-cell lysates were immunoprecipitated with anti-Kaiso 6F mouse monoclonal antibody,[55] anti-p53 mouse monoclonal antibody (CST-2524, which recognizes both wt and mutant-p53), anti-p53 rabbit monoclonal antibody (Abcam-ab32049 that recognizes only mutant-p53) or normal rabbit IgG antibody (Santa Cruz Technology) for 2 h or overnight at 4 °C. The immunoprecipitates were collected by incubation with 50 μl Protein A agarose beads that were subsequently washed five times with lysis buffer before proceeding to SDS-PAGE and IB analysis.

Immunoblot and densitometry analysis. IB analysis was performed as previously described.[26] Overnight incubations were performed at 4 °C using the following primary antibodies at their respective dilutions; Kaiso-specific rabbit polyclonal (gift from Dr. Reynolds; 1:5000), mouse monoclonal antibody against c-Myc (SantaCruz (9E10); 1:500), rabbit polyclonal antibody against Cyclin D1 (US Biological (144418); 1:5000), p120^{ctn}-15D2 specific mouse monoclonal (gift from Dr. Reynolds; 1:1000[56]), Bax-specific rabbit monoclonal (1:500; CST-5023), PUMA-specific rabbit monoclonal (1:500; CST-12450), p53-specific rabbit polyclonal (1:2000; Abcam-ab32049), cleaved PARP-specific rabbit monoclonal (1:1000; CST-5625), BRCA1-specific rabbit polyclonal (1:2000; Abcam-ab131360) and mouse anti-β-actin monoclonal (1:50 000; Sigma Aldrich). IB images were obtained using the Bio-Rad ChemiDoc MP imaging system (Bio-Rad Laboratories, Mississauga, ON, Canada). The optical densities of Kaiso, c-Myc, Cyclin D1, p120-1, p120-3, p53, Bax, PUMA and β-actin signals were quantified and analyzed using the Image Lab software (Bio-Rad), while the relative ratio of Kaiso/β-actin, c-Myc/β-actin, Cyclin D1/β-actin, p120-1/β-actin, p120-3/β-actin, p53/β-actin, Bax/β-actin and PUMA/β-actin were calculated as indicated using Microsoft Excel. Graphical representation of each respective value was accomplished using GraphPad Prism software.

Gene expression analysis of GEO data sets. Gene expression analyses were conducted on five publicly available data sets obtained using Affymetrix HG-U133 plus 2.0 gene chip arrays (Affymetrix, Santa Clara, CA, USA). The transcript profiles of these data sets were deposited in the GEO database under accession numbers GSE20685, GSE21653, GSE16446, GSE19615 and GSE9195.[57–62] All samples used for this study were normalized with frozen robust multi-array analysis[63] and then the distance-weighted discrimination method[64] was used to remove technical variation from the data sets that were to be combined. The combined data sets correlation coefficients for pair-wise comparisons of samples using Affymetrix house-keeping probe sets were computed, and only samples exhibiting a correlation higher than 0.95 with at least half of the data set were selected for further classification. This resulted in a cohort containing 894 tumor samples, which was subsequently used for generating Kaplan–Meier survival curves and performing log-rank analysis.

Gene expression analysis of TCGA data sets. TCGA Level 3 IlluminaHiSeq_RNASeqV2 expression (Illumina, Inc., San Diego, CA, USA) and associated clinical data were downloaded for all available patients from the Broad GDAC Firehose repository (https://gdac.broadinstitute.org/) on 16 September 2016 ($n = 1212$). In all further analyses this data set is referred to as 'TCGA dataset'. We selected tumor samples only ($n = 1,094$), and their RSEM-quantified gene expression values were log2-transformed and used for further analyses to represent gene expression. For identification of ER, PR and ERBB2 status and for overall survival information we used the downloaded clinical data. All data processing was performed using R software.[65]

Survival analysis. Survival analysis and visualization of the Kaplan–Meier curves were performed using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA). For statistical tests P-value < 0.05 indicated significance.

Statistical analyses. All statistical analyses were performed as previously described[26] using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ values were considered statistically significant and data are presented as means ± S.E.M.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis).

APPENDIX

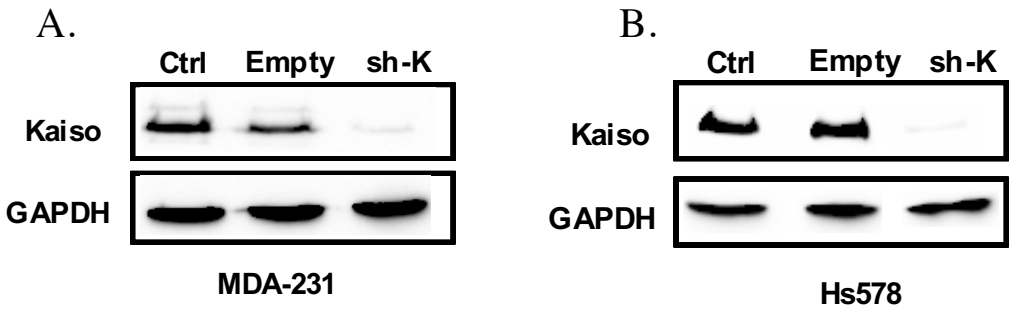

Figure 1A: Kaiso siRNA-mediated knockdown in MDA-231 and Hs578T cells. (A) MDA-231 and (B) Hs578T cells were transfected with the Kaiso-specific siRNA retroviral vector (sh-K) and its control vector (empty). After Puromycin selection, protein lysate was isolated and western blot analysis was performed with antibodies against Kaiso and GAPDH (loading control).

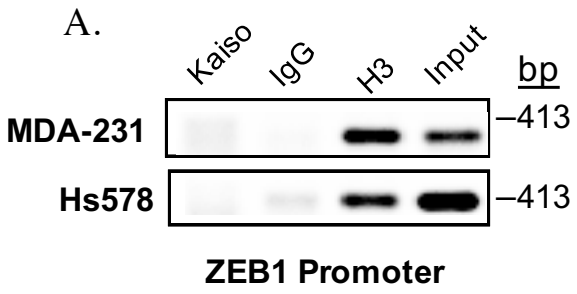

Figure 2A: Kaiso does not interact with the ZEB1 promoter region. (A) Using ChIP-PCR analysis, there appears to be no enrichment of Kaiso at the ZEB1 promotor region in MDA-231 and Hs578T TNBC cells.

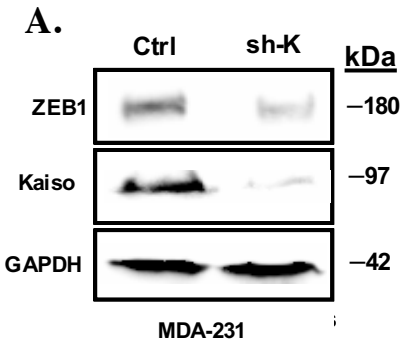

Figure 3A: Kaiso depletion attenuates ZEB1 expression in MDA-231 cells. 35μg of protein extracts from (**A**) Kaiso-depleted (sh-K), control (ctrl) MDA-231 cells were used to perform WB analysis using antibodies against ZEB1, Kaiso and GADPH as loading control.

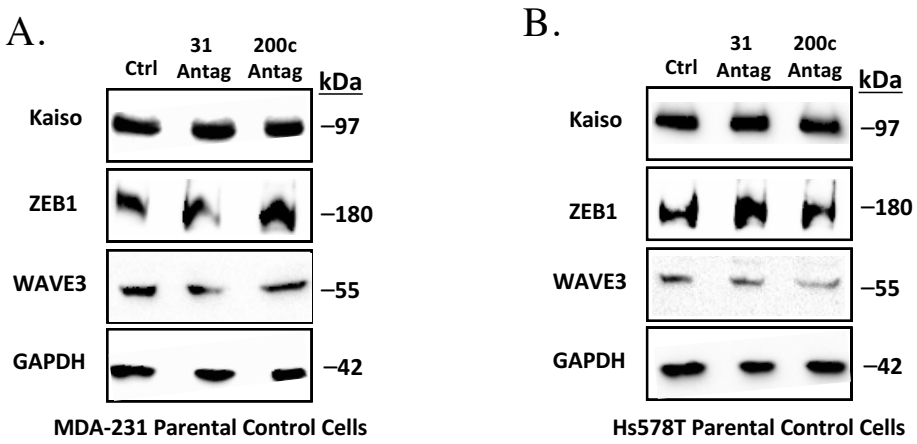

Figure 4A: MiR-31 and miR-200c inhibition does not affect ZEB1 or WAVE3 expression in control MDA-231 and Hs578T cells. **(A)** Control MDA-231 and **(B)** Hs578T cells were transfected with antagomiRs (antag), against miR-31 and miR-200c. 35µg of protein was isolated and used to perform WB analysis using antibodies against ZEB1, Kaiso, WAVE3 and GADPH as loading control.

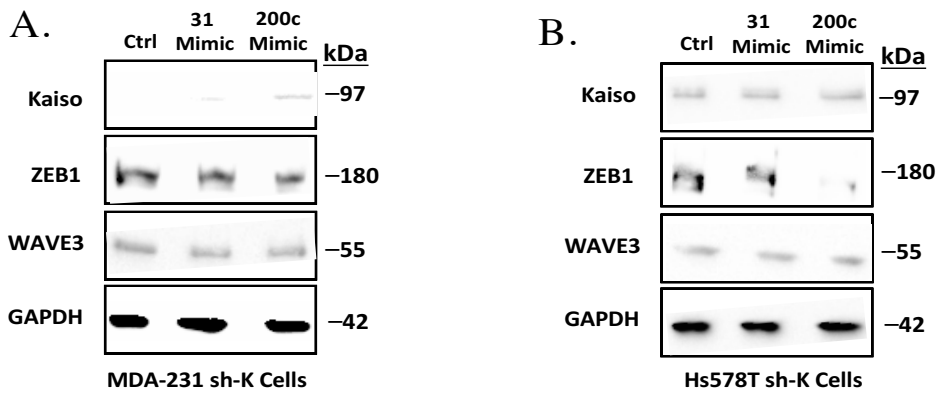

Figure 5A: miR-200c overexpression attenuates ZEB1 expression in Hs578T Kaiso-depleted cells (A) Kaiso-depleted MDA-231 and **(B)** Hs578T cells were transfected with mimics of miR-31 and miR-200c. miR-31 and miR-200c overexpression does not affect WAVE3 expression in Kaiso-depleted TNBC cells. 35 μg of protein was isolated and used to perform WB analysis using antibodies against ZEB1, Kaiso and GADPH as loading control.

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