BIOLOGICAL ROLES FOR KAISO IN TRIPLE NEGATIVE BREAST CANCERS

UNRAVELLING THE BIOLOGICAL ROLES OF KAISO IN TRIPLE NEGATIVE BREAST CANCERS

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ABSTRACT

Recent studies indicate a correlation between high expression of the POZ-ZF transcription factor Kaiso, and the aggressiveness of the **t**riple **n**egative **b**reast **c**ancer (TNBC) subtype. However, little is known about the biological roles of Kaiso in TNBC tumorigenesis and metastasis, which laid the foundation for this thesis. To elucidate Kaiso's role in TNBC, we generated stable Kaiso depletion in two well-established TNBC cell lines – MDA-MB-231 and Hs578T – using RNA interference technology. Intriguingly, we observed that Kaiso depletion delayed the tumor onset of MDA-MB-231 but not Hs578T cells, and led to the reduced expression of the c-Myc oncoprotein in MDA-MB-231 but not Hs578T cells. We postulate that this reduction in c-Myc expression is partly responsible for the delayed tumor onset observed in MDA-MB-231 cells. Additionally, loss of Kaiso expression resulted in increased apoptosis of both MDA-MB-231 and Hs578T cells *in vitro* and *in vivo*, which was accompanied by reduced expression of the DNA repair protein BRCA1. Remarkably, bioinformatic analysis revealed that high Kaiso and BRCA1 mRNA expression correlates with the reduced survival rates of TNBC patients.

Further characterization of the Kaiso-depleted cells revealed that loss of Kaiso expression strongly inhibited the metastatic abilities of MDA-MB-231 and Hs578T cells. Importantly, Kaiso depletion led to decreased **TGFβ-r**eceptor **I** and **II** (TGFβRI and II) expression that is essential for the activation of the TGFβ

signaling cascade. Concomitantly, suppressing Kaiso led to reduced TGFβ signaling. As increased TGFβRI expression is independently associated with the poor prognostic outcome of breast tumors, and the TGFβ signaling pathway is highly involved in breast tumor metastasis, we hypothesize that Kaiso functions together with TGFβRI and the TGFβ signaling cascade to promote TNBC metastasis.

An additional goal of this thesis was to investigate the role of Kaiso in the prevalence of TNBC in **w**omen of **A**frican **a**ncestry (WAA) compared to Caucasian women – since increased Kaiso expression is implicated in the poor survival outcomes of breast cancer patients of African ancestry relative to their Caucasian counterparts. Using tissue microarray and immunohistochemical analyses, we revealed for the first time a high nuclear expression of Kaiso in TNBC tissues of WAA (Nigerian, Barbadian, African American) compared to TNBC tissues of Caucasian women. Collectively, these findings unveiled functional oncogenic roles for Kaiso in the tumorigenesis and metastasis of TNBC, and revealed a plausible link between high Kaiso expression, high African ancestry and the predisposition of young WAA to TNBC.

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LIST OF ABBREVIATIONS*
***Commonly used scientific abbreviations not included**

5-aza-dC	5-**aza**-2'-**d**eoxy**c**ytidine
α-SMA	α-**s**mooth **m**uscle **a**ctin
AA	**A**frican **A**merican
ALDH1	**Al**dehyde **de**h**y**drogenase **1**
ALDH1A1	**Al**dehyde **de**h**y**drogenase **1** family, member **A1**
AREB	**A**nimal **r**esearch **e**thics **b**oard
ARVCF	**A**rmadillo **r**epeat gene deleted in **v**elo**c**ardio**f**acial syndrome
BCa	**B**reast **Ca**ncer
Bcl-6	**B c**ell **l**ymphoma **6**
BTB	**B**road complex, **T**ramtrak, **B**ric à brac
CA	**C**aucasian **A**merican
CBD	**C**atenin **b**inding **d**omain
CTCF	**C**CC**TC**-binding **f**actor
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
DTT	**D**i**t**hio**t**hreitol
DWD	**D**istance-**w**eighted **d**iscrimination
E-cadherin	**E**pithelial **cadherin**
ECL	**E**nhanced **c**hemi**l**uminescence
ECM	**E**xtra**c**ellular **m**atrix
EDTA	**E**thylene**d**iamine**t**etra**a**cetate
EGF	**E**pidermal **g**rowth **f**actor
EGFR	**E**pidermal **g**rowth **f**actor **r**eceptor
EMT	**E**pithelial to **m**esenchymal **t**ransition
ERK	**E**xtracellular signal-**r**egulated **k**inase

EtBr	**Et**hidium **br**omide
FBS	**F**etal **b**ovine **s**erum
FFPE	**F**ormalin-**f**ixed and **p**araffin-**e**mbedded
FITC	**F**luorescein **i**so**th**io**c**yanate
fRMA	**F**rozen **r**obust **m**ulti-**a**rray
GEO	**G**ene **e**xpression **o**mnibus
GSK3β	**G**lycogen **s**ynthase **k**inase **3** **b**eta
H&E	**H**ematoxylin and **e**osin
H-score	**H**istochemical **score**
HDAC	**H**istone **d**e**ac**etylase
HIC-1	**H**ypermethylated **i**n **c**ancer **1**
HRP	**H**orse **r**adish **p**eroxidase
IHC	**I**mmuno**h**isto**c**hemistry
JMD	**j**uxta-**m**embrane **d**omain
KCl	Potassium(**K**) **c**h**l**oride
KBS	**K**aiso **b**inding **s**ite
LiCl	**Li**thium **c**h**l**oride
LUTH	**L**agos **U**niversity **T**eaching **H**ospital
meCpG	**Me**thylated **CpG**
MET	**M**esenchymal to **e**pithelial **t**ransition
MgCl	**Mg**nesium **c**h**l**oride
MH	**M**ad-**H**omology
MIZ1	**M**sx-**i**nteracting-**z**inc finger **1**
MMP	**M**atrix **m**etallo**p**roteinases
mKaiso	**m**urine **Kaiso**
MTG-16	**M**yeloid **t**ranslocation **g**ene **16**
NaCl	Sodium (**Na**) **c**h**l**oride
N-CoR	**N**uclear receptor **co-r**epressor-1
NSG	**N**OD **S**CID **g**amma

Oligos	**Oligo**nucleotide**s**
p120^{ctn}	**p120 c**a**t**e**n**in
PBS	**P**hosphate **b**uffered **s**aline
PBS-T	**PBS-T**ween 20
PI3K	**p**hosphatidyl**i**nositol-**3**-**k**inase
Poly dI-dC	**Poly d**eoxy**i**nosinic – **d**eoxy**c**ytidylic acid
POZ	**Po**x virus and **z**inc finger
pRS	**pR**etro**S**uper
PUMA	**p**53 **u**pregulated **m**odulator of **a**poptosis
QEH	**Q**ueen **E**lizabeth **H**ospital
RIPA	**R**adio **i**mmuno**p**recipitation **a**ssay
RMA	**R**obust **m**ulti-**a**rray
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
TAE	**T**ris-**a**c**e**tate EDTA buffer
TBE	**T**ris-**b**orat**e** EDTA buffer
TBS	**T**ris **b**uffered **s**aline
TBS-T	**TBS-T**ween 20
TCGA	**T**he **c**ancer **g**enome **a**tlas
TE	**T**ris-**E**DTA
TGFβ	**T**ransforming **g**rowth **f**actor β
TMA	**T**issue **m**icro**a**rray
Tris-Cl	**Tris c**h**l**oride
TSA	**T**richo**s**tatin **A**
xKaiso	***X****enopus* **Kaiso**
YPTS	**Y**ale **p**athological **t**issue **s**ervices

YTMA	**Y**ale **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 Synopsis

The slave trade era witnessed a huge migration of many West Africans to the Caribbean and North America (Mannix and Cowley 1962), where their descendants are now settled, with many having a more heterogeneous genetic makeup than their ancestors. Surprisingly, the origins of **b**reast **ca**ncer (BCa) – a very common disease diagnosed in females worldwide – can possibly be traced back to Africa, with the earliest documented case described in the "Edwin Smith Surgical Papyrus" (Lakhtakia 2014). Unfortunately, BCa is now considered a major health issue not only in Africa but worldwide (Bray et al. 2004; Coughlin and Ekwueme 2009; Forbes 1997), and is a leading cause of cancer-related deaths in females (Ferlay et al. 2015). Although the past 20 years have seen a decline in BCa mortality rates in developed countries, BCa incidence and related mortality rates are continually on the rise in developing countries (Ferlay et al. 2015). In terms of definition, BCa is described as an extremely heterogeneous and multifactorial disease with diverse subtypes – including luminal A, luminal B, HER2-enriched, Claudin-low and basal-like – that are characterized by distinct molecular profiles and response to therapy (Perou et al. 2000; Sørlie et al. 2001). The Claudin-low and basal-like BCas are often biologically characterized as triple-negative breast cancers due to their lack of three key proteins used to clinically classify BCa (Anders and Carey 2008; Foulkes et al. 2010). Triple-

negative BCas account for a disproportionate number of BCa-related deaths due to their highly aggressive and metastatic nature (Oakman et al. 2010).

The exact etiology of **t**riple **n**egative **b**reast **c**ancer (TNBC) is currently unknown, but likely risk factors include high parity and body mass index (Anders and Carey 2008; Foulkes et al. 2010). Intriguingly, TNBC is prevalent in younger women, and premenopausal **w**omen of **A**frican **a**ncestry (WAA) compared to women of other ethnicities (Carey et al. 2006; Lund et al. 2009), but the cause of this racial disparity is currently unknown. However, mounting evidence suggests that there may be a genetic predisposition to TNBC in WAA (Stark et al. 2010) that may explain the racial disparity in TNBC prevalence. Recently, we and others discovered that the transcription factor Kaiso is highly expressed in TNBCs. Notably, high Kaiso expression correlates with TNBC aggressiveness and the disparity in survival outcomes of BCa patients of African ancestry compared to their Caucasian counterparts (Jones et al. 2014; Vermeulen et al. 2012). Despite these findings, the clinical relevance of Kaiso expression in TNBC aggressiveness and racial disparity in prevalence was unknown, and these questions provided the basis for the studies described in this thesis.

1.2 Triple negative breast cancer: Origin, features and treatment options

1.2.1 Origin: The female mammary epithelium

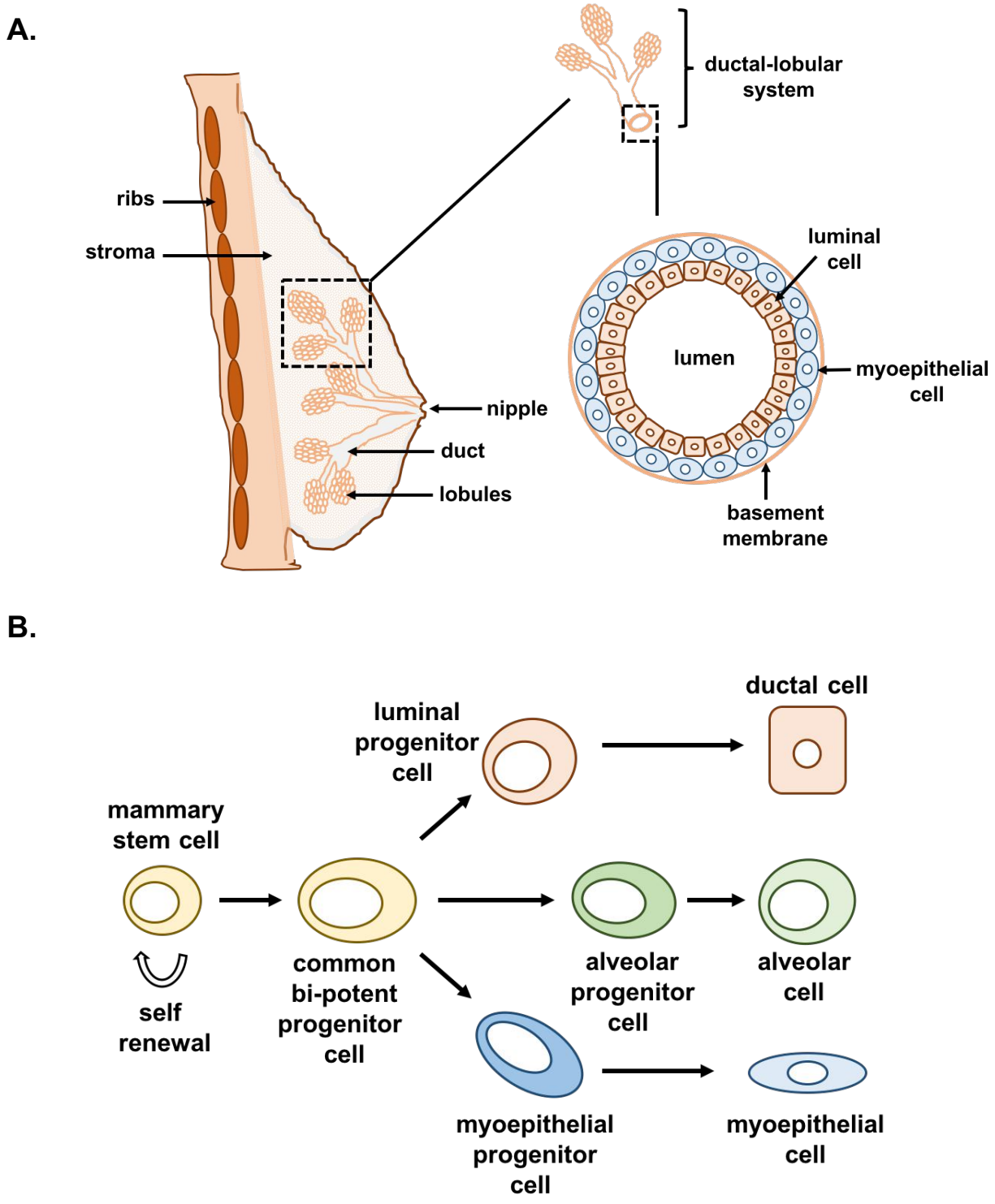
The adult female breast is a complex, specialized organ that is physiologically tasked with the synthesis and delivery of milk to babies after birth (Medina 1996). Structurally, the female breast consists of two main compartments: the mammary parenchyma and the stroma (**Figure 1.1a**). The mammary parenchyma is primarily involved in milk production (lobes/lobules/alveoli) and transportation (ducts/ductules), while the stroma is comprised of an enriched multicellular environment that supports the development and function of the parenchyma (Medina 1996; Pandya and Moore 2011; Polyak and Kalluri 2010; Shekhar et al. 2003). In terms of structure, the mammary parenchyma is organized into a ductal-lobular system that is characterized by ~15 – 20 lobes connected by a complex network of ducts (Guinebretière et al. 2005; Pandya and Moore 2011). Each ductal-lobular structure contains a bi-layered epithelium: an inner ductal/alveolar luminal epithelial cell layer lining the lumen, and a discontinuous outer basal myoepithelial cell layer adjacent to the basement membrane (Guinebretière et al. 2005; Owens et al. 2013; Pandya and Moore 2011) (**Figure 1.1a**).

The growth and differentiation of the mammary epithelium occurs mainly after birth in females (Gjorevski and Nelson 2011; Watson and Khaled 2008). In the adult female, mammary epithelial cells continually undergo sequential surges of

proliferation and apoptosis during estrus cycles, pregnancy, lactation and involution (Borellini and Oka 1989; Macias and Hinck 2012; Navarrete et al. 2005; Nazario et al. 1995; Pike et al. 1993; Watson et al. 2006). Therefore, it is not surprising that the majority of mammary neoplasms arise in the mammary epithelium (Medina 1996). There is strong evidence of a differentiation hierarchy in the mammary epithelium, with both luminal epithelial and myoepithelial cells postulated to arise from a common bi-potent progenitor mammary stem cell, reviewed in (Visvader 2009).

Although there is much controversy surrounding the particular cell type in the normal breast from which triple negative tumors arise, there is some indication that most of these tumors originate from luminal-progenitor cells that are postulated to differentiate into ductal cells (Lim et al. 2009a) (**Figure 1.1b**). Indeed, the majority of TNBCs are ductal in origin, and are frequently histologically classified as invasive ductal carcinomas. However other rare histological TNBC subtypes also exist: e.g. adenoid cystic, metaplastic and medullary carcinomas, reviewed in (Irshad et al. 2011; Sasaki and Tsuda 2009). Additionally, most triple-negative tumors exhibit molecular characteristics affiliated with cancer stem cells, e.g. high CD44 and ALDH1 expression (Collina et al. 2015; Ma et al. 2017) or mammary stem cells, which suggest that a fraction of these tumors may also arise from the much posited mammary stem cells (Foulkes et al. 2010).

Figure 1.1: **Schematic illustration of the female mammary organ. (A)** The female mammary organ consists mostly of the mammary parenchyma (ducts and lobules) and stroma. An enlarged portion of the ductal-lobular structure of the mammary parenchyma reveals a bi-layered epithelium comprised of an inner ductal luminal epithelial cell layer lining the lumen, and a discontinuous outer basal myoepithelial cell layer resting on the basement membrane. **(B)** Illustration of the differentiation hierarchy of the mammary epithelium, which indicates that both luminal epithelial and myoepithelial cells arise from a common bi-potent progenitor mammary stem cell. The luminal progenitor cell that stems from the common progenitor cell population can further differentiate into a ductal cell. Model adapted from (Visvader, 2009).


A.
ductal-lobular system
ribs
stroma
nipple
duct
lobules
luminal cell
lumen
myoepithelial cell
basement membrane
B.
ductal cell
luminal progenitor cell
mammary stem cell
self renewal
common bi-potent progenitor cell
alveolar progenitor cell
alveolar cell
myoepithelial progenitor cell
myoepithelial cell

1.2.2 Molecular and clinical features of triple negative breast tumors

The term triple-negative BCa was first coined over a decade ago and accounts for ~15% of all breast cancers (Foulkes et al. 2010). Triple-negative tumors as the name implies lack expression of three key proteins – **e**strogen **r**eceptors (ER), **p**rogesterone **r**eceptors (PR), and the **h**uman **e**pidermal growth factor **r**eceptor (HER) 2 – that are used to immunohistochemically classify breast tumors (Foulkes et al. 2010). Most triple-negative breast tumors also exhibit a high frequency of p53 and BRCA1 mutations, and thus an unstable genome due to the roles of wild-type p53 and BRCA1 in DNA repair and genomic integrity (Gonzalez-Angulo et al. 2011; Manié et al. 2009; Shah et al. 2012; Wong-Brown et al. 2015). In addition to the aforementioned genetic profile, most TNBCs also exhibit distinct molecular profiles including increased expression of **e**pidermal **g**rowth **f**actor **r**eceptor (EGFR) and the c-Myc oncoprotein (Burness et al. 2010; Network 2012). The heterogeneous genetic profiles of triple negative breast tumors have led to the stratification of TNBC into several molecular subgroups including basal-like, Claudin-low, luminal androgen receptor and mesenchymal (Lehmann et al. 2011; Perou 2010; Prat et al. 2010; Rakha et al. 2009). Clinically, TNBCs are typically characterized by a younger age at onset, high histopathological grade, large tumor size, increased frequency of early recurrence and a high risk of visceral (lung, brain) metastasis compared to other BCa subtypes (Dent et al. 2009; Ismail-Khan and Bui 2010).

A study of ~1600 breast cancer patients diagnosed at the Women's College Hospital, Toronto from 1987 – 1997, revealed that the mean age at diagnosis for TNBC patients was 53 years compared to 57.7 years for non-TNBC patients (Dent et al. 2007). Furthermore, triple-negative breast tumors were found to be more often of a higher grade (66%), and affiliated with a larger mean (3 cm) tumor size than non-TNBC tumors, which were associated with a smaller mean (2.1 cm) tumor size and had a decreased probability (28%) of being high grade (Carey et al. 2006; Dent et al. 2007). Compared to patients with other BCa subtypes, TNBC patients also had a more aggressive clinical course of the disease; this was often associated with an increased probability of distant recurrence (33.9% versus 20.4% for other BCa subtypes) within the first 3 years (Carey et al. 2006; Dent et al. 2007). Finally, TNBC patients were observed to be more likely to die from the disease within the first five years than patients diagnosed with other BCa subtypes (Carey et al. 2006; Dent et al. 2007), a finding that was corroborated by an independent study of Eastern and Central North Carolina BCa patients (Carey et al. 2006).

1.2.3 Established treatment options for triple negative breast cancers (TNBC)

The decreased rate of survival of TNBC patients is primarily associated with the aggressive and metastatic nature of the disease, as well as lack of effective targeted therapies. Currently, the mainstay of treatment for TNBC is surgery,

radiation and/or chemotherapy (Foulkes et al. 2010; Wahba and El-Hadaad 2015). Most triple-negative tumors are sensitive to chemotherapy regimens containing anthracyclines, taxanes and/or platinum-based agents, which is often associated with an overall good **p**athologic **c**omplete **r**esponse (pCR) rate in the corresponding patients (Carey et al. 2007; Liedtke et al. 2008; Silver et al. 2010). TNBC patients who attain good pCR are reported to have fewer distant relapses, and excellent distant metastasis-free and overall survival. Conversely, TNBC patients with partial pCR, and thus residual disease, experience a high rate of distant relapses, coupled with reduced distant metastasis-free and overall survival (Carey et al. 2007; Foulkes et al. 2010; O'Reilly et al. 2015; Pareja Fresia et al. 2016). This is most likely due to the increased metastatic propensity of triple-negative tumors, as metastasis is responsible for 90% of all cancer-related deaths (Mehlen and Puisieux 2006).

1.3 The transforming growth factor-β signaling cascade and TNBC metastasis

1.3.1 The canonical TGFβ signaling pathway

The **t**ransforming **g**rowth **f**actor β (TGFβ) cytokine is the prototypic member of a large family of signaling proteins, each with cell-type specific and context-dependent functions (Akhurst and Hata 2012; Weiss and Attisano 2013). Since its discovery, TGFβ has been implicated in many important cellular processes including but not limited to cell proliferation, apoptosis, differentiation and

migration, reviewed in (Giehl and Menke 2006; Moses and Serra 1996; Schuster and Krieglstein 2002; Seoane 2006). In mammals, TGFβ exists as one of three homologous isoforms (TGFβ1, 2 and 3) with distinct expression patterns (Akhurst and Hata 2012; Barcellos-Hoff and Ewan 2000). Each TGFβ isoform, is synthesized and secreted into the extracellular environment as a latent dimeric precursor protein, comprised of the mature bioactive cytokine non-covalently bound to its propeptide – the **l**atency-**a**ssociated **p**eptide (LAP). Disruption of the TGFβ – LAP connection via several mechanisms including proteolysis, liberates the biologically active TGFβ (Miyazono et al. 1991; Saharinen et al. 1999). This allows association of the bioactive TGFβ with its signaling cognate serine/threonine kinase receptors – **TGFβ r**eceptors (TGFβR) type I and II – that are present on the membrane of multiple cell types (Massagué 1998). In the basal state, TGFβRII exists in a phosphorylated form and is thus designated as a constitutively active kinase. In contrast, TGFβRI exist in an un-phosphorylated form, and is associated with the immunophilin **FK**506-**b**inding **p**rotein (FKBP12) that binds to specific residues (Leu-Pro) adjacent to the TGFβRI un-phosphorylated **G**lycine-**S**erine rich (GS) domain (Massagué 1998). Consequently, TGFβRI is kept from ligand-independent signaling and phosphorylation/activation by the constitutively active TGFβRII kinase.

The principal downstream effectors of the canonical TGFβ signaling cascade are the Smad proteins, in particular the **r**eceptor-regulated (R)-Smad (Smad2/3) and

the **co**mmon-mediator (Co)-Smad4 proteins. Smad proteins are homologues of the *Caenorhabditis elegans* SMA (from the *sma* gene which indicates **sma**ll body size) proteins and the *Drosophila* MAD (**m**others **a**gainst **d**ecapentaplegic) proteins (Savage et al. 1996; Sekelsky et al. 1995), and derived their name from a combination of the two (SMA and MAD). In the absence of TGFβ signaling, un-phosphorylated Smad2/3 monomers are mainly localized in the cytoplasm where they exist in an inactive auto-inhibitory state due to the physical interaction of their MH1 and MH2 (**M**ad-**H**omology **1** and **2**) domains (Massagué 1998).

The TGFβ signaling cascade is initiated upon binding of bioactive TGFβ to its cognate receptors – TGFβRII and TGFβRI. Interaction of bioactive TGFβ with its cognate receptors have been shown to occur in a two-step process as demonstrated by genetic complementation studies in TGFβ-resistant cell mutants lacking either TGFβRI or TGFβRII, reviewed in (Massagué 1998). First, mature TGFβ directly associates with the ligand-binding domain of the constitutively active TGFβRII kinase that can bind TGFβ in the absence of TGFβRI (Massagué 1998; Wrana et al. 1992). Next, TGFβRI is recruited into the TGFβRII-bound TGFβ complex, which results in the formation of a hetero-tetrameric ligand-receptor complex consisting of the bioactive TGFβ dimer and pairs of TGFβRI and TGFβRII proteins (Heldin et al. 1997; Massagué 1998), see (**Figure 1.2**). TGFβ-induced formation of the ligand-receptor complex stimulates a conformational change in TGFβRI that results in the dissociation of FKBP12

(Chen et al. 1997b; Massagué 1998; Wrana et al. 1992) (**Figure 1.2**). This is then accompanied by TGFβRII-mediated phosphorylation of serine/threonine residues present in the TGFβRI GS domain, which results in TGFβRI activation.

The activated TGFβRI then binds and phosphorylates Smad2/3 – localized to the membrane by **S**MAD **a**nchor for **r**eceptor **a**ctivation or SARA (Tsukazaki et al. 1998) – at a characteristic C-terminal SXS motif (Seoane 2006; Shi and Massagué 2003). Upon phosphorylation, Smad2/3 dissociates from SARA, forms homo- and heterodimeric complexes with each other, and subsequently heterotrimeric complexes with Smad4. The heterotrimeric Smad complexes then translocate to the nucleus where they regulate the expression of a large repertoire of TGFβ target genes in concert with other DNA-binding proteins and nuclear cofactors e.g. the coactivator p300 (Heldin et al. 1997; Massagué 2012; Shi and Massagué 2003) (**Figure 1.2**). The capacity of the heterotrimeric Smad transcription complex to associate with diverse DNA-binding proteins in TGFβ-stimulated cells is largely responsible for the numerous cellular processes controlled by the canonical TGFβ signaling cascade (Massagué et al. 2005). Notably, TGFβ signals can also be transduced within the cell via non-canonical (non-Smad) intracellular proteins such as **p**hosphatidyl**i**nositol-**3**-**k**inase (PI3-K) and various **m**itogen-**a**ctivated **p**rotein **k**inases (MAPK) e.g. **e**xtracellular signal-**r**egulated **k**inase **1/2** or ERK1/2 (Zhang 2009). However, the canonical Smad

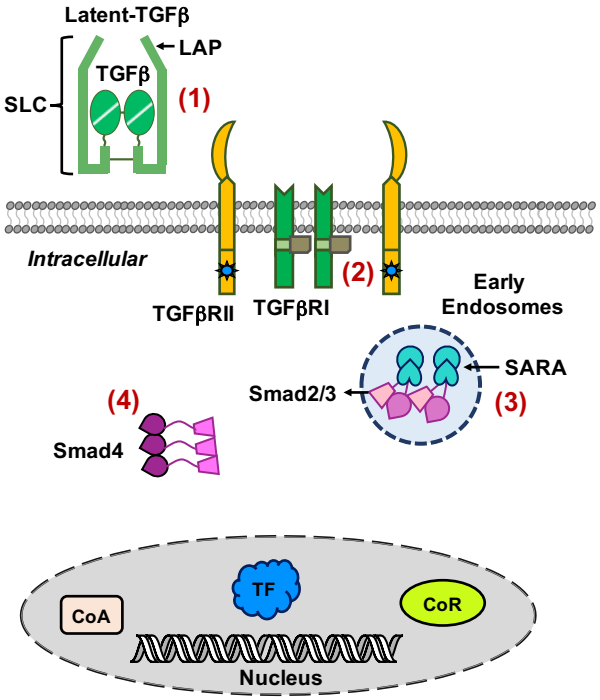
proteins represent the primary route of TGFβ signal propagation from the cell membrane to the nucleus (Massagué 2000).

1.3.2 TGFβ inhibits cell proliferation in normal breast cells

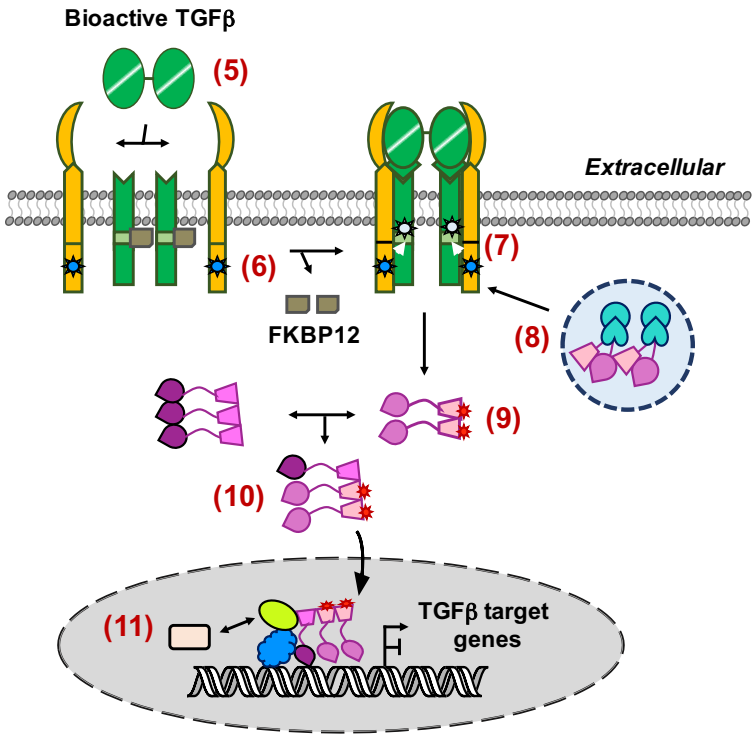
A principal function of the TGFβ signaling cascade in non-transformed mammary epithelial cells is the inhibition of cell cycle progression – particularly the transition from growth (G1) to synthesis (S) phase – and thus cell proliferation. One of the first reports implicating TGFβ in mammary epithelial proliferation demonstrated that slow-releasing TGFβ pellets embedded in the mouse mammary parenchyma rapidly inhibited epithelial proliferation and growth of emerging ductal end buds (Silberstein and Daniel 1987). The epithelium-specific nature of TGFβ inhibitory effects on the actively growing end buds was later demonstrated in a subsequent study where exogenous TGFβ was shown to impede DNA synthesis in the growing epithelium, but not in the stroma surrounding the developing end buds (Daniel et al. 1989). Further evidence of TGFβ's anti-proliferative functions in non-transformed mammary epithelium was gained from studies in mouse models, where TGFβ1 overexpression in the mammary glands of virgin and pregnant transgenic mice was shown to suppress ductal epithelial proliferation and development (Pierce et al. 1995).

Figure 1.2: The canonical TGFβ signaling pathway. TGFβ ligands are synthesized and secreted into the extracellular environment as latent precursors in a **s**mall **l**atent **c**omplex (SLC) that is comprised of the mature TGFβ dimeric protein non-covalently attached to its pro-peptide **l**atency-**a**ssociated **p**eptide (LAP). In this biologically inactive form (**1**), TGFβ is unavailable for binding to its cognate receptors – TGFβRI and TGFβRII. In unstimulated cells, TGFβRI is bound by the immunophilin FKBP12 at residues adjacent to its GS domain – small lemon box within TGFβRI (**2**) while the TGFβ downstream effectors – the receptor-regulated Smad2/3 are bound to SARA and localized in endosomes within the cytoplasm (**3**). The common-mediator Smad4 is also localized to the cytoplasm most often as trimers (**4**). Upon TGFβ activation via various mechanisms including proteolysis by matrix metalloproteinases, the bioactive TGFβ ligand binds to TGFβRII (**5**), which allows for the recruitment of TGFβRI and its release from FKBP12 (**6**) that is accompanied by its phosphorylation (white arrows) and subsequent activation by TGFβRII (**7**). Activated TGFβRI phosphorylates Smad2/3 that is translocated to the membrane by SARA (**8**) upon activation of TGFβ signaling. Phosphorylated Smad2/3 dissociates from SARA (**9**) and forms heterotrimeric complexes with Smad4 (**10**), which translocate to the nucleus where they regulate the expression of a large repertoire of TGFβ target genes in coalition with other **t**ranscription **f**actors (TF) and nuclear cofactors including **coa**ctivators (CoA) or **cor**epressors (CoR) (**11**). Original artwork.

TGFβ Pathway off



TGFβ Pathway on



In complementary studies, heterozygous deletion of *TGFβ1* resulted in increased proliferation of the mammary epithelium and consequently enhanced ductal and lobulo-alveolar development during puberty and pregnancy respectively in the *TGFβ1*$^{+/-}$ transgenic mice (Ewan et al. 2002). Interestingly, in this study, the TGFβ1 depletion phenotype in the mammary epithelium was only elicited in the presence of the steroid hormones estrogen and progesterone that are major regulators of mammary gland development (Ewan et al. 2002). This observation suggested that TGFβ growth-suppressive functions in normal mammary epithelial cells are influenced in part by the estrogen/progesterone signaling cascade.

The anti-proliferative functions of the canonical TGFβ signaling cascade are accomplished by the concurrent activation of the **c**yclin-**d**ependent **k**inase (CDK) inhibitors (*CDKN2B* and *CDKN1A*), and repression of the growth-promoting transcription factors (c-*MYC*, *ID1*, *ID2* and *ID3*) by distinct Smad transcription complexes (Chen et al. 2002; Siegel and Massagué 2003). Interestingly, c-Myc can inhibit *CDKN2B* and *CDKN1A* expression (Jung and Hermeking 2009; Seoane et al. 2001; Staller et al. 2001), however, c-Myc repression of both CDK inhibitors is relieved by TGFβ-mediated suppression of c-*MYC* expression (Frederick et al. 2004; Kubiczkova et al. 2012). Thus, TGFβ inhibition of c-*MYC* expression is critical for its cytostatic responses (Chen et al. 2001; Claassen and Hann 2000; Warner et al. 1999). Correspondingly, attenuation of TGFβ-mediated c-*MYC* inhibition, or aberrant overexpression of c-Myc in mammary epithelial

cells abolishes the growth-suppressive functions of the TGFβ pathway (Chen et al. 2001; Gomis et al. 2006; Singh et al. 2010). Remarkably, the growth-inhibitory functions of TGFβ are attenuated in many mammary tumors (Gomis et al. 2006; Kretzschmar 2000) even though mutational inactivation of core TGFβ pathway constituents (e.g. Smad4) is rare in these tumors (Padua and Massague 2009). Recent reports attribute the aberrant TGFβ cytostatic responses observed in many mammary tumors – including those belonging to the TNBC subtype – to a loss in the formation of the Smad transcription complex that facilitates *c-MYC* repression (Chen et al. 2001; Gomis et al. 2006). Interestingly, several proteins including the C/EBPβ transcription factor implicated in the formation of the *c-MYC* inhibitory Smad transcription complex – and thus TGFβ cytostatic responses – are lost or inhibited in malignant mammary cells (Gomis et al. 2006). For instance, loss of C/EBPβ expression (Johansson et al. 2013), or an increased expression of its inhibitory isoform (C/EBPβ-LIP) attenuates TGFβ growth-inhibitory functions (Gomis et al. 2006) and switches TGFβ to a promoter of invasion and metastasis in aggressive breast cancer cells (Johansson et al. 2013).

1.3.3 TGFβ Promotes TNBC cell metastasis

As previously mentioned, the TGFβ signaling cascade regulates multiple other cellular processes in addition to cell proliferation. Thus, abolition of TGFβ anti-proliferative responses in mammary carcinomas, including triple-negative tumors,

permits the corrupt utilization of other TGFβ-regulated physiological processes (Padua and Massague 2009). Indeed, numerous studies have demonstrated that TGFβ initially functions as a potent growth-suppressor in the early stages of mammary tumorigenesis. However, as mammary tumors become refractory to TGFβ growth-suppressive functions, TGFβ becomes a powerful driver of invasion and metastasis, reviewed in (Serra and Crowley 2003; Zarzynska 2014). TGFβ roles in the metastasis of mammary tumors have been well examined (Bandyopadhyay et al. 2006; Ganapathy et al. 2010; Kang et al. 2005; Padua et al. 2008; Tian et al. 2003; Yin et al. 1999; Yu and Stamenkovic 2004). Remarkably, many of these studies utilized established TNBC cell lines to investigate the mechanisms of TGFβ-regulated mammary tumor metastasis, and thus inadvertently provided evidence of TGFβ metastatic functions in TNBC cells. One study found that TGFβ promotes lung-specific metastasis of TNBC cells via the induction of *ANGPTL4* (**a**n**g**io**p**oietin-**l**ike **4)** in the corresponding primary tumor cells (Padua et al. 2008), while a different study demonstrated that TGFβ-stimulated parathyroid hormone-related protein production fostered bone metastasis of TNBC cells (Yin et al. 1999). Consistent with these findings, tumors obtained from TNBC patients display an enrichment of TGFβ signaling proteins, which correlates with the aggressiveness of these tumors (Ding et al. 2016).

There is strong evidence that TGFβ further facilitates the metastasis of TNBC cells via cooperation between downstream TGFβ effectors (Smad proteins) and

other proteins such as oncogenic Ras and mutant-p53 (Adorno et al. 2009). In addition, TGFβ is thought to promote the metastasis of transformed mammary cells in part via its potent induction of the **e**pithelial-to-**m**esenchymal **t**ransition (EMT) program (Pang et al. 2016; Papageorgis 2015; Singh and Settleman 2010). EMT is a multifaceted biological phenomenon that ultimately enables a polarized epithelial cell to replace its epithelial characteristics with features affiliated with mesenchymal cells, e.g. reduced cell-cell adhesion and increased migratory/invasive abilities, reviewed in (Christiansen and Rajasekaran 2006; Kalluri and Weinberg 2009). At the molecular level, EMT is associated with the activation of mesenchymal proteins such as the intermediate filament Vimentin, and the EMT-inducing transcription factors ZEB1 and Slug. These transcription factors facilitate EMT in part via downregulation of several epithelial proteins including the tumor suppressor E-cadherin, which is a key mediator of epithelial cell-cell adhesion (Christofori 2006; Thiery et al. 2009; Tomaskovic-Crook et al. 2009).

1.4 The E-cadherin-catenin complex and epithelial cell adhesion

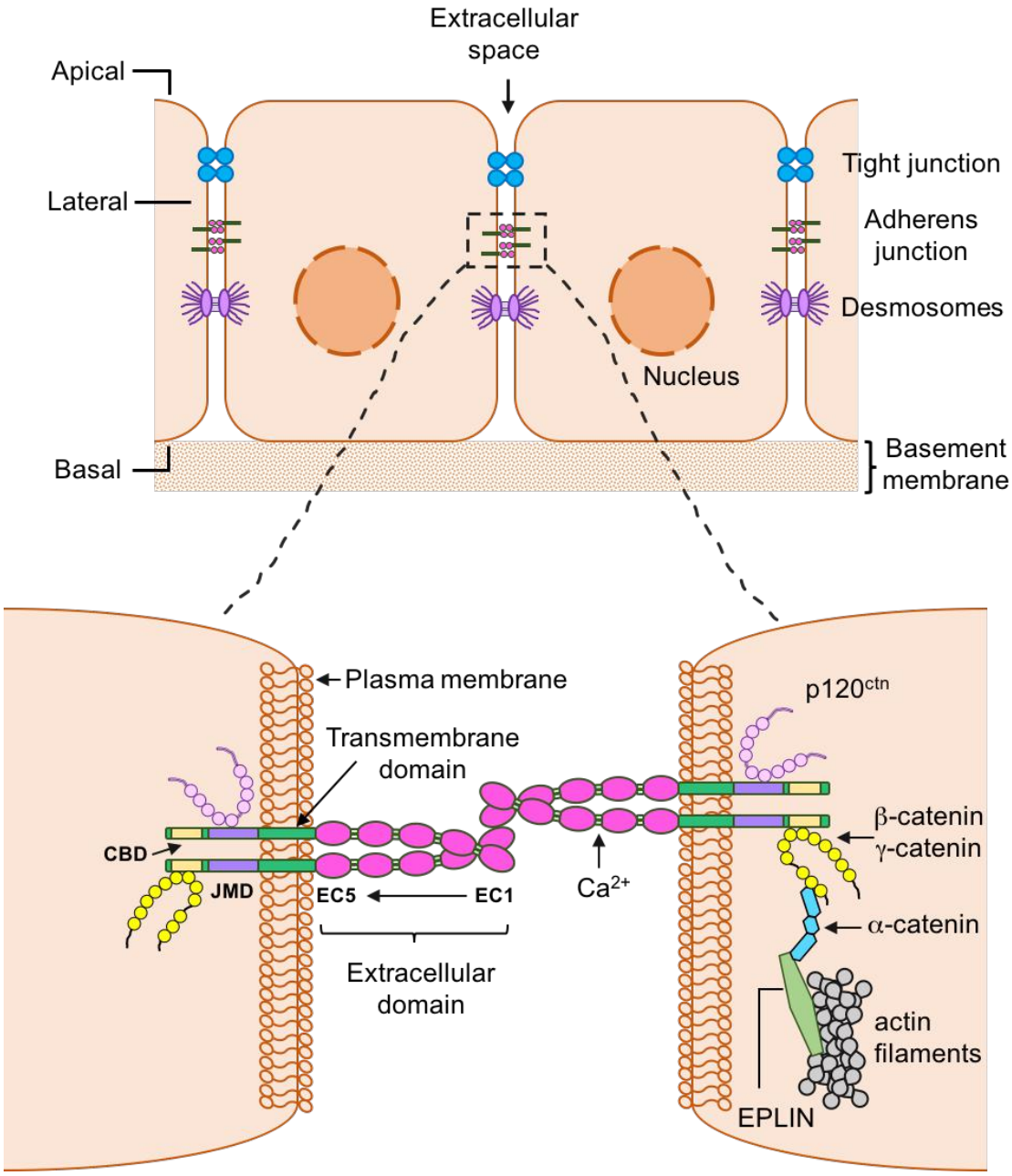
1.4.1 E-cadherin mediates epithelial cell adhesion via interaction with catenins

A typical epithelial cell is characterized by apical, lateral and basal plasma membrane domains (Huang et al. 2012), and is held together via distinct intercellular adhesive networks – tight junctions, *adherens* junctions and

desmosomes – that reside in the lateral domain (Giepmans and van Ijzendoorn 2009) (**Figure 1.3**). Epithelial cell-cell adhesion is principally facilitated by classical cadherins, which are the fundamental membrane constituents of *adherens* junctions (Van den Bossche et al. 2012). Classical cadherins belong to a large family of single-span transmembrane glycoproteins that facilitate calcium-dependent cell adhesion in almost all compact tissues of multicellular organisms (Ivanov et al. 2001). Structurally, the classical cadherins are characterized by two important domains: (i) an amino-terminal ectodomain comprised of five **e**xtracellular **c**adherin (EC) repeats that mediate calcium-dependent homotypic interactions with cadherin molecules on adjoining cells, and (ii) a highly conserved carboxyl-terminal cytodomain that associates with the actin cytoskeleton via distinct catenin proteins (Beavon 2000; Halbleib and Nelson 2006; Ivanov et al. 2001). This section will focus on **e**pithelial (E)-cadherin – the most widely studied of all the classical cadherins – and its role in cell adhesion.

E-cadherin belongs to the type 1 classical cadherin family and is essential for the establishment and maintenance of epithelial tissue integrity and cell-cell adhesion (Chen et al. 1997a). Like most type 1 family members, E-cadherin contains five EC repeats in its extracellular domain that facilitates homotypic interactions between adjacent cells, and binding sites in its intracellular domain for association with the actin cytoskeleton via catenins (Halbleib and Nelson 2006; Ivanov et al. 2001; Perez-Moreno et al. 2003) (**Figure 1.3**).

Figure 1.3: Schematic illustration of the E-cadherin–catenin complex. Cadherin-dependent cell-cell adhesion in epithelial cells is mediated by E-cadherin. The mature E-cadherin molecule possesses an extracellular domain that extends from the cell surface, a single membrane-spanning domain, and a highly conserved intracellular cytoplasmic domain that interacts with catenin cofactors. The extracellular domain is comprised of five cadherin repeats (EC1 – EC5) that facilitate calcium (Ca^{2+})-dependent homophilic cis- and trans-interactions with cadherin molecules on adjoining cells while the cytoplasmic domain contains specific sites (JMD and CBD) that directly binds $p120^{ctn}$ and β-catenin/γ-catenin respectively. The stability of E-cadherin-mediated cell-cell contacts is dependent on Ca^{2+} binding to two highly conserved "calcium binding" sites located between adjoining EC repeats. α-catenin anchors the E-cadherin-catenin complex to actin filaments of the cytoskeleton via direct interaction with β-catenin and actin-binding proteins such as EPLIN. Original artwork.


Extracellular space
Apical
Lateral
Basal
Tight junction
Adherens junction
Desmosomes
Nucleus
Basement membrane
Plasma membrane
Transmembrane domain
CBD
JMD
EC5
EC1
Ca2+
Extracellular domain
p120ctn
β-catenin
γ-catenin
α-catenin
actin filaments
EPLIN

Almost all catenins with the exception of the structurally distinct α-catenin, contain a centralized **Arm**adillo (Arm) domain comprised of ~ 9-12 Arm repeats that participate in protein-protein interactions (McCrea and Gu 2010). Vertebrate Armadillo-containing catenins are divided into three subfamilies named after key representative members. These include: plakophilin (plakophilin-1, -2, -3), β-catenin (β- and γ-catenin), and p120-catenin (p120, δ-catenin, ARVCF, p0071) subfamilies (McCrea and Gu 2010). Plakophilin family members primarily interact with desmosomal cadherins whereas the β-catenin and p120-catenin (hereafter $p120^{ctn}$ or p120) family members primarily associate with classical cadherins including E-cadherin (McCrea and Gu 2010).

β-catenin interacts with the distal cytoplasmic tail of E-cadherin via the **c**atenin-**b**inding **d**omain (CBD) while $p120^{ctn}$ associates with the more membrane proximal region of E-cadherin (Gall and Frampton 2013; Reynolds et al. 1994) via the **j**uxta**m**embrane **d**omain (JMD) – which is implicated in cadherin clustering and stabilization (Yap et al. 1998). β-catenin functions to strengthen the E-cadherin-catenin complex by indirectly tethering E-cadherin to actin filaments through its direct interaction with α-catenin (Abe and Takeichi 2008; Halbleib and Nelson 2006; Ivanov et al. 2001; Perez-Moreno et al. 2003). Unlike β-catenin, $p120^{ctn}$ does not associate with α-catenin (Daniel and Reynolds 1995) or participate in the anchorage of E-cadherin to the cytoskeleton. Instead $p120^{ctn}$ functions as a master regulator of E-cadherin's stability and turnover at the cell

membrane (Davis et al. 2003b; Ireton et al. 2002). Indeed, ectopic $p120^{ctn}$ expression in p120-deficient cells resulted in increased E-cadherin's stability and protein levels (Ireton et al. 2002), while downregulation of $p120^{ctn}$ in p120-expressing cells led to the rapid degradation of E-cadherin, as well as α- and β-catenins (Davis et al. 2003b). Interestingly, the effects of $p120^{ctn}$ on E-cadherin stability is attributed to its interaction with distinct binding sites on the E-cadherin JMD including those involved in clathrin-dependent endocytosis and Hakai-mediated ubiquitination of E-cadherin (Ishiyama et al. 2010; Miyashita and Ozawa 2007).

Despite the integral structural role of β-catenin in the linkage between the E-cadherin-catenin complex and the actin cytoskeleton, β-catenin is dispensable for strong cell-cell adhesion. This was demonstrated by several studies which showed that E-cadherin deletion mutants lacking the β-catenin binding domain or fused directly to α-catenin were efficient in mediating cell-cell adhesion in the absence of β-catenin (Gottardi et al. 2001; Nagafuchi et al. 1994). In contrast, $p120^{ctn}$ is essential for strong cell-cell adhesion (Ireton et al. 2002; Ishiyama et al. 2010; Thoreson et al. 2000). This finding was confirmed by studies utilizing E-cadherin-deficient or p120-deficient cells (Ireton et al. 2002; Thoreson et al. 2000). Ectopic expression of an intact E-cadherin, but not a mutant E-cadherin (incapable of coupling p120) in E-cadherin-deficient cell lines was found to sufficiently promote strong cell adhesiveness (Thoreson et al. 2000). Similarly,

ectopic $p120^{ctn}$ expression in the p120-deficient SW48 cells rescued the aberrant epithelial morphology and impaired E-cadherin-mediated cell adhesion observed in these cells (Ireton et al. 2002).

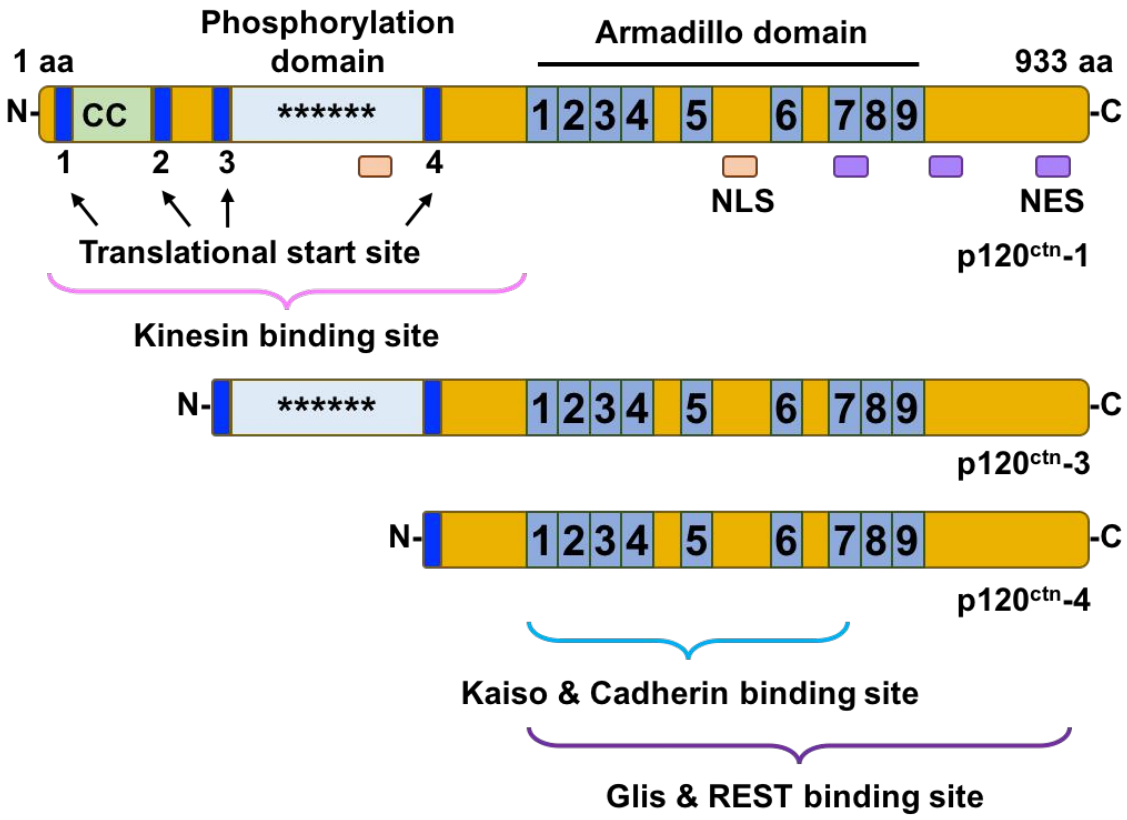
1.4.2 E-cadherin loss alters p120-catenin ($p120^{ctn}$) subcellular localization

In the absence of E-cadherin, both β-catenin and $p120^{ctn}$ localize to the cytoplasm (Sarrio et al. 2004; Thoreson et al. 2000; Valenta et al. 2012). However, unlike unbound cytoplasmic β-catenin that is rapidly targeted for degradation by the destruction complex, and is thus short-lived (Kimelman and Xu 2006), free cytoplasmic $p120^{ctn}$ is typically stable (Thoreson et al. 2000). Multiple $p120^{ctn}$ isoforms (1-4) derived by the alternate use of four translation initiation codons have been identified with distinct expression patterns and functions (Mo and Reynolds 1996). The longest $p120^{ctn}$ isoform ($p120^{ctn}$-1) is predominantly expressed in motile cells such as fibroblasts and macrophages (Mo and Reynolds 1996). Conversely, the shorter $p120^{ctn}$-3 isoform lacking the coiled-coil amino(NH_2)-terminal domain present in $p120^{ctn}$-1, is primarily expressed in epithelial cells that are typically immobile (Aho et al. 2002; Mo and Reynolds 1996). Synonymous with their expression patterns in highly motile versus immobile cells, cytoplasmic $p120^{ctn}$-1 but not $p120^{ctn}$-3 is implicated in cell motility and invasiveness via its inhibition of RhoA (Anastasiadis et al. 2000; Noren et al. 2000) and/or activation of the related Rho-GTPases Rac1 and Cdc42 (Grosheva et al. 2001).

Both cytoplasmic p120^{ctn}-1 and p120^{ctn}-3 are capable of shuttling between the cytoplasm and the nucleus (Daniel et al. 2002; Roczniak-Ferguson and Reynolds 2003; van Hengel et al. 1999). Notably, p120^{ctn} subcellular localization is influenced by its proximal (NH_2)-terminal domains (see **Figure 1.4**). In E-cadherin-deficient cells, p120-1 that contains the full proximal NH_2-terminal domain, localizes predominantly to the cytoplasm, while p120-3 and -4 that lack either a portion (coiled-coil region) or the entire NH_2-terminal domain (coiled-coil and phosphorylation regions; see **Figure 1.4**) respectively, localizes primarily to the nucleus of these cells (Roczniak-Ferguson and Reynolds 2003). p120^{ctn} subcellular localization is also modulated by its interaction with various cytoplasmic proteins – including α-tubulin and Kinesin (Roczniak-Ferguson and Reynolds 2003; Yanagisawa et al. 2004). Intriguingly, p120^{ctn} can also directly interact with certain nuclear proteins – including the transcription factors Glis2 (**Gli**-**s**imilar **2**), REST (**RE**1-**S**ilencing **t**ranscription factor) and Kaiso, which suggests roles for p120^{ctn} in the regulation of gene expression (Daniel and Reynolds 1999; Hosking et al. 2007; Lee et al. 2014). Indeed, p120^{ctn} indirectly regulates the expression of several genes including *Oct4* and *Wnt11* through its direct interaction with REST and Kaiso respectively (Kim et al. 2004; Lee et al. 2014). The remaining sections of this introduction will focus on the nuclear p120^{ctn}-binding partner Kaiso.

Figure 1.4: Schematic diagram of p120-catenin and its structural and functional domains.

p120-catenin ($p120^{ctn}$) consists of an amino(N)-terminal **c**oiled-**c**oil (CC) and phosphorylation region (asterisks) that is important in the integration of upstream signals from serine, tyrosine and non-receptor tyrosine kinases, as well as the regulation of $p120^{ctn}$ function and subcellular localization. The amino-terminal domain also contains the translation start sites (1-4), a putative **n**uclear **l**ocalization **s**ignal (NLS), and can interact with several proteins including the microtubule-affiliated motor protein kinesin. The CC region is present solely in p120-isoform 1. p120-isoform 3 contains the phosphorylation region while p120-isoform 4 lacks both the CC and phosphorylation regions. The Armadillo domain encompasses nine Armadillo repeats (1-9; (Hong et al. 2016)) – each comprised of 42 amino acids. This domain consists of a putative NLS and **n**uclear **e**xport **s**ignal (NES), and mediates protein-protein interactions with multiple proteins including Glis2, REST, Kaiso and the classical cadherins often in a mutually exclusive manner. The carboxy(C)-terminal tail on the other hand contains two putative NES implicated in the nuclear export of $p120^{ctn}$ (van Hengel et al. 1999).


Phosphorylation domain
Armadillo domain
1 aa
933 aa
N-
CC
******
1 2 3 4 5 6 7 8 9
-C
1
2
3
4
NLS
NES
Translational start site
p120ctn-1
Kinesin binding site
N-
******
1 2 3 4 5 6 7 8 9
-C
p120ctn-3
N-
1 2 3 4 5 6 7 8 9
-C
p120ctn-4
Kaiso & Cadherin binding site
Glis & REST binding site

1.5 The p120^{ctn}-binding partner Kaiso is a dual-specificity transcription factor

1.5.1 The POZ-ZF transcription factor Kaiso

Kaiso is encoded by the **z**inc finger and **BTB** domain containing-**33** (*ZBTB33*) gene located on chromosome Xq24. It was first identified as a p120^{ctn}-specific binding partner in a yeast two-hybrid screen for unique p120^{ctn} binding partners using full length p120^{ctn} as bait (Daniel and Reynolds 1999). Notably, the epithelial p120-3 isoform interacts with more affinity to Kaiso than the mesenchymal p120-1 isoform (Daniel and Reynolds 1999; Liu et al. 2014). Subsequent analysis of the deduced Kaiso amino acid sequence led to the classification of Kaiso as a member of the **B**road complex, **T**ramtrak, **B**ric á Brac/**Po**x virus and **Zi**nc-Finger (BTB/POZ) superfamily of **z**inc **f**inger (ZF) transcription factors (hereafter termed POZ-ZF proteins) (Daniel and Reynolds 1999). Further characterization revealed that Kaiso is also the founding member of a subfamily of zinc finger methyl-CpG binding proteins comprised of the Kaiso-like ZBTB38 and ZBTB4 proteins (Daniel et al. 2002; Filion et al. 2006; Prokhortchouk et al. 2001). Despite the structural similarity between the proteins, ZBTB38 and ZBTB4 do not interact with p120^{ctn}, which highlight the unique nature of the Kaiso – p120^{ctn} interaction (Filion et al. 2006). Intriguingly, p120^{ctn} interacts with Kaiso via its Arm repeats 1-7, which is the same region required for p120^{ctn} binding to E-cadherin (Daniel and Reynolds 1999). Surprisingly however, Kaiso does not associate with the cadherin-catenin complex and more

importantly, $p120^{ctn}$'s interaction with Kaiso and E-cadherin occur in a mutually exclusive manner (Daniel and Reynolds 1999).

1.5.2 Kaiso localizes to both the nucleus and cytoplasm

Although the majority of normal human tissues exhibit little to no nuclear Kaiso localization (Soubry et al. 2005), Kaiso predominantly localizes to the nucleus in cultured human tumor cells (Soubry et al. 2005) – a phenomenon that has been attributed to its highly conserved nuclear localization signal (Kelly et al. 2004a). In addition to its nuclear localization, Kaiso has also been observed to localize to the cytosol of normal and malignant human tissues (Soubry et al. 2005; Vermeulen et al. 2012). The cytoplasmic localization of Kaiso especially in cancerous cell lines has been credited in part to Kaiso's interaction with $p120^{ctn}$, particularly $p120^{ctn}$-isoform 3 (Jiang et al. 2012; Zhang et al. 2011). Correspondingly, Kaiso's cytoplasmic localization almost always overlaps with cytosolic $p120^{ctn}$ in certain human tumor tissues (Soubry et al. 2005; Vermeulen et al. 2012). However, Kaiso's subcellular localization is also dynamic and appears to be regulated by the cell cycle (Soubry et al. 2010) and the tumor microenvironment in transformed cells (Soubry et al. 2005).

1.5.3 Kaiso is a dual-specificity transcription factor

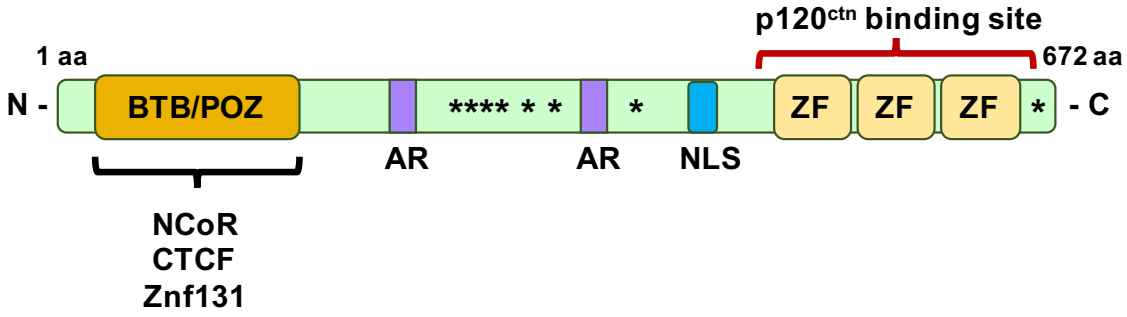
Like most POZ-ZF transcription factors, Kaiso possesses a highly conserved hydrophobic NH_2-terminal BTB/POZ protein-protein interaction domain that

facilitates homo- and heterodimeric interactions with other POZ and non-POZ proteins (Daniel 2007). Consistently, Kaiso homodimerizes and heterodimerizes with other proteins such as the **n**uclear **co**-**r**epressor (NCoR), the vertebrate insulator **C**CC**TC**-binding **f**actor (CTCF) and the **zin**c **f**inger protein-**131** (Znf131) via its POZ domain (Defossez et al. 2005; Donaldson et al. 2010; Kim et al. 2002; Yoon et al. 2003). At its C-terminus, Kaiso contains three Krüppel-like C_2H_2 zinc finger motifs that mediate DNA binding (**Figure 1.5**). Intriguingly, and unlike many of the previously characterized POZ-ZF proteins (that only bind DNA at a specific consensus DNA sequence), Kaiso exhibits dual-specificity DNA binding. Specifically, Kaiso recognizes and binds DNA via the consensus sequence-specific **K**aiso **b**inding **s**ite (KBS) – TC**CTGCNA**; core sequence emboldened (Daniel and Reynolds 1999; Daniel et al. 2002), the unmethylated palindromic TCT**CGCG**AGA sequence (Blattler et al. 2013), and at **me**thylated **CpG** (meCpG) dinucleotides either independently or in the context of the palindromic TCT**CGCG**AGA motif (Prokhortchouk et al. 2001; Raghav et al. 2012). Remarkably, Kaiso interacts with $p120^{ctn}$ not via its protein-protein interaction POZ domain, but via its DNA-binding domain specifically at sites flanking its zinc fingers (Daniel and Reynolds 1999). Since the Kaiso ZF domain (**Figure 1.5**) is crucial for efficient DNA-binding, and formation of a stable Kaiso-DNA complex (Buck-Koehntop et al. 2012), it is not surprising that $p120^{ctn}$ inhibits Kaiso DNA-binding ability, and consequently its transcriptional activity (Daniel et al. 2002; Kelly et al. 2004b; Kim et al. 2004; Park et al. 2005; Spring et al. 2005).

1.5.4 Kaiso functions as a transcriptional repressor and activator

With the exception of MIZ1 that functions as both a transcriptional repressor and activator, most characterized POZ-ZF proteins (e.g. Bcl-6, PLZF, HIC-1) act as transcriptional repressors, and elicit their repressive functions by binding to sequence-specific DNA targets, reviewed in (Kelly and Daniel 2006). Like most POZ-ZF proteins Kaiso has been primarily characterized as a transcriptional repressor (Daniel et al. 2002; Kim et al. 2004; Park et al. 2005; Prokhortchouk et al. 2001; Spring et al. 2005), with confirmation of Kaiso's repressive abilities attained mostly from studies in *Xenopus* and mammalian cells (Kim et al. 2004; Park et al. 2005; Spring et al. 2005). Several lines of evidence garnered from both *in vivo* and *in vitro* studies in *Xenopus* and mammalian cells indicate that Kaiso can repress gene expression in either a KBS-specific (Kim et al. 2004; Park et al. 2005; Spring et al. 2005), or meCpG-specific manner (Liu et al. 2014; Prokhortchouk et al. 2001), as well as in both a KBS-specific and meCpG-specific manner (Donaldson et al. 2012). Notably, $p120^{ctn}$ inhibition of Kaiso transcriptional activity has only been observed to occur when Kaiso mediates gene repression via KBS-specific sites (Kelly et al. 2004b; Kim et al. 2004; Park et al. 2005; Spring et al. 2005).

Figure 1.5: **Schematic representation of Kaiso and its functional domains.** Kaiso contains the highly conserved BTB/POZ protein-protein interaction domain at its amino (N)-terminus and three C_2H_2 **z**inc **f**inger (ZF) DNA binding domain at its carboxy (C)-terminus. Kaiso also comprise two **a**cidic **r**egions (AR) implicated in transcriptional activation, a **n**uclear **l**ocalization **s**ignal (NLS) located between its BTB/POZ and ZF domains, and multiple putative serine/threonine phosphorylation sites (asterisks).


p120ctn binding site
1 aa
672 aa
N -
BTB/POZ
**** * *
*
ZF
ZF
ZF
*
- C
AR
AR
NLS
NCoR
CTCF
Znf131

The mechanism via which Kaiso regulates gene silencing at sequence-specific and methylation-dependent sites has been fairly characterized, and involves Kaiso's recruitment of macromolecular corepressor complexes, the constituents of which can comprise N-CoR, **s**ilencing **m**ediator of **r**etinoid and **t**hyroid receptor (SMRT), **h**istone **d**e**ac**etylases (HDAC) and **m**yeloid **t**ranslocation **g**ene **16** (MTG16) proteins (Raghav et al. 2012; Yoon et al. 2003). In one study, Kaiso-mediated methylation-specific repression of the MTA2 gene was shown to be HDAC-dependent via Kaiso's recruitment of the N-CoR/HDAC3 complex. Treatment of cells with either the DNA methyltransferase 5-aza-dCT or the HDAC inhibitor trichostatin A attenuated Kaiso's repression of MTA2, with both treatments having a synergistic outcome on MTA2 expression (Yoon et al. 2003). In another study, Kaiso was found to recruit the MTG16 corepressor to the *MMP7* promoter in a sequence-specific manner, and this was crucial for Kaiso-mediated repression of *MMP7* (Barrett et al. 2012). Both Kaiso and MTG family members recruit HDAC-dependent corepressor complexes to mediate repression (Davis et al. 2003a; Yoon et al. 2003), which intimated that the Kaiso-MTG16 complex may also regulate *MMP7* expression in a HDAC-dependent manner.

In addition to Kaiso's function as a transcriptional repressor, increasing evidence suggest a role for Kaiso as a transcriptional activator that may be dependent on its protein interacting partners (Defossez et al. 2005; Koh et al. 2014; Rodova et al. 2004). The first evidence of Kaiso's transcriptional activating functions was

reported by Rodova and colleagues in 2004. They showed that Kaiso activated rather than repressed a *rapsyn* promoter-reporter construct containing the Kaiso sequence-specific KBS site in a Kaiso-δ-catenin dependent manner (Rodova et al. 2004). Since then, other studies have supported a role for Kaiso in gene activation. For example, Koh and colleagues demonstrated that Kaiso activates the **a**poptotic **p**rotease-**a**ctivating **f**actor **1** (APAF-1), the cell cycle arrest (*CDKN1A*/*p21*) and apoptosis-related (*BBC3/PUMA* and *BAX*) genes in a p53-dependent manner (Koh et al. 2015; Koh et al. 2014). **In this thesis, I found that Kaiso bound the promoter regions of BRCA1, TGFβRI and II, and also showed that Kaiso depletion decreased BRCA1 (Chapter 3), TGFβRI and II (Chapter 4) expression at the transcript and protein level, which was restored upon ectopic Kaiso expression in the Kaiso-depleted cells**. These studies further implicate a role for Kaiso as a transcriptional activator and are consistent with recent reports that Kaiso associates predominantly with highly active promoter regions *in vivo* (Blattler et al. 2013).

1.6 Emerging roles for Kaiso in normal and malignant mammalian cells

1.6.1 Roles for Kaiso in normal mammalian cells

Although the transcriptional functions of Kaiso have been extensively characterized, and more putative Kaiso target genes are being elucidated, there is still very little known about Kaiso's physiological functions in mammals. To begin to elucidate Kaiso's functions in normal tissues, Kaiso expression patterns

were examined in multiple mouse tissues, and this revealed that Kaiso is expressed at the transcript level in skeletal muscles, heart, spleen, lung, liver, kidney, testis (Daniel and Reynolds 1999) and brain (Daniel and Reynolds 1999; Della Ragione et al. 2006). A subsequent study further revealed that Kaiso is also expressed at the protein level in several normal murine tissues including the skin, urinary bladder, spleen, brain, thymus, testes, retina, colon, small intestine and mammary glands (Shumskaya et al. 2015) and normal human tissues including the breast (Vermeulen et al. 2012), ovary, colon and prostate (Soubry et al. 2005), which suggests potential biological roles for Kaiso in numerous mammalian tissues.

Interestingly, Kaiso expression was observed either predominantly localized to the cytoplasm of the abovementioned human tissues (Soubry et al. 2005), or primarily localized to the nucleus of all the aforementioned murine tissues with the exception of the retina where Kaiso localized primarily to the cytoplasm (Shumskaya et al. 2015), which intimated unique biological roles for Kaiso in distinct vertebrate species. Additionally, Kaiso protein expression was only detected in a subset of cell types in the various tissues analyzed particularly in distinct epithelial cells of murine intestines and mammary glands (Shumskaya et al. 2015), as well as in the epithelial cells of human prostate and colon tissues (Soubry et al. 2005). This indicated that Kaiso may function to modulate epithelial cell homeostasis in these tissues. Indeed, aberrant Kaiso overexpression has

been observed in transformed epithelial cells of intestines, prostate and mammary glands (Jones et al. 2014; Jones et al. 2012; Pierre et al. 2015b; Prokhortchouk et al. 2006; Vermeulen et al. 2012).

A pivotal study by Prokhortchouk and colleagues sought to shed some light on the possible physiological roles of Kaiso in mouse tissues by generating Kaiso knockout mice. Analysis of the effect of Kaiso loss on the development of diverse mouse tissues and organs revealed that Kaiso-null tissues and organs exhibited no visible deformities or developmental abnormalities (Prokhortchouk et al. 2006). This was in stark contrast to the crude structural developmental defects and phenotypes observed in Kaiso-depleted *Xenopus* embryos (Kim et al. 2004; Park et al. 2005; Ruzov et al. 2004; Ruzov et al. 2009). These studies thus implied that distinct physiological roles exist for Kaiso in different vertebrate species. Notably, the most similar Kaiso-like protein ZBTB4 that recognizes and binds the same DNA sites as Kaiso *in vitro* (Filion et al. 2006) is expressed in mice but lacks a *Xenopus* homologue. This suggests that there may be a functional redundancy conveyed by ZBTB4 in mice, which might explain the lack of an overt phenotype in the Kaiso-null mice.

Analysis of the effect of Kaiso knockout in generation-3 Kaiso-null progeny revealed that persistent Kaiso loss resulted in abnormal spleen enlargement and large diffused germinal centers (Koh et al. 2013). The Kaiso-null phenotype was

linked to increased cell proliferation as a result of enhanced *Bcl-6* and *c-Myc* expression, and reduced expression of the cell cycle arrest genes – *p27* (*CDKN1B*), *p21 (CDKN1A)* and *Gadd45a* (Koh et al. 2013). This study thus hinted at a role for Kaiso in the suppression of cell proliferation in normal organs like the spleen. Moreover, it suggested that Kaiso expression levels in certain organs like the spleen may promote or attenuate immune responses since the spleen exhibit both local and systemic immune functions (Mebius and Kraal 2005). This notion is supported by the pro-inflammatory phenotype (increased neutrophil infiltration and activation – a well-known immune response (Mócsai 2013; Rosales et al. 2016)) observed upon specific overexpression of Kaiso in murine intestines (Chaudhary et al. 2013; Pierre et al. 2015b). Intestinal-specific Kaiso overexpressing mice (hereafter referred to as Kaiso transgenics or $Kaiso^{Tg/+}$) also exhibited reduced cell proliferation (Chaudhary et al. 2013), which further confirmed roles for Kaiso in cell proliferation. Additionally, $Kaiso^{Tg/+}$ displayed increased differentiation of epithelial progenitor cells into secretory cell types (Paneth, Goblet, and enteroendocrine), a phenotype that was later linked to Kaiso's regulation of several Notch signaling pathway components including Notch1 and Dll-1 in intestinal cells (Robinson et al. 2017). More importantly, Kaiso overexpression led to secretory cell and crypt hyperplasia (Chaudhary et al. 2013; Robinson et al. 2017), which implied that Kaiso expression levels must be maintained within a defined physiological range in normal cells, as significantly high Kaiso levels may predispose to tumorigenesis. Consistent with this theory,

increased Kaiso expression has been observed in advanced-grade colon and breast tumors compared to their low-grade counterparts (Pierre et al. 2015b; Vermeulen et al. 2012).

1.6.2 Roles for Kaiso in malignant mammalian cells

Numerous studies have revealed several roles for Kaiso in transformed cells (Jones et al. 2016; Jones et al. 2014; Jones et al. 2012; Lopes et al. 2008; Ogden et al. 2008; Pierre et al. 2015a; Pierre et al. 2015b; Prokhortchouk et al. 2006; Vermeulen et al. 2012). The earliest evidence of Kaiso's involvement in carcinogenesis came from studies by Prokhortchouk *et al.* where Kaiso deficiency was shown to delay the onset of intestinal neoplasia in progeny derived from a cross between Kaiso-null (*Kaiso*$^{-/y}$) and the *Apc*$^{Min/+}$ mouse model of colorectal cancer (Prokhortchouk et al. 2006). The *Apc*$^{Min/+}$ mouse model is characterized by germline mutations in the *APC* gene, hyperactive Wnt/β-catenin signaling and multiple intestinal tumors (Bienz and Clevers 2000; Galiatsatos and Foulkes 2006).

Remarkably, Prokhortchouk and colleagues were the first to suggest a positive rather than a negative impact of Kaiso expression on the Wnt signaling cascade, and the first to suggest a pro-tumorigenic role for Kaiso in intestinal neoplasia. These findings were quite surprising as previous studies had characterized Kaiso as a predominantly negative regulator of Wnt signaling responses (Kim et al.

2004; Park et al. 2005; Spring et al. 2005). However, since subsequent studies reported that Kaiso's regulation of Wnt signaling was bimodal (both positive and negative) (Iioka et al. 2009), it was conceivable that Kaiso could indeed positively regulate the Wnt cascade in intestinal tumorigenesis. Indeed, recent studies from our lab demonstrated that intestinal-specific Kaiso overexpressing $Apc^{Min/+}$ mice, i.e. ($Kaiso^{Tg/+}$; $Apc^{Min/+}$), exhibited increased expression of Wnt target genes, had increased polyp numbers, and shorter survival compared to parental $Apc^{Min/}$ mice (Pierre et al. 2015b). These findings reinforced a pro-tumorigenic role for Kaiso in intestinal carcinogenesis. In addition to the studies by Prokhortchouk *et al.* and Pierre *et al.* implicating Kaiso in intestinal tumorigenesis, multiple studies have demonstrated roles for Kaiso in other cancers including prostate, lung, and breast cancer (Jiang et al. 2012; Jones et al. 2014; Jones et al. 2012).

The mechanism via which Kaiso influences tumorigenesis is still being elucidated. However, several of the putative Kaiso target genes identified to date – *BCL6*, *MYC*, *CCND1, CDKN2A*, *MMP7*, *DLL1*, *JAG1*, *MTA2, S100A4*, *CDH1*, *HIF-1α* – (Barrett et al. 2012; Dai et al. 2011; Daniel et al. 2002; Donaldson et al. 2012; Jiang et al. 2012; Jones et al. 2012; Koh et al. 2013; Lopes et al. 2008; Ogden et al. 2008; Park et al. 2005; Pierre et al. 2015a; Prokhortchouk et al. 2001; Robinson et al. 2017; Spring et al. 2005; Yoon et al. 2003), are inextricably linked to tumour onset, proliferation, progression and metastasis (Adachi et al. 1999; Covington and Fuqua 2014; Dai et al. 2014; Gabay et al. 2014; Huang et

al. 2011; Lloyd et al. 1998; Musgrove et al. 2011; Onder et al. 2008; Schwab et al. 2012; Wu et al. 2014). Kaiso has also been implicated in the regulation of tumor suppressive miRNAs (Wang et al. 2016), and cancer-related events like inflammation (Chaudhary et al. 2013; Coussens and Werb 2002). These findings coupled with Kaiso's regulation of oncogenic signaling pathways like the Wnt signaling cascade, have provided some insight into Kaiso's molecular and physiological functions in tumorigenesis.

For example, Kaiso was found to mitigate *cyclin D1*–mediated proliferation of HCT-116 colon cancer cells; Kaiso-depletion resulted in increased *cyclin D1* expression, which concomitantly enhanced the proliferation of these cells (Donaldson et al. 2012). In another study, Kaiso was shown to contribute to methylation-dependent silencing of the tumour-suppressor genes *CDKN2A, MGMT* and *HIC1* in human colon cancer cells, and its depletion sensitized these cells to cell cycle arrest (Lopes et al. 2008). Wang *et al.* further showed that Kaiso promotes the migration and invasion of **p**rostate **ca**ncer (PCa) cells via repression of **miR**NA-**31** (miR-31); Kaiso-depletion resulted in increased miR-31 expression, and decreased cell migration and invasiveness of PCa cells (Wang et al. 2016). Moreover, PCa cell migration and invasion was restored upon depletion of the elevated miR-31 in the Kaiso-depleted PCa cells. **In this thesis, we demonstrate that Kaiso-depletion attenuates the proliferation, invasion and metastasis of triple negative breast cancer cells in part via the reduction of**

c-Myc expression, as well as other gene responses associated with mutant-p53, and oncogenic TGFβ signaling (Chapter 3, 4 and 5). Collectively, these studies reveal functional roles for Kaiso in several cancers, and suggest both tumor promoting and suppressive roles for Kaiso in carcinogenesis that are likely due to Kaiso's ability to dual-regulate (activate or repress depending on cellular context) genes involved in tumorigenic processes.

1.6.3 Kaiso subcellular localization and prognostic features in cancer cells

Most emerging studies correlate increased Kaiso levels with the aggressiveness and progression of several tumors including prostate and breast cancers (Jones et al. 2014; Jones et al. 2012; Vermeulen et al. 2012). Notably, Kaiso's subcellular localization also correlates with the poor prognostic features of various cancers (Cofre et al. 2012; Dai et al. 2009; Jones et al. 2014; Jones et al. 2012; Vermeulen et al. 2012; Wang et al. 2012; Zhang et al. 2011). For example, increased cytoplasmic Kaiso correlates with advanced grade, lymph node metastases and reduced overall survival in non-small cell lung cancer patients (Dai et al. 2009); advanced grade tumors of thymoma patients; high-grade and/or invasive tumors of male and female pancreatic cancer patients (Jones et al. 2016; Wang et al. 2012). On the other hand, increased nuclear Kaiso correlates with lymph node positivity of pancreatic ductal adenocarcinomas (Jones et al. 2016) as well as advanced grade, lymph node metastases and poor overall

survival outcomes in prostate and breast cancer patients (Jones et al. 2014; Jones et al. 2012).

Correlation analyses of Kaiso expression patterns with tumor type/grade and prognosis in a cohort of 477 human invasive breast cancer cases revealed a link between cytoplasmic Kaiso expression and invasive lobular carcinomas (Vermeulen et al. 2012). Conversely, nuclear Kaiso expression characterized invasive ductal carcinomas, and was positively correlated with high-grade, ERα negativity, EGFR-overexpression, BRCA1-associated, HER2-driven, basal and triple negative breast cancers (Vermeulen et al. 2012). High nuclear Kaiso expression also correlated significantly with high-grade tumors of African American (AA) male prostate cancer patients compared to their Caucasian counterparts (Jones et al. 2012), and with poor survival outcomes of AA female breast cancer patients relative to their Caucasian counterparts (Jones et al. 2014). These findings hinted for the first time a role for high Kaiso expression in the racial disparity associated with prostate and breast cancers. **We report in Chapter 6 that nuclear Kaiso is highly expressed in TNBC tissues from women of African ancestry (WAA) compared to Caucasian women. This finding implicates Kaiso in the prevalence, and possibly in the high mortality rates associated with TNBC in WAA** (Dietze et al. 2015).

1.7 Summary of intent

TNBC is highly prevalent in premenopausal WAA (Agboola et al. 2012; Carey et al. 2006; Lund et al. 2009; Stark et al. 2010), and is associated with a poorer prognosis compared to other BCa subtypes due to its highly proliferative and metastatic nature, and lack of targeted-therapies (Foulkes et al. 2010). Recently, increased Kaiso expression was found to correlate with TNBC aggressiveness (Vermeulen et al. 2012), and the racial disparity in breast cancer outcomes (Jones et al. 2014). However, the functional significance of Kaiso's expression in TNBC tumorigenesis or prevalence in WAA remained unknown. Most studies indicate a pro-tumorigenic role for Kaiso in other tumors (Jiang et al. 2012; Jones et al. 2012). Thus, **we hypothesized that Kaiso functions as a tumor promoter in TNBC.** Since Kaiso is highly expressed in TNBCs, we opted to deplete Kaiso expression in several established TNBC cell lines in order to determine the functional significance of Kaiso expression in the tumorigenesis and metastasis of TNBC. The goals of this thesis were to:

(i) Characterize the effects of stable Kaiso-depletion on the proliferation, survival and invasion of TNBC cells *in vitro* and *in vivo*

(ii) Define the mechanism by which Kaiso-depletion influences the proliferation, survival and invasion of TNBC cells

(iii) Analyze and correlate Kaiso expression patterns in a cohort of WAA- and Caucasian women (CW)-TNBC tissues.

To address these objectives, I generated and characterized three stable Kaiso-depleted TNBC cell lines (MDA-MB-231, Hs578T and MDA-MB-157) using cell culture experimental systems. Next, I performed xenograft studies by injecting control and Kaiso-depleted TNBC cells into the fourth mammary fat pad of **N**OD **S**CID **G**amma (NSG) mice and examined the effect of Kaiso-depletion on tumor growth and metastasis. I then examined Kaiso protein expression patterns in TNBC tissues obtained from Nigerian, Barbadian, African American and Caucasian women. Nigeria and Barbados represent homogenous populations of WAA from West Africa and the Caribbean respectively with a high TNBC occurrence (~65% and ~25% respectively) ((Adisa et al. 2012; Carey et al. 2006; Stark et al. 2010), Dr. Desiree Skeete, personal communication with Dr. Juliet Daniel). African Americans represent a heterogeneous population of WAA from the USA, with ~22% of TNBC incidence rates while Caucasian women represent a non-WAA American population with ~10% TNBC prevalence rates (DeSantis et al. 2016; Stark et al. 2010).

Remarkably, Kaiso depletion resulted in the delayed tumor onset of MDA-MB-231 but not Hs578T cells and increased apoptosis of MDA-MB-231, Hs578T and MDA-MB-157 cells (**Chapter 3**). Loss of Kaiso expression also inhibited the ability of MDA-MB-231 and Hs578T cells to spread and establish tumor masses in the lungs (**Chapter 4**). Remarkably, Kaiso depletion attenuated the capacity of the highly metastatic MDA-MB-231 cells to invade large lung blood vessels and

establish macrometastases at other distal organs (**Chapter 5**). Our studies also revealed high nuclear Kaiso expression in TNBC tissues from WAA compared to Caucasian women (**Chapter 6**). Collectively, these findings shed significant light on the varied roles of Kaiso in oncogenic-related processes, and the importance of Kaiso expression in the tumorigenesis and metastasis of TNBC. Our observations also raise the exciting possibility that Kaiso could be utilized as a diagnostic or prognostic biomarker for TNBC in WAA and more importantly that Kaiso could be a plausible therapeutic target for the treatment of TNBCs.

CHAPTER 2: MATERIALS AND METHODS

Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from ATCC (Manassas, VA, USA), while Hs578T, MDA-MB-157 (hereafter MDA-157) and ZR75.1 were a generous gift from Dr. John Hassell (McMaster University, Hamilton, Canada). All cell lines were cultured in **D**ulbecco's **M**odified **E**agle's **M**edium (DMEM; Lonza BioWhittaker®, Walkersville, MD, USA) supplemented with 10% **F**etal **B**ovine **S**erum (FBS; Hyclone, Logan, Utah, USA), 0.1 mg/mL penicillin/streptomycin and 0.25 µg/mL Fungizone (Invitrogen, Grand Island, NY, USA). Cells were passaged every 2-3 days and grown in a 5% CO_2 humidified incubator at 37°C. For TGFβ treatments, cells were treated with 10 ng/mL of TGFβ1 (R&D Systems, Minneapolis, MN, USA) for 24 or 48 hours. For **5-aza**-2'-**d**eoxy**c**ytidine (5-aza-dC) treatment, cells were treated with 10 µM 5-aza-dC (Sigma, MO, USA) daily for three consecutive days before proceeding to chromatin immunoprecipitation experiments.

Generation of stable Kaiso-depleted cell lines

Kaiso depletion was achieved using Kaiso-specific shRNAs (sh-Kaiso) cloned into a **pR**etro**S**uper (pRS) vector that targeted the following mRNA sequences, 5'– AAAAGATCATTGTTACCGATT – 3' or 5' – TTTTAACATTCATTCTTGGGAGAAG – 3'. 6 µg of pRS-sh-Kaiso plasmid or control vector (pRS-Kaiso scrambled) were transfected into the breast tumor cell

lines (MDA-231, Hs578T and MDA-157) using the Turbofect transfection reagent (Thermo Scientific, MA, USA) according to the manufacturer's instructions. 48 hours post-transfection, cells were treated with Puromycin (Invitrogen) at 0.8 µg/mL for MDA-231 cells, 1.5 µg/mL for Hs578T cells and 1.0 µg/mL for MDA-157 cells, to select for stable Kaiso knockdown. Selection media was replaced every 2 or 3 days for approximately three weeks until individual colonies were formed. Individual colonies were isolated and collected using 10 mm Pyrex cloning cylinders (Corning Incorporated, NY, USA) gently coated with high vacuum grease (DOW Corning Corporation, MI, USA), and expanded until each clone was confluent enough to grow in 100 mm culture plates. Individual clones were subsequently analyzed for optimal Kaiso depletion using immunoblot analysis of whole cell lysates. Clones exhibiting efficient Kaiso knockdown were selected for RT-PCR analysis and further studies.

Rescue experiments

A pCDNA3 vector expressing the **m**urine **Kaiso** (mKaiso) cDNA coding sequence that is not targeted by the Kaiso-specific shRNA was used for Kaiso rescue experiments. Transient transfection of the pCDNA3 mKaiso vector into MDA-231 **sh-K**aiso clone **1** (sh-K1) was achieved using the Turbofect transfection reagent (Thermo Scientific) as per the manufacturer's protocol. 48 hours post transfection, cells were treated with Puromycin (Invitrogen) at 0.8 µg/mL and Geneticin (Invitrogen) at 1000 µg/mL to select for efficient Kaiso overexpression.

Three weeks post transfection, whole cell lysates were obtained from the pCDNA3-mKaiso and pCDNA-empty (control vector that does not contain the mKaiso cDNA coding sequence) transfected cells and subjected to immunoblot analysis of interested proteins. For rescue of TGFβ signaling, MDA-231 and Hs578T sh-K1 cells were transfected with the constitutively active mutant form of TGFβR1 (TGFβR1^{T204D}) hereafter TβRI-204D (a kind gift from Dr. Gerard Blobe, Duke University Medical Center, USA) using Turbofect transfection reagent according to the manufacturer's instructions. Three weeks post-transfection, total protein was isolated from control Kaiso-depleted (MDA-231-sh-K1 and Hs578T-sh-K1) and experimental (MDA-231-sh-K1 and Hs578T sh-K1 transfected with TβRI-204D) cells, and used for western blot analysis of interested proteins.

Reverse transcription – polymerase chain reaction (RT-PCR)

Control and Kaiso-depleted cells were washed twice with cold 1X **p**hosphate **b**uffered **s**aline (PBS) before performing RNA isolation using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples were quantified and stored at -20°C prior to use. Isolated mRNA was subjected to cDNA synthesis and RT-PCR analysis of genes of interest (see Table 2.1 for list of primers) using the Superscript one-step RT-PCR with Platinum Taq kit (Invitrogen) according to the manufacturer's protocol.

Table 2.1: List of primers used for RT-PCR with their annealing temperatures (temp.) and product size

Target Gene	Annealing Temp. (°C)	Product Size (bp)	Sequence (5' – 3')	
Kaiso	55.0	248	Fwd	TGCCTATTATAACAGAGTCTTT
			Rev	AGTAGGTGTGATATTTGTTAAAG
E-cadherin	63.0	313	Fwd	CACCCTGGCTTTGACGCCGA
			Rev	AAACGGAGGCCTGATGGGGCG
ZO-1	60.0	415	Fwd	CGGGAAGTTACGTGGCGAA
			Rev	CTCCATTGCTGTGCTCTTGG
Vimentin	63.0	491	Fwd	TACGTGACTACGTCCACCCG
			Rev	ATCTCCTCCTGCAATTTCTCCC
Slug	63.0	280	Fwd	AGACCCCCATGCCATTGAAG
			Rev	CTTCTCCCCCGTGTGAGTTC
ZEB1	53.0	52	Fwd	AGAATTCACAGTGGAGAGAAGCC
			Rev	CGTTTCTTGCAGTTTGGGCATT
β-actin	55.0	116	Fwd	CTCTTCCAGCCTTCCTTCCT
			Rev	AGCACTGTGTTGGCGTACAG

RT-PCR reactions were performed using the Eppendorf–Thermal cycler (Eppendorf, NY, USA) and the following RT-PCR conditions: reverse transcription at 50°C for 30 minutes, followed by initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at the respective temperatures indicated in Table 2.1 for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. 10 µL of each RT-PCR reaction was electrophoresed on a 1.0% agarose gel with 0.5 µg/mL **et**hidium **br**omide (EtBr), at 120 V for 30 minutes in 1X TAE solution. Images were

obtained using the Bio-RAD ChemiDoc MP imaging system (Bio-RAD Laboratories, Philadelphia, PA, USA).

Quantitative RT-PCR analysis

The GeneJet RNA-plus isolation kit (Macherey-Negel, PA, USA) was used to isolate RNA (1 µg) from untreated and TGFβ1-treated control & Kaiso-depleted cells. The RNA was then reverse transcribed to cDNA using the qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD, USA) according to the manufacturer's instructions. The obtained cDNA was then amplified using the PerfeCta SYBR Green SuperMix ROX (Quanta BioSciences) with the primers indicated in Tables 2.2 (for Chapter 3 & Appendix) and Table 4 (within the article reported in Chapter 4) according to the manufacturer's protocol. The Applied Biosystems Prism 7900HT sequence detection system was used for all quantitative RT-PCR reactions, which were performed in triplicate. The expression of each target was determined using a standard curve that was generated using 5-fold serial dilutions of cDNA reverse transcribed from a combination of RNA isolated from each control and experimental sample. The expression levels of each target gene were then normalized to the expression levels of β-actin. Statistical significance (using unpaired, two-tailed student's t-test and one-way **an**alysis **o**f **va**riance (ANOVA) with Tukey's test where appropriate) was determined with data obtained from at least three independent trials. Corresponding figures were plotted using GraphPad Prism software.

Table 2.2: List of primers used for qRT-PCR with their annealing temperatures

Target	Annealing Temperature (°C)	Sequence (5' – 3')	
Bax	60.0	Fwd	GCCCTTTTGCTTCAGGGTTT
		Rev	GCAATCATCCTCTGCAGCTC
PUMA	55.0	Fwd	AGCAGGGCAGGAAGTAACAA
		Rev	CCCTGGGGCCACAAATCT
BRCA1	62.0	Fwd	CTCGCTGAGACTTCCTGGAC
		Rev	TCAACTCCAGACAGATGGGAC
c-MYC	60.0	Fwd	AGGGAGATCCGGAGCGAATA
		Rev	GTGGACTTCGGTGCTTACCT
C/EBPβ	62.0	Fwd	TTTGTCCAAACCAACCGCAC
		Rev	GCATCAACTTCGAAACCGGC
β-actin	55.0	Fwd	CTCTTCCAGCCTTCCTTCCT
		Rev	AGCACTGTGTTGGCGTACAG

Chromatin immunoprecipitation (ChIP) and ChIP-PCR experiments

Control and experimental MDA-231, Hs578T and MDA-157 cells were cultured to achieve a confluency of 80% after which cells were washed twice with 5 mL cold 1X PBS. To crosslink protein-DNA complexes, cells were incubated in 10 mL of serum-free DMEM containing 37% formaldehyde (1.42 % final concentration) with gentle rocking for 10 minutes at room temperature. After which cells were washed twice with 5 mL cold 1X PBS and crosslinks quenched with 125 mM glycine at room temperature for 5 minutes. Following incubation in 125 mM glycine, cells were washed twice with 5 mL cold 1X PBS, harvested using cell

scrapers and centrifuged at 2000 rpm for 5 minutes at 4°C. Pelleted cells were re-suspended in 2 mL cell lysis buffer (0.5% NP-40 buffer, 85 mM KCl, 5 mM PIPES pH 8.0 and Complete Mini Protease Inhibitor Cocktail Tablets (1 tablet/10 mL buffer) (Roche, QC, USA)), dounced ten times with a homogenizer and incubated on ice for 15 minutes to enable cell lysis. Lysed cells were pelleted by centrifuging at 5000 rpm for 5 minutes at 4°C, and the nuclear pellets re-suspended in 250 µL nuclear lysis buffer (10 mM EDTA, 50 mM Tris-Cl pH 8.1, 1% SDS and Complete Mini Protease Inhibitor Cocktail Tablets (1 tablet/10 mL buffer) (Roche)), prior to incubation on ice for 10 minutes. Nuclear lysates were then sonicated on ice with five rounds of 15 pulses each at 90% duty cycle and 5% maximum power output, and centrifuged at 13,200 rpm for 10 minutes at 4°C to clear lysates. Prior to immunoprecipitation, lysates were pre-cleared with 5 µL rabbit IgG (Abcam; ab37415) and non-specific proteins precipitated with Protein A Sepharose beads blocked with 50 µL salmon sperm DNA (Rockland Immunochemicals Inc., Limerick, PA, USA; MB-103-0025).

Approximately 15 µg of cleared chromatin was subsequently incubated with 8 µg of mouse anti-Kaiso monoclonal antibody (Daniel et al. 2001), 4 µg of rabbit anti-Histone H3 polyclonal antibody (Abcam; ab1791) and 4 µg of mouse anti-negative control IgG antibody (Abcam; ab18413) overnight at 4°C. After incubation, the formed antibody-protein-DNA complexes were precipitated with 50 µL Protein A Sepharose beads at 4°C for 1 hour.

Table 2.3: List of primers used for ChIP-PCR with their annealing temperatures and product size

Target	Annealing Temperature (°C)	Product Size (bp)	Sequence (5' – 3')	
Bax	64.0	94	Fwd	CTAATTCCTTCTGCGCTGGG
			Rev	GTCCAATCGCAGCTCTAATGC
PUMA	64.0	161	Fwd	GATCGAGACCATCCTGGCTA
			Rev	CGATCTCAGCAAACTGCAAG
BRCA1	64.0	245	Fwd	AGGGCTCTCTCATCCTGTCA
			Rev	TGTCCGCCATGTTAGATTCA
TGFβR1 [-1035/-1008 KBS]	65.0	120	Fwd	AGGGCAAATTGGGACTGGAG
			Rev	GAGGCCTGCAACTTGCTCTA
TGFβR2 [-35/-29 KBS]	65.0	77	Fwd	CAGCTGAAAGTCGGCCAAAG
			Rev	AGCCCCTAGCTCTCTCGTAG
TGFβR1-neg [-2960/-2725 KBS]	63.0	235	Fwd	GGAGCCTGGGAAATTGACAT
			Rev	CTCCAGTGCCTTGTACCCTG
TGFβR2-neg [-2642/-2274 KBS]	63.0	185	Fwd	TCATGGCAAAGACCACCAGT
			Rev	TTGCCCAAGTTCCTCCAGAT
c-Myc	64.0	141	Fwd	ATAGCGATTGGTTGCTCCCC
			Rev	GAGTTTGCAGCTCAGCGTTC
C/EBPβ	65.0	520	Fwd	GCCTCTGGGACTGGCAATAG
			Rev	ACGACTCCCCTCTTAAGCCA

bp = base pair

The immunoprecipitated complexes were then washed once for 10 minutes with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA and 1% NP-40), once for 10 minutes with high salt buffer (500 mM NaCl, 50 mM Tris pH 8.0, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA and 1% NP-40), once for 5 minutes with lithium chloride (250 mM LiCl, 50 mM Tris pH 8.0, 1% NP-40, 1 mM EDTA and 0.5% deoxycholate), and twice for 10 minutes each with **T**ris-**E**DTA (TE) buffer pH 8.0 (10 mM Tris, 1 mM EDTA). Protein-DNA crosslinks were subsequently reversed by incubating samples overnight at 65°C after treatment with RNase A (50 µg/mL) and proteinase K (250 µg/mL).

Three rounds of phenol chloroform extraction and ethanol-salt precipitation was then used to recover the precipitated chromatin. Recovered chromatin was suspended in RNase/DNase-free water and used to conduct the endpoint PCR experiments described in Chapters 3, 4 and Appendix with the primers listed in Table 2.3. For these experiments, 1 µL of recovered DNA from each immunoprecipitated chromatin sample was utilized in a PCR reaction containing 1X PCR buffer, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µL Taq polymerase (Invitrogen), 0.4 mM forward and reverse primers and RNase/DNase sterile distilled water to a final reaction volume of 20 µL; with the following PCR conditions – initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at the respective temperatures indicated in Tables 2.3 for 1 minute and extension at 72°C for 1 minute, with a

final extension at 72°C for 10 minutes. 10 µL of each PCR reaction was electrophoresed on a 1% agarose gel with 0.5 µg/mL EtBr, at 120 V for 30 minutes in 1X TAE solution. Images were obtained using the Bio-RAD ChemiDoc MP imaging system (Bio-RAD Laboratories) and each reaction was performed at least three times.

Electrophoretic mobility shift assay (EMSA)

Double stranded **oligo**nucleotide**s** (oligos) that span the specified KBS in the TGFβR1 and TGFβR2 promoters (see Chapter 4) were generated to determine the direct binding affinity of Kaiso to the TGFβR1 and TGFβR2 promoters. To assess the requirement of the KBS for Kaiso binding, four mutated forms of the **w**ild**t**ype (wt) oligos reported in Chapter 4 were also generated. The GST-Kaiso fusion protein (KaisoΔPOZ – that retains the DNA-binding ZF domain but is deficient in the POZ protein-protein interaction domain) was utilized to assess Kaiso binding to the wt and mutated TGFβR1 and TGFβR2 oligos. Kaiso fusion proteins were expressed from the pGEX-5X-1 vectors (Amersham) generated by Dr. Abena Otchere. The GST-only protein served as a negative control. The oligos were biotin labeled using a Biotin 3'-End DNA Labeling kit (Pierce Biotechnology, Rockford, IL, USA) as per the manufacturer's protocol. TGFβR1 probes containing a CpG dinucleotide (KBS2-4) were also methylated by incubating 500 ng of each TGFβR1 oligo with 200U of SssI methyltransferase (M.SssI; New England Biolabs, Ipswich, MA, USA) in a 250 µL reaction

containing 1X NEB buffer and 640 µM S-adenosyl methionine for 2 hours at 37°C. Inactivation of the enzyme was achieved by incubation at 65°C for 20 minutes, followed by purification of the methylated oligos with standard phenol-chloroform extraction and ethanol precipitation.

Following biotinylation, complementary oligonucleotides were annealed by heating at 90°C for 1 minute, and then allowed to cool slowly to room temperature. The reaction was then frozen and stored at −20 °C until use. Binding reactions were performed using 100 fmol of biotinylated double-stranded DNA probe and 200 ng of purified protein in 20 µL of binding buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 25% Glycerol, 1 mM **di**thio**t**hreitol (DTT) and Halt protease phosphatase inhibitor cocktail). To eliminate nonspecific binding, reaction mixtures were first incubated with 2 µg of **poly d**eoxy**i**nosinic-**d**eoxy**c**ytidylic acid (poly dI-dC) on ice for one hour. Reaction mixtures containing biotinylated double-stranded DNA probe were then incubated at room temperature for 30 minutes. For competition assays, a 100-fold excess (10 pmol) of unlabeled (cold) DNA was added. Reaction mixtures were subsequently loaded onto a 4.8% non-denaturing polyacrylamide gel and electrophoresed in 0.5 X TBE at 100 volts at 4°C. Nucleic acids were transferred onto a nylon membrane in 0.5 X TBE and crosslinked using a 312 nm UV lamp for 10 minutes. Visualization was performed utilizing a horseradish peroxidase-conjugated streptavidin Chemiluminescent Nucleic Acid Detection Module kit (Pierce

Biotechnology) and hyperfilm (GE Healthcare, Mississauga, ON, Canada; #28906839) according to the manufacturer's protocol. Where applicable, experiments were performed in triplicate.

Protein isolation

Control and experimental cells cultured to 90% confluency were washed twice with 5 mL cold 1X PBS and lysed with 500 µL ice-cold NP40 lysis buffer (0.5% NP40, 50 mM Tris, 150 mM NaCl, 0.5% sodium orthovanadate (Sigma, MO, USA) and Complete Mini Protease Inhibitor Cocktail Tablets (Roche) – 1 tablet/10 mL buffer) on ice for 10 minutes. Whole cell lysates were collected into a cold microfuge tube, vortexed briefly and centrifuged at 13,200 rpm for 10 minutes at 4°C. The resulting supernatant was transferred into fresh pre-chilled microfuge tubes and total protein quantified using a Bradford assay.

Immunoprecipitation

Total protein (1 mg) isolated from parental MCF-7, MDA-231 and Hs578T (as described above) were immunoprecipitated with 4 µg of anti-Kaiso 6F mouse monoclonal antibody (Daniel et al. 2001), 2 µg of anti-p53 mouse monoclonal antibody (**C**ell **S**ignalling **T**echnology (CST), MA, USA; 2524 – which recognizes both **w**ild**t**ype (wt) and mutant-p53), 2 µg of anti-p53 rabbit monoclonal antibody (Abcam – ab32049; that recognizes only mutant-p53) or 4 µg of normal rabbit IgG antibody (**S**anta **C**ruz **B**iotechnology (SCB), CA, USA; sc –2027) for 2 hours or

overnight at 4°C. The immunoprecipitates were collected by incubation with 50 µL Protein A agarose beads and washed 5 times for 30 seconds at 4°C with 1000 µL lysis buffer before proceeding to western blot analysis.

Western blot (WB) and densitometry analysis

30 µg or 50 µg of total protein suspended in 2X Laemmli Sample Buffer was denatured by boiling for 5 minutes prior to electrophoresis on a 6% or 12% **s**odium **d**odecyl **s**ulphate (SDS) polyacrylamide gel. Resolved proteins were transferred onto a nitrocellulose membrane (PerkinElmer Life Sciences, MA, USA) using either a TE77XP Hoefer semi-dry transfer apparatus (Hoefer Inc., Richmond, CA, USA) or an Amersham semi-dry transfer apparatus (Amersham Biosciences Corp., San Francisco, CA, USA). Non-specific binding was prevented by blocking the nitrocellulose membranes with 3% skimmed milk in 1X **t**ris **b**uffered **s**aline (TBS) at pH 7.4 for 30 minutes or 1 hour at room temperature. Blocked membranes were then incubated overnight at 4°C with the primary antibodies listed in Table 2.4 at their indicated dilutions in 3% skimmed milk/TBS. The respective primary antibodies were used to probe for proteins of interest reported in Chapters 3, 4, 5 and the Appendix. Following the primary antibody overnight incubations, membranes were washed five times for 5 minutes each in 1X TBS or 1X **TBS**-0.1% **T**ween-20 (TBS-T) with gentle shaking to remove unbound primary antibodies.

Secondary antibody incubations were done at room temperature for 2 hours with either horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-mouse secondary antibody (Jackson Immuno Research Laboratories, PA, USA), each at a 1:10,000 dilution in 3% skimmed milk/TBS. After secondary antibody incubations, membranes were washed as described above, processed and visualized using Clarity Western Enhanced Chemiluminescence Substrate and the Bio-Rad ChemiDoc MP imaging system (Bio-RAD Laboratories, ON, Canada) according to the manufacturer's instructions. The optical densities of Kaiso, c-Myc, Cyclin D1, p120-1, p120-3, p53, Bax, PUMA and β-actin signals reported in Chapter 3, were quantified and analyzed using the Image Lab^{TM} 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 protein levels were calculated as indicated using Microsoft Excel and graphed using GraphPad Prism software.

Immunocytochemistry/immunofluorescence (ICC/IF)

MDA231 cells were seeded on 22 mm^2 cover slips (ThermoFisher Scientific, MA, USA) in 6-well dishes to achieve 60 – 80% confluency. Cells were subsequently washed twice with 2 mL of cold 1X PBS and fixed with 1 mL of ice-cold 100% methanol for 10 minutes at -20°C.

Table 2.4: List of antibodies and their respective dilutions used for western blot (WB) analysis

Antibody	Source (Catalogue #)	Species/Clonality	Dilution
Kaiso	Dr. Albert Reynolds (NA)	Rabbit/Polyclonal	1:5000
E-cadherin	BD Biosciences (610182)	Mouse/Monoclonal	1:1000
ZO-1	Invitrogen (40-2200)	Rabbit/Polyclonal	1:4000
Vimentin	CST (D21H3-XP)	Rabbit/Monoclonal	1:1000
Slug	CST (C19G7)	Rabbit/Monoclonal	1:1000
ZEB1	SCB (H-102)	Rabbit/Polyclonal	1:1000
TGFβR1	CST (3712)	Rabbit/Monoclonal	1:1000
TGFβR2	SCB (sc-400)	Rabbit/Polyclonal	1:2000
phospho-Smad2 (Ser465/467)	CST (D43B4-XP)	Rabbit/Monoclonal	1:800
Smad2/3	CST (3102)	Rabbit/Monoclonal	1:1000
HA probe (12CA5)	SCB (sc-57592)	Mouse/Monoclonal	1:200
phospho-Akt	CST (4060)	Rabbit/Monoclonal	1:1000
Akt	CST (4691)	Rabbit/Monoclonal	1:1000
c-Myc	(SCB (9E10)	Mouse/Monoclonal	1:500
Cyclin D1	US Biological (144418)	Rabbit/Polyclonal	1:5000
p120 (15D2)	Dr. Albert Reynolds (NA)	Mouse/Monoclonal	1:1000
Bax	CST (5023)	Rabbit/Monoclonal	1:500
PUMA	CST (12450)	Rabbit/Monoclonal	1:500
mutant p53	Abcam (ab32049)	Rabbit/Monoclonal	1:2000
cleaved PARP	CST (5625)	Rabbit/Monoclonal	1:1000
BRCA1	Abcam (ab131360)	Rabbit/Polyclonal	1:2000
β-actin	Sigma (A5441)	Mouse/Monoclonal	1:50,000
C/EBPβ (H-7)	SCB (sc-7962)	Mouse/Monoclonal	1:500
GAPDH (14C10)	CST (2118)	Rabbit/Monoclonal	1:5000

NA = not applicable

Fixed cells were washed immediately with 2 mL of cold 1X PBS, and blocked with 1 mL of 3% skimmed milk in 1X PBS for 30 minutes at **r**oom **t**emperature (RT) before incubation at RT for 2 hours with the following antibodies: rabbit anti-Kaiso polyclonal (1:3000; gift from Dr. Reynolds) and mouse anti-p120 15D2 monoclonal (1:500; gift from Dr. Reynolds) at the specified dilutions in 3% milk/1X PBS. For negative control, cells were incubated in 3% milk/1X PBS in the absence of primary antibody. Following primary antibody incubations, cells were rinsed twice with 2 mL of cold 1X PBS and briefly immersed in 3% milk/1X PBS before incubation in the dark at RT for 1 hour with the appropriate secondary antibodies (Alexafluor 488-conjugated goat anti-mouse or Alexafluor 594-conjugated goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, Oregon)) at a dilution of 1:500 in 3% milk/1X PBS. Nuclear staining was achieved by simultaneous incubation with Hoechst (Sigma) at a dilution of 1:300 in 3% skimmed milk/PBS containing secondary antibodies. Cells were then washed twice with cold 1X PBS, and once with double-distilled water. Cells were subsequently mounted with Poly-Aquamount (PolyScience Inc. Warrington, USA) and imaged using a Carl Zeiss Axiovert 200, inverted fluorescent microscope.

Immunohistochemistry (IHC)

Harvested tissues from the mice xenograft metastatic studies fixed in 10% formalin were embedded in paraffin (at the John Mayberry Histology Facility, McMaster University, ON, Canada) before the preparation of 5 μm thick tissue

sections on slides that were either stained with H&E or Masson's trichrome at the John Mayberry Histology Facility, McMaster University, or the primary antibodies indicated in Tables 2.5. Prior to staining with primary antibodies, tissues were 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% and 70%). Antigen retrieval was achieved by boiling (~95°C) slides at low power (10%) for 15 – 20 minutes in a low pH buffer (pH 6.0) solution (or high pH buffer (pH 9.0) solution (DAKO, Glostrup, Denmark) for the Nigerian, Barbadian, AA and CA TNBC tissues used in Chapter 6). Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in 1X PBS, while non-specific staining was blocked by incubating tissues in a blocking solution comprised of 1X PBS and 5% or 10% normal donkey or goat serum (depending on the species the secondary antibody was raised in) for 1 hour at room temperature. Endogenous biotin, biotin receptors, and avidin binding sites on tissues were blocked using the Avidin/Biotin blocking kit (Vector Labs, CA, USA) preceding primary antibody incubations. Tissues were then incubated overnight at 4°C with the respective primary antibodies indicated in Tables 2.5 diluted in a solution containing 1X PBS and 1% normal donkey or goat serum. Tissues were subsequently washed two times for 10 minutes each in 1X PBS solution containing 0.05% **T**ween-20 (PBS-T) and one time for 10 minutes each in 1X PBS.

Secondary antibody incubations were performed at room temperature for 2 hours with biotinylated goat anti-mouse, donkey anti-mouse or donkey anti-rabbit antibody at a dilution of 1: 1000. Washes were performed as described above. To visualize immunostaining, tissues were incubated in Vectastain (Vector Labs) for 30 minutes and **d**i**a**mino**b**enzidine (DAB) (Vector Labs) for 2 - 10 minutes (depending on time taken to achieve adequate colour development). Tissues were subsequently counterstained with Harris hematoxylin (Sigma), differentiated in acid ethanol (0.3% HCl in 70% ethanol) and blued in Scott's tap water substitute. Dehydration of tissues was achieved 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).

Negative controls were attained by excluding primary antibody. IHC-stained images were acquired using the Aperio Slide scanner (Leica Biosystems, ON, Canada). Kaiso-stained TMA tissues were scored blindly by two Pathologists, and their scores averaged to give a final mean score value that was used for all subsequent analyses in Chapter 6. The scoring intensity was designated as 0, 1, 2 or 3 representing no, mild, moderate, or marked/intense staining.

Table 2.5: List of primary antibodies used for immunohistochemistry

Antibody	Antibody type	Source/Company & catalogue number	Dilution
Chapter 3			
Ki-67	Mouse monoclonal	BD Biosciences; 550609	1:50
PCNA	Rabbit monoclonal	CST; D3H8P	1:30 000
c-Myc	Rabbit monoclonal	Abcam; ab32072	1:100
Cyclin D1	Rabbit monoclonal	CST; 2978	1:100
Cleaved caspase 3	Rabbit monoclonal	CST; 9661	1:50
Chapter 4			
Kaiso 12H	Mouse monoclonal	Daniel *et al.* 2001	1:800
phospho-Smad2 (Ser465/467)	Rabbit monoclonal	CST; D43B4-XP	1:200 - MDA-231 xenografts 1:50 - Hs578T xenografts
Chapter 5			
Kaiso 6F	Mouse monoclonal	Daniel *et al.* 2001	1:500
Vimentin	Rabbit monoclonal	CST; 5741	1:500
E-cadherin	Mouse monoclonal	BD Biosciences; 610182	1:50
MMP-2	Rabbit polyclonal	CST; 4022	1:1000
MMP-9	Rabbit polyclonal	CST; 3852	1:1000
Chapter 6			
Kaiso 6F	Mouse monoclonal	Daniel *et al.* 2001	1:10000
Human cytokeratin clones (AE1/AE3)	Mouse monoclonal	Dako, Carpinteria, CA, USA; M3515	1:500

The staining intensity scores for each stained tissue was then calculated using the formula for the modified **h**istochemical **score** (H-score) system which is 3x (percentage of cells with maximum/marked intensity staining) + 2x (percentage of cells with moderate intensity staining) + 1x (percentage of cells with mild intensity staining) to give a final score value ranging from 0 – 300. Clinical observations

and histological analysis of H&E and Masson's trichome stained tissue sections reported in Chapter 4 and 5 were performed by Dr. Jacek Kwiecien, Pathologist, Central Animal Facility, McMaster University, using a Nikon Eclipse 50 light microscope.

Phase-contrast microscopy

Control (ctrl) and Kaiso-depleted (sh-K) MDA-231 and Hs578T cells were grown in 60 mm plates in a humidified chamber at 37°C and 5% CO_2 until they achieved 90-100% confluency. Cells were washed with 3 mL of sterile 1X PBS and fresh media was added to cells prior to obtaining phase contrast images. 10X and 40X phase-contrast images of ctrl and sh-K cells were obtained using the Zeiss Axiovert 200 microscope (Carl Zeiss Canada Ltd., ON, Canada).

Cell proliferation assay

Equal numbers (1 x10^4) of ctrl and sh-K MDA-231 and Hs578T cells were grown in 6-well plates for 3 days. Direct cell counts were obtained each day using the Bio-RAD TC10 automated cell counter and averaged using Microsoft Excel. Graphical representation of counts was achieved using GraphPad Prism software.

MTT assay

Equal numbers (1 x10^4) of ctrl and Kaiso-depleted (sh-K) MDA-231 and Hs578T cells were grown in 96-well plates for 24 hours. Cells were then immediately treated with MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; Sigma) for 2 hours. The precipitated formazan crystals were subsequently dissolved with 100 µL of **dim**ethyl **s**ulf**o**xide (DMSO) 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).

Clonogenic cell survival assay

5 x10^2 ctrl and Kaiso-depleted (sh-K) MDA-231 and Hs578T cells were cultured in 60 mm dishes (without agar) in triplicate and allowed to grow and form colonies for 10-14 days. After the incubation period, colonies were stained with 0.5% Gentian Violet diluted in methanol and counted. Graphical representation of counts (percentage of cells that survived) was achieved using GraphPad Prism software.

Soft agar assay

5 x10^4 control and Kaiso-depleted (sh-K1 and sh-K2) MDA-231 and Hs578T cells were cultured in 0.3% agarose in 60 mm dishes already containing a base layer of 0.7% solidified agarose. These cells were then allowed to grow and form colonies for ~10 – 12 days. Following incubation, resultant colonies were stained

with 0.05% Gentian Violet diluted in methanol, and microscopic (10X) images of colonies obtained using the Zeiss Axiovert 200 microscope (Carl Zeiss). Visible colonies were counted using the ImageJ software and graphical representation of counts achieved using GraphPad Prism software.

Caspase 3 assay

The Caspase 3 colorimetric assay kit was procured from Abcam (Boston, MA, USA), and the assay performed according to the company's guidelines. MDA-231 and Hs578T ctrl and Kaiso-depleted (sh-K1 and sh-K2) cells were cultured for 24 hours prior to performing the assay. As positive controls, MDA-231 and Hs578T control cells were treated for 4 hours with 200 µm Cisplatin (Sigma Aldrich, St. Louis, MO, USA) before harvesting the cells. The treated cells were then washed once with 1X PBS, trypsinized with 1X Trypsin and de-activated with 1X DMEM media. Cells were then counted using the Bio-RAD TC10 automated cell counter. Next, 1 x 10^6 ctrl, sh-K1 and sh-K2 MDA-231 and Hs578T cells, and 1 x 10^6 ctrl MDA-231 and Hs578T cells treated with Cisplatin were re-suspended in 50 µL of chilled cell lysis buffer. The re-suspended cell lysate was then incubated for 10 minutes on ice, followed by centrifugation for 1 minute at 13,000 rpm. The resultant supernatant (cytosolic extract) was subsequently transferred into a new tube, quantified using the Bio-RAD assay, and ~ 200 µg of protein/ 50 µL cell lysis buffer transferred to 96-well plates in duplicate per cell condition. 50 µL reaction buffer (2X) containing 10 mM DTT was added to each 96-well containing

an experimental sample (in duplicate). This was immediately followed by the addition of 5 µL of 4 mM DEVD-p-NA substrate (200 µM final concentration). The ensuing mixture was incubated for 2 hours at 37°C, and optical density readings of the mixture obtained at 405 nm using the SpectraMax® Plus 384 Microplate reader (Molecular Devices). Background readings were obtained from 96-wells containing 50 µL of cell lysis buffer without cell lysates, prior to the addition of 2X reaction buffer containing 10 mM DTT and 5 µL of 4 mM DEVD-p-NA substrate.

Annexin V-FITC staining assay

The **F**luorescein **i**so**t**hio**c**yanate (FITC)–conjugated Annexin V apoptosis detection kit was purchased from Abcam and staining performed according to the manufacturer's instructions. Equal numbers (1 x10^5) of ctrl and sh-K MDA-231, Hs578T and MDA-157 cells, sh-K-empty and sh-K-mKaiso MDA-231 and Hs578T cells, as well as MCF-7 (empty and mKaiso) cells were re-suspended in 500 µL of 1X binding buffer (Abcam) after being washed with 1X PBS and trypsinized with 1X Trypsin (Invitrogen). These cells were subsequently stained with 5 µL of Annexin V-**FITC** and 5 µL of **P**ropidium **i**odide (PI) and incubated for 10 minutes prior to analysis by Flow cytometry. Data was acquired using the LSRFortessa flow cytometer (BD Biosciences) and analyzed with the FlowJo version 9 software.

Ethics statement and mouse xenograft studies

All experiments with **N**OD **S**CID **G**amma (NSG) mice described in this study were approved by the **A**nimal **R**esearch **E**thics **B**oard (AREB) at McMaster University, ON, Canada (AUP Number 14-05-14) and performed in accordance with AREB guidelines. Female NSG mice were a generous gift from Dr. John Hassell (McMaster University). To study the effect of Kaiso depletion on the metastasis of TNBC cells, 4.5 x 10^6 MDA-231 and Hs578T control and sh-K cells were re-suspended in 200 µL of DMEM-serum free media/Matrigel mixture and injected under the fourth mammary fat pad on the right abdominal side of 6-8 week old female NSG mice (n = 10 each) and allowed to form tumors. Non-invasive monitoring of mice was performed weekly, and increased to 2-3 times weekly upon tumor appearance and growth. Tumor growth was monitored externally with Vernier calipers, and tumor volume (in mm^3) measured using the following formula (length/2 x $width^2$) 2-3 times weekly (Bhola et al. 2013). Mice were euthanized when tumor volume reached the endpoint of 2500-3300 mm^3 (to allow for subsequent dissemination of tumors). Tissues were perfused and fixed in 10% formalin prior to harvest and histological examination, and necropsies performed by a Pathologist to detect macro-metastases.

Experimental metastasis studies

For experimental metastasis studies, 5 x10^5 MDA-231 ctrl and sh-K cells, 1 x10^6 Hs578T ctrl and sh-K cells, and 1 x 10^6 MDA-231 Ctrl, **sh-K**aiso **e**mpty (sh-K-E)

and **T**GFβ**R1**^{204D} (sh-K-TR1^{204D}) cells were re-suspended individually in 1X PBS (200 µL) and injected into the tail veins of ~ 6-week-old female NSG mice (n = 5/cell line). Mice were euthanized 5 – 6 weeks post injection and tissues perfused and fixed in 10% formalin prior to harvest. Harvested tissues were embedded in paraffin at the John Mayberry Histology Facility, McMaster University before the preparation of 5 µm thick tissue sections on slides that were subsequently **h**ematoxylin and **e**osin (H&E) stained at the John Mayberry Histology Facility, McMaster University.

Retrospective study patient population

A total of 137 patient samples were utilized in this study and encompassed 28 Nigerian, 46 Barbadian, 20 African American and 43 Caucasian American TNBC tissues. Archived **f**ormalin-**f**ixed and **p**araffin-**e**mbedded (FFPE) tumor tissue blocks of Nigerian TNBC patients diagnosed at the **L**agos **U**niversity **T**eaching **H**ospital (LUTH), Nigeria from 2011 – 2013, and Barbadian TNBC patients diagnosed at the **Q**ueen **E**lizabeth **H**ospital (QEH), Barbados from 2002 – 2011 respectively, were obtained from the Pathology department at the respective hospitals after approval by LUTH and QEH Ethics Committees. The FFPE tissue blocks were then shipped to the Developmental Histology Lab at the **Y**ale **P**athological **T**issue **S**ervices (YPTS), Yale University (Connecticut, New Haven, USA), where tissue sections of each block were H&E stained for histological confirmation.

Representative tumor areas of each Nigerian and Barbadian FFPE specimen were then selected for the construction of a pilot **t**issue **m**icro**a**rray (TMA) comprised of Nigerian and Barbadian TNBC tissues. ER, PR and HER2 status of the Nigerian and Barbadian tissues were confirmed by immunohistochemistry conducted at LUTH (in the case of the Nigerian tissues), or at the Department of Pathology at QEH, Barbados, the Human Tissue Resource Center (Chicago, IL, USA) or the Immunohistochemistry lab at the University of Miami, Miller School of Medicine (Clinical Research Building, Miami, FL, USA) – for Barbadian tissues. Any tissue specimen with less than 1% staining for ER and PR was scored as negative; Similarly, 0 or +1 scoring for HER2 was considered negative. Available clinico-pathological information including age, tumor grade and lymph node involvement were retrieved from the hardcopy medical/pathology reports at LUTH and QEH.

TNBC tissue samples of African American (AA) and Caucasian American (CA) patients diagnosed at the Yale-New Haven Hospital, Connecticut, New Haven, USA from 1996 – 2004, were obtained by purchasing the **Y**ale **T**issue **M**icroarray **347** (YTMA-347) that was generated at the Developmental Histology Lab, (YPTS, Yale University, USA). The status of ER, PR and HER2 on these tissues was determined by IHC at the Developmental Histology Lab. The clinico-pathological features of all patients utilized in this study are reported in Chapter 6. It is

noteworthy that some clinical/pathological information was missing from all the TNBC cohorts (Nigerian, Barbadian, AA and CA) utilized in this study. Likewise, some scoring information for Kaiso was not obtained due to tissue damage from either poor tissue preservation, or poor tissue quality.

Gene expression analysis of TCGA and GEO datasets

For gene expression analysis of **T**he **C**ancer **G**enome **A**tlas (TCGA) datasets performed in Chapter 3, Level 3 IlluminaHiSeq_RNASeqV2 expression and associated clinical data were downloaded for all available patients from the Broad GDAC Firehose repository (https://gdac.broadinstitute.org/) on September 16th, 2016 (n=1,212). Only tumor samples (n=1,094) were selected for subsequent analysis, with their log2-transformed RSEM-quantified gene expression values used to denote gene expression. ER, PR and ERBB2 status, and overall survival information were obtained from the downloaded clinical data, and all data processing performed in R software (Team 2014). For the gene expression analysis of **G**ene **e**xpression **o**mnibus (GEO) datasets performed in Chapter 3, five (5) publicly available datasets, obtained using Affymetrix HG-U133 plus 2.0 gene chip arrays (Affymetrix, Santa Clara, CA, USA) were utilized. The transcript profiles of these datasets were deposited in the Gene Expression Omnibus under accession numbers GSE20685, GSE21653, GSE16446, GSE19615, and GSE9195 (Desmedt et al. 2011; Kao et al. 2011; Li et al. 2010; Loi et al. 2010; Sabatier et al. 2011a; Sabatier et al. 2011b).

For the gene expression analysis conducted in Chapter 4, Level 3 IlluminaHiSeq_RNASeqV2 expression and associated clinical data was downloaded for all available patients from TCGA data-portal, on 19th March 2014 (n=977). RSEM-quantified gene expression values were used to represent gene expression (Li and Dewey 2011). For consistency, transcript levels of the genes ESR1 and ERBB2 were used to assign ER and HER2 status to each patient. Transcript profiling data from the GEO dataset, GSE20685 (n=327), was performed on Affymetrix U133 Plus 2.0 gene chips and downloaded from the GEO website. **R**obust **M**ulti-**A**rray (RMA) was used to pre-process the dataset and gene expression values were calculated based on median expression of all probe sets mapping to a given gene based on Unigene ID. All genomic data processing was completed using R software.

All samples used for this study were normalized with **f**rozen **R**obust **M**ulti-**A**rray analysis (fRMA) (McCall et al. 2010) and then the DWD (**D**istance-**W**eighted **D**iscrimination) method (Benito et al. 2004) was used to remove technical variation from the datasets that were to be combined. The combined datasets 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 dataset were selected for further classification. This resulted in a cohort containing 894 tumor samples,

which were subsequently used for generating Kaplan-Meier survival curves and performing log-rank analysis. All Kaplan-Meier survival analyses and curve generation was performed by means of the GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA). p-value < 0.05 indicated statistical significance.

Statistical analysis

All statistical tests were completed using GraphPad Prism statistical software, and $p<0.05$ indicated significance. Data are presented as means ± **s**tandard **e**rror of the **m**ean (SEM). Unpaired Student's t test was used for statistical analysis of two datasets. Statistical analysis of more than two datasets was performed using one-way **an**alysis **o**f **va**riance (ANOVA) with either Tukey test or Kruskal-Wallis test with Dunn's multiple comparison test. Chi-square test was used to compare the differences in the clinico-pathological features of Nigerian, Barbadian, African American and Caucasian American patients reported in Chapter 6.

CHAPTER 3: KAISO, TNBC CELL GROWTH AND SURVIVAL

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

Preface

This chapter consists of the published article entitled: ***"Kaiso depletion attenuates the growth and survival of triple negative breast cancer cells."*** by Bassey-Archibong BI, Rayner LG, Hercules SM, Aarts CW, Dvorkin-Gheva A, Bramson JL, Hassell JA, and Daniel JM. (***Cell Death Dis. 2017; 8(3): e2689. doi:10.1038/cddis.2017.92***) in its original form.

In this article, we characterize the effects of Kaiso depletion on the growth and survival of **t**riple **n**egative **b**reast **c**ancer (TNBC) cells. We found that Kaiso depletion resulted in reduced cell proliferation and increased apoptosis of TNBC cells *in vitro* and *in vivo*, an effect possibly mediated by Kaiso's interaction with mutant-p53 and regulation of the proliferation-associated genes – *c-Myc* and *CCND1*, and apoptosis-related genes *Bax* and *PUMA*. We also found that Kaiso depletion attenuated anchorage dependence in a cell type dependent manner. Importantly, we found a statistically significant correlation between high Kaiso and BRCA1 mRNA expression and poor overall survival in TNBC patients.

Contributions:

BI Bassey-Archibong wrote the manuscript and conducted the experiments for the data represented 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 and SM Hercules conducted the experiments for Figure 1D and Figure 2B. BI Bassey-Archibong, SM Hercules and C Aarts conducted the experiments for Figure 3C-D. LG Rayner conducted the experiments for Figure 3A. BI Bassey-Archibong and LG Rayner conducted the experiments for Figure 4E and 6D. Dr. 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. Dr. JM Daniel also provided significant intellectual guidance throughout the study. All other authors assisted with reagents/resources (Dr. J Bramson and Dr. J Hassell), optimization of experimental procedures for the manuscript, 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.
Cell Death and Disease (2017) **8,** e2689; doi:10.1038/cddis.2017.92; published online 23 March 2017

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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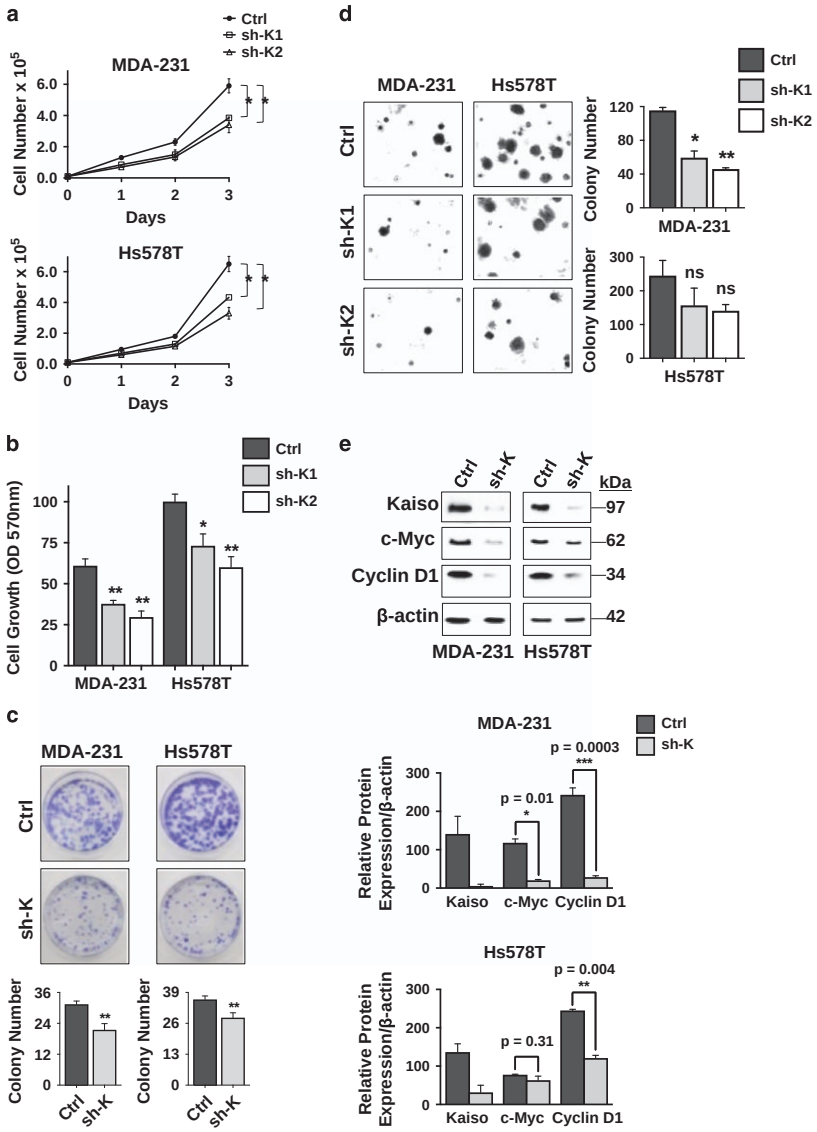

Figure 1 Kaiso depletion attenuates the proliferation of TNBC cells. Growth curve (**a**), MTT assays (**b**) and colony formation assays (**c**) were conducted on Ctrl and Kaiso-depleted (sh-K1 and sh-K2, hereafter referred to as sh-K) cells, and reveal that Kaiso depletion inhibits proliferation of 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]

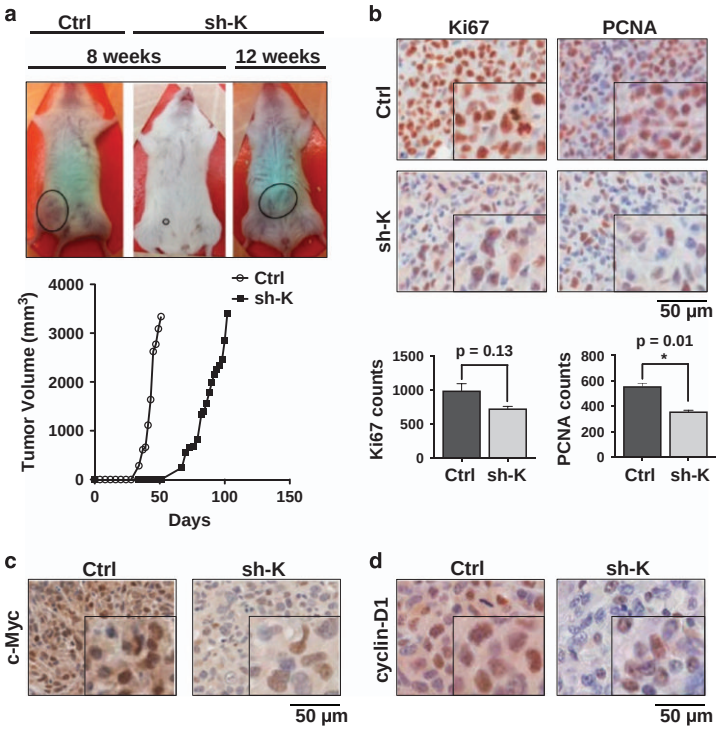

Figure 2 Kaiso-depleted MDA-231 cells exhibit delayed tumor onset in mouse xenografts. (**a**) Kaiso-depleted MDA-231 xenografts (sh-K) are delayed ~ 3 weeks in tumor onset and development compared to control (Ctrl) MDA-231 xenografted tumors as seen by time-course analysis of the tumor volume of Ctrl 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

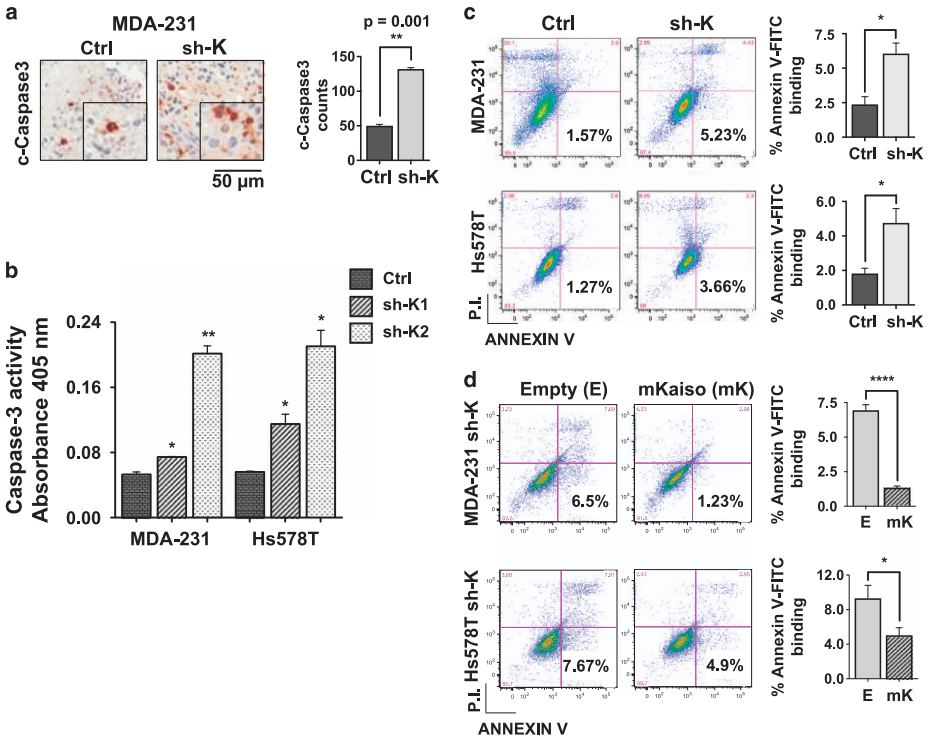

Figure 3 Kaiso depletion results in increased apoptosis of TNBC cells. (**a**) IHC-stained images of control (Ctrl) and Kaiso-depleted (sh-K) MDA-231 tumor tissues with cleaved (c)-Caspase 3 antibody show that Kaiso-depletion results in increased c-Caspase 3 expression in MDA-231 tumor tissues. (**b**) 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)

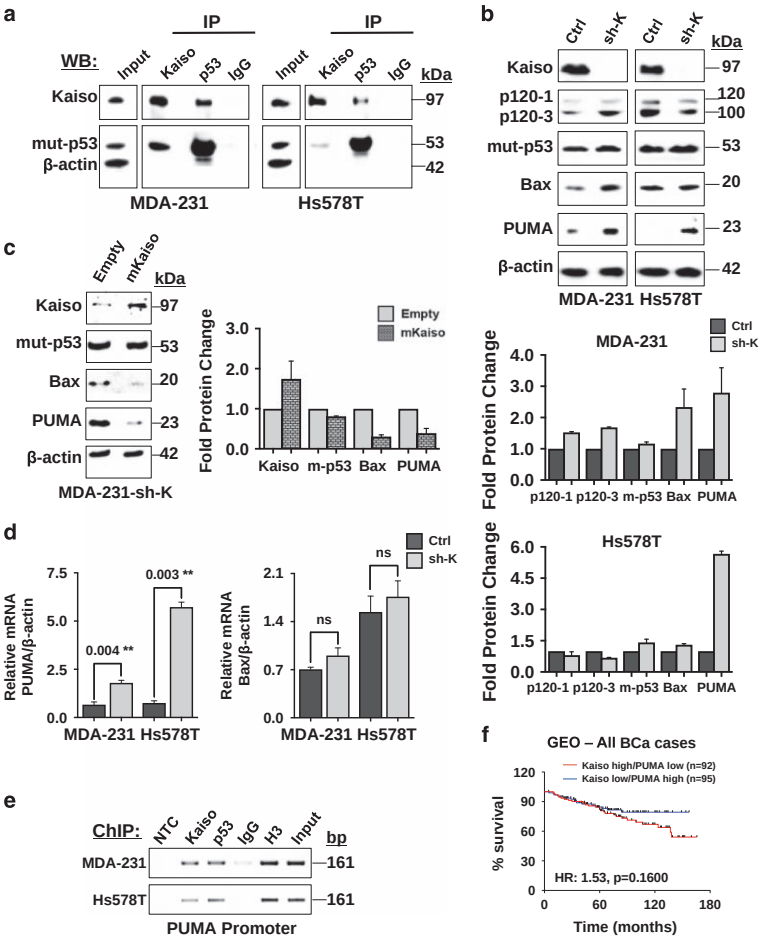

Figure 4 Kaiso depletion increases expression of pro-apoptotic proteins in TNBC cells lacking wt p53. (**a**) Kaiso co-precipitates with mutant p53 in MDA-231 and Hs578T cell lysates. Parental MDA-231 and Hs578T cells were subjected to immunoprecipitation with anti-Kaiso, anti-p53 and anti-IgG antibodies, 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.

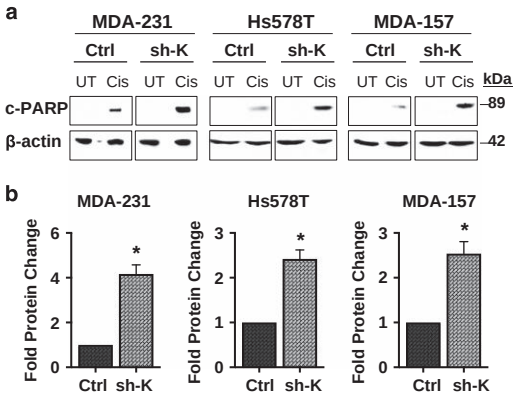

Figure 5 Kaiso depletion enhances the sensitivity of TNBC cells to Cisplatin. (**a**) Kaiso-depletion sensitizes TNBC cells to Cisplatin treatment, as demonstrated by the increased cleaved-PARP expression observed in Kaiso-depleted MDA-231, Hs578T and MDA-157 cells treated with Cisplatin (Cis) for 48 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

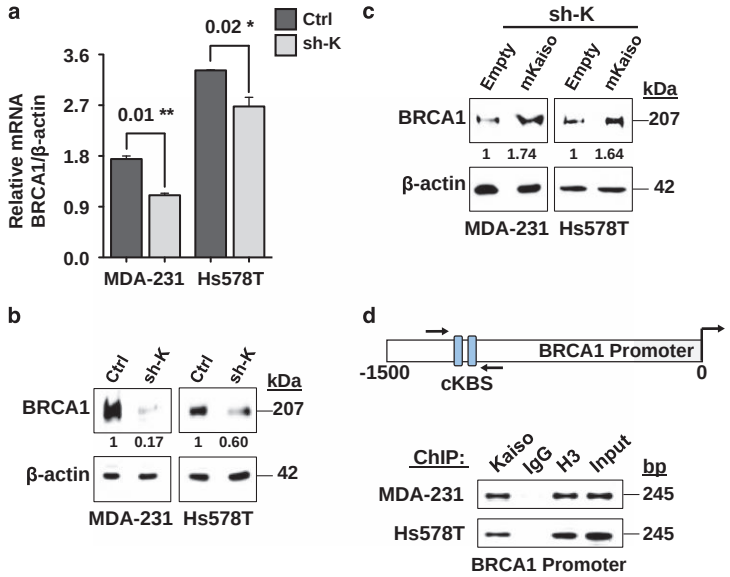

Figure 6 Kaiso depletion attenuates BRCA1 expression in sporadic TNBC cells. (**a**) BRCA1 mRNA expression was significantly reduced in Kaiso-depleted MDA-231 and Hs578T cells compared to controls as measured by qRT-PCR. (**b**) WB analysis with a BRCA1-specific antibody shows decreased BRCA1 protein 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. $^{*}P<0.05$, $^{**}P<0.01$

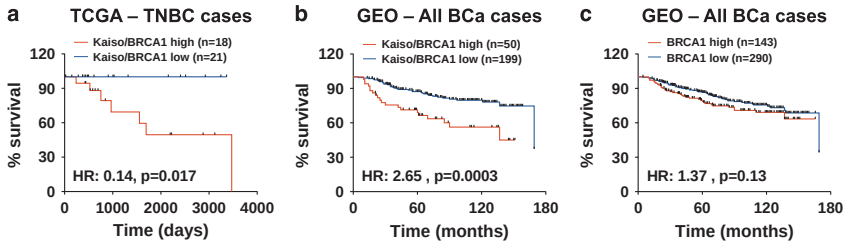

Figure 7 High Kaiso and BRCA1 expression correlates with poor prognosis in BCa patients. Transcript profiles of patients from the TCGA and GEO (GSE20685, GSE21653, GSE16446, GSE19615 and GSE9195) BCa data sets were pooled and segregated into Kaiso/BRCA1 high or Kaiso/BRCA1 low, and BRCA1 high or BRCA1 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

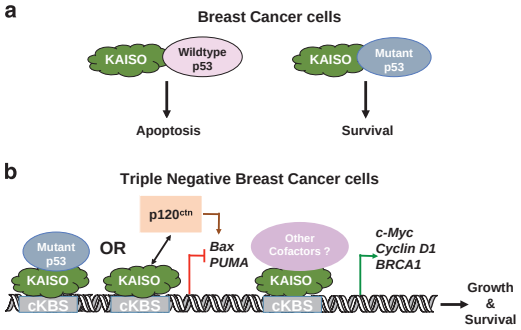

Figure 8 Schematic diagram of proposed model for Kaiso's role in TNBC. (**a**) Kaiso interacts with both wt p53 and mutant p53 in BCa cells and this differential interaction may modulate Kaiso's function in apoptosis. (**b**) In TNBC cells lacking wt p53 (but expressing mutant p53), Kaiso might directly 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 x10^5) 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 $\pm$ S.E.M.

Conflict of Interest

The authors declare no conflict of interest.

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1. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med* 2010; **363**: 1938–1948.
2. Brouckaert O, Wildiers H, Floris G, Neven P. Update on triple-negative breast cancer: prognosis and management strategies. *Int J Womens Health* 2012; **4**: 511–520.
3. O'Reilly EA, Gubbins L, Sharma S, Tully R, Guang MH, Weiner-Gorzel K *et al.* The fate of chemoresistance in triple negative breast cancer (TNBC). *BBA Clin* 2015; **3**: 257–275.
4. Hill SJ, Clark AP, Silver DP, Livingston DM. BRCA1 pathway function in basal-like breast cancer cells. *Mol Cell Biol* 2014; **34**: 3828–3842.
5. Hongthong K, Ratanaphan A. BRCA1-associated triple-negative breast cancer and potential treatment for ruthenium-based compounds. *Curr Cancer Drug Targets* 2016; **16**: 606–617.
6. Varna M, Bousquet G, Plassa LF, Bertheau P, Janin A. TP53 status and response to treatment in breast cancers. *J Biomed Biotechnol* 2011; **2011**: 284584.
7. Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H. BRCA1 regulates p53-dependent gene expression. *Proc Natl Acad Sci USA* 1998; **95**: 2302–2306.
8. Wong-Brown MW, Meldrum CJ, Carpenter JE, Clarke CL, Narod SA, Jakubowska A *et al.* Prevalence of BRCA1 and BRCA2 germline mutations in patients with triple-negative breast cancer. *Breast Cancer Res Treat* 2015; **150**: 71–80.
9. Gonzalez-Angulo AM, Timms KM, Liu S, Chen H, Litton JK, Potter J *et al.* Incidence and outcome of BRCA mutations in unselected patients with triple receptor-negative breast cancer. *Clin Cancer Res* 2011; **17**: 1082–1089.
10. Silver DP, Richardson AL, Eklund AC, Wang ZC, Szallasi Z, Li Q *et al.* Efficacy of neoadjuvant Cisplatin in triple-negative breast cancer. *J Clin Oncol* 2010; **28**: 1145–1153.
11. Olivier M, Langerød A, Carrieri P, Bergh J, Klaar S, Eyfjord J *et al.* The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. *Clin Cancer Res* 2006; **12**: 1157–1167.
12. Walerych D, Napoli M, Collavin L, Del Sal G. The rebel angel: mutant p53 as the driving oncogene in breast cancer. *Carcinogenesis* 2012; **33**: 2007–2017.
13. Shibue T, Takeda K, Oda E, Tanaka H, Murasawa H, Takaoka A *et al.* Integral role of Noxa in p53-mediated apoptotic response. *Genes Dev* 2003; **17**: 2233–2238.
14. Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M *et al.* Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 2004; **303**: 1010–1014.
15. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001; **7**: 683–694.
16. Bullock AN, Fersht AR. Rescuing the function of mutant p53. *Nat Rev Cancer* 2001; **1**: 68–76.
17. Aas T, Børresen AL, Geisler S, Smith-Sørensen B, Johnsen H, Varhaug JE *et al.* Specific P53 mutations are associated with *de novo* resistance to doxorubicin in breast cancer patients. *Nat Med* 1996; **2**: 811–814.
18. Lim LY, Vidnovic N, Ellisen LW, Leong CO. Mutant p53 mediates survival of breast cancer cells. *Br J Cancer* 2009; **101**: 1606–1612.
19. Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P *et al.* Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer. *PLoS ONE* 2012; **7**: e37864.

20. Jones J, Wang H, Zhou J, Hardy S, Turner T, Austin D *et al.* Nuclear kaiso indicates aggressive prostate cancers and promotes migration and invasiveness of prostate cancer cells. *Am J Pathol* 2012; **181**: 1836–1846.
21. Daniel JM, Reynolds AB. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol Cell Biol* 1999; **19**: 3614–3623.
22. Roczniak-Ferguson A, Reynolds AB. Regulation of p120-catenin nucleocytoplasmic shuttling activity. *J Cell Sci* 2003; **116**: 4201–4212.
23. van de Ven RA, Tenhagen M, Meuleman W, van Riel JJ, Schackmann RC, Derksen PW. Nuclear p120-catenin regulates the anoikis resistance of mouse lobular breast cancer cells through Kaiso-dependent Wnt11 expression. *Dis Model Mech* 2015; **8**: 373–384.
24. Park JI, Kim SW, Lyons JP, Ji H, Nguyen TT, Cho K *et al.* Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell* 2005; **8**: 843–854.
25. Spring CM, Kelly KF, O'Kelly I, Graham M, Crawford HC, Daniel JM. The catenin p120ctn inhibits Kaiso-mediated transcriptional repression of the beta-catenin/TCF target gene matrilysin. *Exp Cell Res* 2005; **305**: 253–265.
26. Bassey-Archibong BI, Kwiecien JM, Milosavljevic SB, Hallett RM, Rayner LG, Erb MJ *et al.* Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells. *Oncogenesis* 2016; **5**: e208.
27. Pozner A, Terooatea TW, Buck-Koehntop BA. Cell-specific Kaiso (ZBTB33) regulation of cell cycle through cyclin D1 and cyclin E1. *J Biol Chem* 2016; **291**: 24538–24550.
28. Prokhortchouk A, Sansom O, Selfridge J, Caballero IM, Salozhin S, Aithozhina D *et al.* Kaiso-deficient mice show resistance to intestinal cancer. *Mol Cell Biol* 2006; **26**: 199–208.
29. Donaldson NS, Pierre CC, Anstey MI, Robinson SC, Weerawardane SM, Daniel JM. Kaiso represses the cell cycle gene cyclin D1 via sequence-specific and methyl-CpG-dependent mechanisms. *PLoS ONE* 2012; **7**: e50398.
30. Koh DI, Yoon JH, Kim MK, An H, Kim MY, Hur MW. Kaiso is a key regulator of spleen germinal center formation by repressing Bcl6 expression in splenocytes. *Biochem Biophys Res Commun* 2013; **442**: 177–182.
31. Koh DI, Han D, Ryu H, Choi WI, Jeon BN, Kim MK *et al.* KAISO, a critical regulator of p53-mediated transcription of CDKN1A and apoptotic genes. *Proc Natl Acad Sci USA* 2014; **111**: 15078–15083.
32. Yi YW, Kang HJ, Kim HJ, Kong Y, Brown ML, Bae I. Targeting mutant p53 by a SIRT1 activator YK-3-237 inhibits the proliferation of triple-negative breast cancer cells. *Oncotarget* 2013; **4**: 984–994.
33. Punj V, Bhattacharyya S, Saint-Dic D, Vasu C, Cunningham EA, Graves J *et al.* Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. *Oncogene* 2004; **23**: 2367–2378.
34. The p53 website. Available at: http://p53.free.fr/Database/Cancer_cell_lines/Breast_cancer.html.
35. Marquette C, Nabell L. Chemotherapy-resistant metastatic breast cancer. *Curr Treat Options Oncol* 2012; **13**: 263–275.
36. Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* 2000; **275**: 23899–23903.
37. Yadav BS, Sharma SC, Chanana P, Jhamb S. Systemic treatment strategies for triple-negative breast cancer. *World J Clin Oncol* 2014; **5**: 125–133.
38. Lopes EC, Valls E, Figueroa ME, Mazur A, Meng FG, Chiosis G *et al.* Kaiso contributes to DNA methylation-dependent silencing of tumor suppressor genes in colon cancer cell lines. *Cancer Res* 2008; **68**: 7258–7263.
39. Dai SD, Wang Y, Miao Y, Zhao Y, Zhang Y, Jiang GY *et al.* Cytoplasmic Kaiso is associated with poor prognosis in non-small cell lung cancer. *BMC Cancer* 2009; **9**: 178.
40. Cofre J, Menezes JR, Pizzatti L, Abdelhay E. Knock-down of Kaiso induces proliferation and blocks granulocytic differentiation in blast crisis of chronic myeloid leukemia. *Cancer Cell Int* 2012; **12**: 28–2867-12-28.
41. Pierre CC, Longo J, Mavor M, Milosavljevic SB, Chaudhary R, Gilbreath E *et al.* Kaiso overexpression promotes intestinal inflammation and potentiates intestinal tumorigenesis in Apc(Min/+) mice. *Biochim Biophys Acta* 2015; **1852**: 1846–1855.
42. Wang H, Liu W, Black S, Turner O, Daniel JM, Dean-Colomb W *et al.* Kaiso, a transcriptional repressor, promotes cell migration and invasion of prostate cancer cells through regulation of miR-31 expression. *Oncotarget* 2016; **7**: 5677–5689.
43. Liu Y, Dong QZ, Wang S, Xu HT, Miao Y, Wang L *et al.* Kaiso interacts with p120-catenin to regulate β-catenin expression at the transcriptional level. *PLoS ONE* 2014; **9**: e87537.
44. Ogden SR, Wroblewski LE, Weydig C, Romero-Gallo J, O'Brien DP, Israel DA *et al.* p120 and Kaiso regulate Helicobacter pylori-induced expression of matrix metalloproteinase-7. *Mol Biol Cell* 2008; **19**: 4110–4121.
45. Rodova M, Kelly KF, VanSaun M, Daniel JM, Werle MJ. Regulation of the rapsyn promoter by kaiso and delta-catenin. *Mol Cell Biol* 2004; **24**: 7188–7196.
46. Daniel JM, Spring CM, Crawford HC, Reynolds AB, Baig A. The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. *Nucleic Acids Res* 2002; **30**: 2911–2919.
47. Buck-Koehntop BA, Martinez-Yamout MA, Dyson HJ, Wright PE. Kaiso uses all three zinc fingers and adjacent sequence motifs for high affinity binding to sequence-specific and methyl-CpG DNA targets. *FEBS Lett* 2012; **586**: 734–739.
48. Kim SW, Park JI, Spring CM, Sater AK, Ji H, Otchere AA *et al.* Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. *Nat Cell Biol* 2004; **6**: 1212–1220.
49. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* 2011; **11**: 558–572.
50. Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE *et al.* Function of the c-Myc oncogenic transcription factor. *Exp Cell Res* 1999; **253**: 63–77.
51. Bae Y-H, Shin J-M, Park H-J, Jang H-O, Bae M-K, Bae S-K. Gain-of-function mutant p53-R280K mediates survival of breast cancer cells. *Genes Genom* 2014; **36**: 171–178.
52. Braicu C, Pileczki V, Irimie A, Berindan-Neagoe I. p53siRNA therapy reduces cell proliferation, migration and induces apoptosis in triple negative breast cancer cells. *Mol Cell Biochem* 2013; **381**: 61–68.
53. Kim E, Deppert W. Transcriptional activities of mutant p53: when mutations are more than a loss. *J Cell Biochem* 2004; **93**: 878–886.
54. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T *et al.* A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006; **10**: 515–527.
55. Daniel JM, Ireton RC, Reynolds AB. Monoclonal antibodies to Kaiso: a novel transcription factor and p120ctn-binding protein. *Hybridoma* 2001; **20**: 159–166.
56. Wu J, Mariner DJ, Thoreson MA, Reynolds AB. Production and characterization of monoclonal antibodies to the catenin p120ctn. *Hybridoma* 1998; **17**: 175–183.
57. Kao KJ, Chang KM, Hsu HC, Huang AT. Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: implications for treatment optimization. *BMC Cancer* 2011; **11**: 143.
58. Desmedt C, Di Leo A, de Azambuja E, Larsimont D, Haibe-Kains B, Selleslags J *et al.* Multifactorial approach to predicting resistance to anthracyclines. *J Clin Oncol* 2011; **29**: 1578–1586.
59. Sabatier R, Finetti P, Cervera N, Lambaudie E, Esterni B, Mamessier E *et al.* A gene expression signature identifies two prognostic subgroups of basal breast cancer. *Breast Cancer Res Treat* 2011; **126**: 407–420.
60. Sabatier R, Finetti P, Adelaide J, Guille A, Borg JP, Chaffanet M *et al.* Down-regulation of ECRG4, a candidate tumor suppressor gene, in human breast cancer. *PLoS ONE* 2011; **6**: e27656.
61. Li Y, Zou L, Li Q, Haibe-Kains B, Tian R, Desmedt C *et al.* Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer. *Nat Med* 2010; **16**: 214–218.
62. Loi S, Haibe-Kains B, Majjaj S, Lallemand F, Durbecq V, Larsimont D *et al.* PIK3CA mutations associated with gene signature of low mTORC1 signaling and better outcomes in estrogen receptor-positive breast cancer. *Proc Natl Acad Sci USA* 2010; **107**: 10208–10213.
63. McCall MN, Bolstad BM, Irizarry RA. Frozen robust multiarray analysis (fRMA). *Biostatistics* 2010; **11**: 242–253.
64. Benito M, Parker J, Du Q, Wu J, Xiang D, Perou CM *et al.* Adjustment of systematic microarray data biases. *Bioinformatics* 2004; **20**: 105–114.
65. Team RC. A language and environment for statistical computing. Austria R Foundation for Statistical Computing: Vienna, 2014.

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CHAPTER 4: KAISO, TGFβ SIGNALING & METASTASIS

Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells

Preface

This chapter features the published article entitled ***"Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells"*** by Bassey-Archibong BI, Kwiecien JM, Milosavljevic SB, Hallett RM, Rayner LG, Erb MJ, Crawford-Brown CJ, Stephenson KB, Bédard PA, Hassell JA and Daniel JM (***Oncogenesis, 2016; 5: e208***) in its original form. This is an open-access article licensed under a Creative Commons Attribution 4.0 International License, which allows unrestricted use, reproduction, and distribution in any format, provided appropriate credit is given to the author and attribution parties, and sources cited.

This work describes the generation and characterization of shRNA-mediated Kaiso depleted TNBC cells that were generated to elucidate Kaiso-depletion effects on the metastasis of TNBC tumors. Kaiso depletion resulted in a mesenchymal-epithelial transition phenotype in the MDA-MB-231 and Hs578T TNBC cells, and attenuated the metastatic abilities of MDA-MB-231 and Hs578T cells in a mouse xenograft model. Notably, Kaiso depletion attenuated TGFβ signaling and TGFβ-dependent transcriptional responses that has been implicated in metastasis. Hence, we postulated that Kaiso expression may promote metastasis in part via the TGFβ signaling cascade and associated pro-tumorigenic responses.

Contributions:

BI Bassey-Archibong generated the Kaiso-depleted TNBC cells, control and Kaiso-depleted TNBC xenograft models, and conducted the experiments

illustrated in Figures 1C-D, Figure 2A, Figures 3B-D, Figures 4A-D, Figures 5A and 5C, and Figure 7A. Dr. J Kwiecien provided the pathological interpretation of the data in Figures 2A-B and Figure 7B. Dr. S Milosavljevic and LG Rayner generated the data for Figure 5D. Dr. R Hallett generated the data for Figures 1A-B, Figure 3A and Figures 6A and B. M Erb generated the data for Figure 5B. Dr. K Stephenson performed the tail-vein injections for Figures 2B and 7B. BI Bassey-Archibong and Dr. JM Daniel conceived the study and co-wrote the manuscript. Dr. JM Daniel also contributed significant intellectual guidance throughout the implementation of this work. All other authors assisted with reagents (Dr. P-A Bedard and Dr. J Hassell), or optimization of experimental procedures (C Crawford-Brown) for the manuscript. All authors edited the manuscript text.

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ORIGINAL ARTICLE

Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells

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Triple-negative breast cancers (TNBCs) represent a subset of breast tumors that are highly aggressive and metastatic, and are responsible for a disproportionate number of breast cancer-related deaths. Several studies have postulated a role for the epithelial-to-mesenchymal transition (EMT) program in the increased aggressiveness and metastatic propensity of TNBCs. Although EMT is essential for early vertebrate development and wound healing, it is frequently co-opted by cancer cells during tumorigenesis. One prominent signaling pathway involved in EMT is the transforming growth factor-β (TGFβ) pathway. In this study, we report that the novel POZ-ZF transcription factor Kaiso is highly expressed in TNBCs and correlates with a shorter metastasis-free survival. Notably, Kaiso expression is induced by the TGFβ pathway and silencing Kaiso expression in the highly invasive breast cancer cell lines, MDA-MB-231 (hereafter MDA-231) and Hs578T, attenuated the expression of several EMT-associated proteins (Vimentin, Slug and ZEB1), abrogated TGFβ signaling and TGFβ-dependent EMT. Moreover, Kaiso depletion attenuated the metastasis of TNBC cells (MDA-231 and Hs578T) in a mouse model. Although high Kaiso and high TGFβR1 expression is associated with poor overall survival in breast cancer patients, overexpression of a kinase-active TGFβR1 in the Kaiso-depleted cells was insufficient to restore the metastatic potential of these cells, suggesting that Kaiso is a key downstream component of TGFβ-mediated pro-metastatic responses. Collectively, these findings suggest a critical role for Kaiso in TGFβ signaling and the metastasis of TNBCs.

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INTRODUCTION

Breast cancer is the most common female cancer and a leading cause of female deaths worldwide.[1] Of the five major breast cancer subtypes,[2,3] the triple-negative breast cancers (TNBCs) have the worst prognosis because of their limited treatment options and highly metastatic nature.[4,5] Several studies suggest a role for the epithelial-to-mesenchymal transition (EMT) program in the metastatic propensity of TNBCs.[6,7] Indeed, increased expression of various EMT proteins (for example, Vimentin, ZEB1) has been reported in many TNBC cases, where they appear to correlate with increased invasiveness and poor disease-free survival.[7,8]

EMT is a complex and tightly regulated process that confers mesenchymal properties (for example, increased motility and invasiveness) to epithelial cells (reviewed in Kalluri and Weinberg[9]). The switch in cellular behavior and characteristics during EMT is accomplished mostly by EMT-associated transcription factors (for example, Snail/Slug, ZEB1/2) that function to promote the loss of epithelial components (for example, E-cadherin) and gain of mesenchymal proteins (for example, Vimentin). These EMT transcription factors are activated by many cytokines or growth factors including the transforming growth factor-β (TGFβ) (reviewed in Puisieux *et al.*[10]).

The TGFβ pathway controls many normal and pathological processes in addition to EMT.[11,12] TGFβ signals are transduced either via the canonical cascade involving Smad proteins (for example, Smad2/3) or the noncanonical cascade involving non-Smad proteins (for example, phosphatidylinositol 3 kinase/AKT, extracellular signal-regulated protein kinase-1/2; reviewed in Zhang[12] and Heldin *et al.*[13]). Depending on the cellular context, TGFβ suppresses or promotes tumor progression in breast cancers (BCa). In early-stage BCa, TGFβ is a potent inhibitor of uncontrolled cell proliferation; however, in advanced BCa, TGFβ promotes metastasis as the cells become refractory to TGFβ growth inhibition.[14] The mechanism underlying the switch in TGFβ function from a tumor suppressor to tumor promoter is not well understood but studies implicate the TGFβ receptors (TGFβR1 and 2) as critical determinants of the functional specificity of the TGFβ signaling cascade.[15–17] A metastasis-associated TGFβ response signature that includes expression of several EMT-associated genes was recently identified in breast tumors,[18] further highlighting the importance of TGFβ signaling in EMT induction and malignant progression of BCa.

Recently, the transcription factor Kaiso was identified as a regulator of E-cadherin expression and EMT in prostate and breast tumors.[19,20] Kaiso is a unique dual-specificity transcription factor that recognizes and binds a consensus Kaiso-binding sequence (KBS), TCCTGCNA, or methylated CpG-dinucleotides.[21] Most Kaiso target genes (for example, *CCND1*, *S100A4*, *MMP7*, *CDH1*) identified

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to date are linked to tumor onset, progression and metastasis.[22–25] Thus, not surprisingly, Kaiso is implicated in various human cancers (breast, colon, lung, prostate), and appears to have both tumor suppressive and promoting roles.[19,20,26–30] Indeed, high Kaiso expression correlates significantly with estrogen receptor-α negativity, basal/TNBCs and poor prognosis in patients with infiltrating BCa.[20,29] More recently, Kaiso was implicated as a potential drug target in glucocorticoid-combined chemotherapy in breast cancer.[30] However, the precise roles and mechanism of action of Kaiso in tumorigenesis remain poorly understood. Here, we report that high Kaiso expression in BCa patients correlates with high expression of the TGFβ signalsome and shorter metastasis-free survival. Silencing Kaiso expression in TNBC cells attenuates TGFβ signaling and TGFβR1 expression, and induces an EMT reversal concomitant with decreased EMT protein expression. More importantly, silencing Kaiso strongly inhibited TNBC cell metastasis in two mouse metastasis models. However, although expression of a constitutively active TGFβR1 in Kaiso-depleted TNBC cells rescued TGFβ signaling, this was insufficient to restore the metastatic abilities of these cells. Our results present the first evidence linking Kaiso to TGFβ signaling and BCa metastasis *in vivo*, and highlight a clinically relevant role for Kaiso in the metastasis of aggressive breast tumors.

RESULTS

High Kaiso expression correlates with poor prognosis in breast cancer patients

Kaiso is highly expressed in several TNBC cell lines (our unpublished data) and nuclear Kaiso expression has been linked with EMT and TNBC aggressiveness.[20,29] To determine the clinical relevance of Kaiso (ZBTB33) expression in aggressive BCa, we analyzed The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) (GSE20685) breast cancer data sets. Consistent with an earlier study,[29] most high Kaiso-expressing tumors lacked the estrogen receptor. However, the highest and most statistically significant Kaiso expression correlated with TNBC cases (Figure 1a). Importantly, Kaplan–Meier survival curves revealed that patients with high Kaiso-expressing tumors (ZBTB33 high) had a poorer overall survival (log-rank test, $P = 0.0052$) and

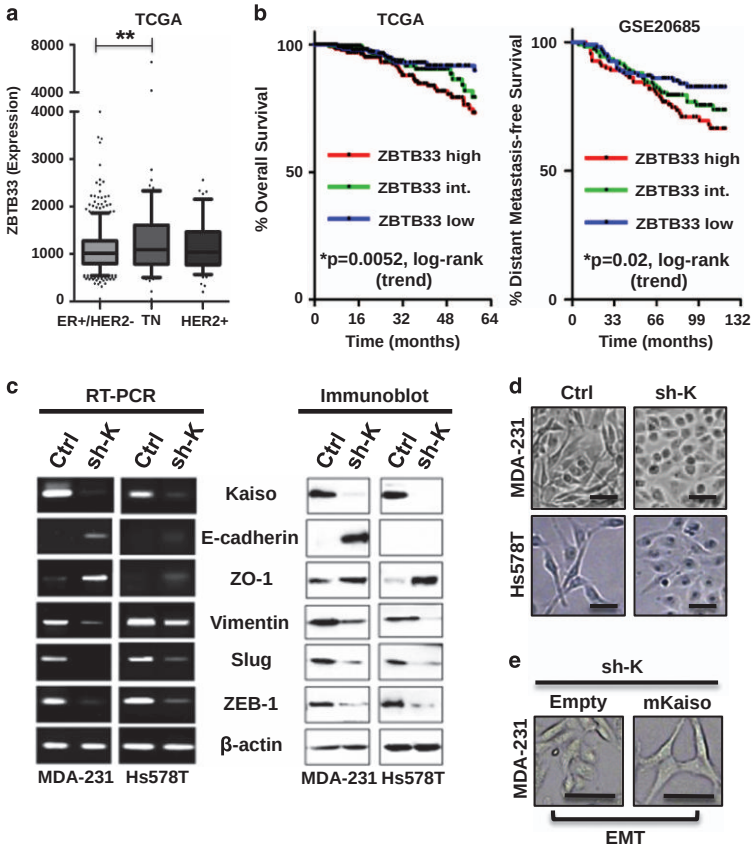

Figure 1. High Kaiso expression correlates with shorter metastasis-free survival and EMT. (**a**) Analysis of the publicly available TCGA breast cancer (BCa) data set revealed that high Kaiso expression correlates with ER (−) negativity and TNBC. $^{**}P < 0.001$. (**b**) Patients from the TCGA ($n = 977$) and the GEO (GSE20685) ($n = 327$) data sets were segregated into Kaiso (ZBTB33)-high, Kaiso-intermediate and Kaiso-low groups based on transcript levels. Kaplan–Meier survival curves revealed a significant negative correlation between high Kaiso expression, overall survival and distant metastasis-free survival in all BCa cases. Statistical significance was determined by log-rank test and *P*-values are indicated. (**c**) RT–PCR and immunoblot analysis of control and Kaiso-depleted MDA-231 and Hs578T cells. (**d**) Phase-contrast images of control and Kaiso-depleted MDA-231 and Hs578T cells. (**e**) Phase-contrast images of Kaiso-depleted MDA-231 cells transfected with either an empty or mKaiso vector. Scale bar, 100 μM.

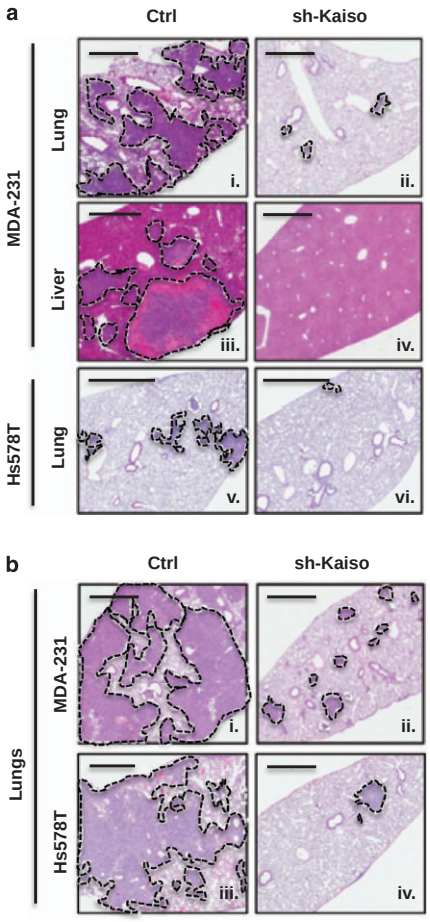

Figure 2. Kaiso depletion inhibits breast tumor cell metastasis to the lungs and liver. (**a** i–vi) Hematoxylin and eosin (H&E) staining of murine lungs and liver revealed that control MDA-231 xenografts formed extensive metastases in lungs (i) and liver (iii), whereas control Hs578T xenografts formed moderate metastases that were limited to the lungs (v) of immune-deficient mice. In contrast, Kaiso-depleted MDA-231 xenografts formed very few metastases in the lungs (ii), and no metastases in the liver (iv) of immune-deficient mice. Kaiso-depleted Hs578T xenografts also formed negligible metastases in the lung (v). (**b**) H&E images showing extensive metastases of control MDA-231 (i) and control Hs578T (iii) in the lungs of NSG mice after tail vein injections compared with few metastases formed by Kaiso-depleted MDA-231 (ii) and Hs578T cells (iv). Scale bar, 1000 μm. Representative images are shown.

shorter distant metastasis-free survival (log-rank test, $P = 0.02$) compared with patients with intermediate or low Kaiso-expressing tumors (Figure 1b) in all BCa cases. These findings suggested a clinically relevant role for Kaiso in TNBC.

Kaiso-depleted TNBC cell lines undergo mesenchymal-to-epithelial transition

As a first step to unraveling the function of Kaiso in TNBC, we generated stable Kaiso depletion in two highly invasive TNBC cell lines (MDA-231 and Hs578T) using two independent Kaiso-specific short hairpin (sh)-RNAs. As Kaiso was linked to EMT,[20] we first confirmed that Kaiso depletion (sh-K1, sh-K2) altered the expression of the EMT proteins E-cadherin and Vimentin (Figure 1c and Supplementary Figure 1). Increased E-cadherin expression was observed in Kaiso-depleted (sh-K) MDA-231 cells but not in Hs578T counterparts (Figure 1c and Supplementary Figure 1). In contrast, Kaiso-depleted MDA-231 and Hs578T cells both exhibited decreased expression of the EMT-inducing transcription factors Slug and ZEB1 but increased expression of the epithelial protein ZO-1 (Figure 1c). These gene expression changes resulted in a concomitant induction of a mesenchymal-to-epithelial transition phenotype in Kaiso-depleted MDA-231 and Hs578T cells (despite Hs578T-sh-K cells lacking any obvious E-cadherin expression) (Figure 1d). Re-expression of a sh-resistant Kaiso complementary DNA (cDNA; mKaiso) in MDA-231-sh-K cells restored the mesenchymal phenotype (Figure 1e). Thus, in addition to directly regulating E-cadherin expression,[19,20] Kaiso may indirectly regulate E-cadherin and EMT via modulation of transcription factors that repress E-cadherin.

Kaiso depletion attenuates the metastasis of TNBC cells

The link of Kaiso to distant metastasis-free survival in BCa patients and EMT (Figure 1)[19,20] led us to question whether Kaiso was essential for TNBC dissemination. Thus, we investigated the effect of Kaiso depletion on TNBC cell metastasis in a mouse model where Kaiso-depleted MDA-231 and Hs578T cells were injected subcutaneously into the mammary fat pads of immuno-compromised mice and allowed to form tumors. In support of our hypothesis, we found that Kaiso-depleted MDA-231 cells exhibited only a few small metastatic foci in the lungs (Figure 2a ii), but no detectable metastases in the liver (Figure 2a iv) or other organs (data not shown) in all xenografted mice ($n = 9$). In contrast, MDA-231 control-injected mice exhibited extensive metastases to the lungs and liver ($n = 6$; Figure 2a i and -iii) as previously shown.[32,33] Similarly, control Hs578T cells exhibited modest metastases that were limited to the lungs of all xenografted mice ($n = 7$; Figure 2a v) compared with Kaiso-depleted Hs578T injected mice that displayed very few metastatic foci in the lungs ($n = 7$; Figure 2a vi). Similar results were obtained in experimental lung metastasis (tail vein injections) studies; control MDA-231 ($n = 5$) and Hs578T ($n = 5$) cells formed large and multiple foci in the lungs of all injected mice (Figure 2b i and iii), whereas Kaiso-depleted MDA-231 ($n = 5$) and Hs578T cells ($n = 5$) formed few foci in the lungs of injected mice (Figure 2b ii and iv). Collectively, these findings highlight for the first time the importance of Kaiso expression on the metastasis of TNBC cells.

Kaiso expression positively correlates with TGFβ signaling protein expression

To successfully undergo metastasis, tumor cells must activate various cellular processes in addition to EMT, to enable their extravasation, survival in the circulatory system and establishment at secondary sites.[34] To elucidate how Kaiso might potentiate the complete metastatic cascade, we analyzed the TCGA BCa dataset to correlate Kaiso expression with other genes implicated in tumor progression and metastasis. We found that high Kaiso expression positively correlates with several TGFβ signaling genes including Smad2, Smad4 and TGFβR1 (Figure 3a). Examination of the expression levels of various TGFβ signaling components in Kaiso-depleted TNBC cells revealed that silencing Kaiso attenuated the expression of TGFβR1 and TGFβR2 at both the transcript and protein levels in both cell lines (Figures 3b and c). However, there were no significant changes in Smad2 or Smad4 expression in either cell line (data not shown). Notably, TGFβR1 and TGFβR2 expression was upregulated following expression of a sh-resistant Kaiso form in Kaiso-depleted MDA-231 cells (Figure 3d).

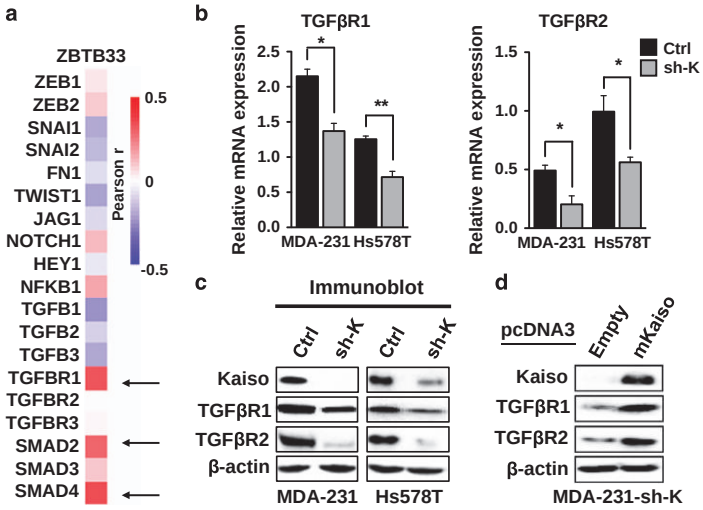

Figure 3. Kaiso expression positively correlates with TGFβ signaling components in triple-negative tumors. (**a**) Heat map showing the positive correlation between Kaiso expression and TGFβ signaling proteins. (**b**, **c**) Kaiso depletion attenuates TGFβR1 and TGFβR2 transcript and protein levels, as assessed by quantitative RT–PCR and immunoblot analysis, that is rescued upon re-expression of a sh-resistant Kaiso cDNA (**d**). β-Actin serves as a loading control. $*P < 0.05$, $**P < 0.005$.

Kaiso depletion attenuates TGFβ signaling and transcriptional responses

The TGFβR1 and TGFβR2 serine/threonine kinases are essential for activation of the TGFβ signaling cascade.[14,35,36] Hence, loss of either the expression or function of TGFβR1 or TGFβR2 perturbs TGFβ signaling.[37–40] As our Kaiso-depleted cells displayed decreased TGFβR1 and TGFβR2 expression, we hypothesized that suppressing Kaiso would attenuate TGFβ signaling. Indeed, Kaiso-depleted MDA-231 and Hs578T cells treated with recombinant human TGFβ1 had negligible levels of phosphorylated Smad2 (p-Smad2) that is indicative of active TGFβ signaling. This was in striking contrast to TGFβ1-treated MDA-231 and Hs578T control cells that exhibited increased p-Smad2 (Figure 4a). Consistent with our *in vitro* results, Kaiso-depleted MDA-231 and Hs578T mouse xenografts displayed reduced p-Smad2 expression *in vivo* compared with control MDA-231 and Hs578T xenografts (Figure 4b). To further validate the role of Kaiso in TGFβ-mediated signaling, we examined Kaiso-depletion effects on TGFβ-target gene expression. We chose *ANGPTL4* that is involved in TGFβ-mediated breast tumor cell homing to lungs[18] as both control MDA-231 and Hs578T cells displayed a proclivity for lung metastasis. Silencing Kaiso significantly reduced TGFβ-induced expression of *ANGPTL4* (Figure 4c). Similarly, Kaiso depletion also attenuated TGFβ induction of *ZEB1* (Supplementary Figure 2) that participates in TGFβ-mediated EMT.[41] Unexpectedly, we observed increased Kaiso (*ZBTB33*) transcript levels in response to TGFβ treatment in both cell lines (Supplementary Figure 3). This increase in Kaiso transcripts was abrogated by Kaiso-specific shRNA in Kaiso-depleted cells (Figure 4c). Persistent TGFβ treatment (1–24 h) also resulted in increased Kaiso protein levels that peaked at ~12 h in both cell lines (Figure 4d). Together, these results hint at a positive feedback loop between Kaiso expression and TGFβ signaling.

Kaiso binds the TGFβR1 and TGFβR2 promoter endogenously

As Kaiso depletion attenuated TGFβR1 and TGFβR2 expression, we next assessed whether Kaiso promotes TGFβ signaling through regulation of TGFβR1 and TGFβR2. We performed electrophoretic mobility shift assay analyses using purified GST-Kaiso-ΔPOZ fusion proteins as previously described,[42,43] and oligonucleotides derived from the *TGFβR1* (KBS 1–4) and *TGFβR2* (KBS1–4) promoters that each contains several KBS and/or CpGs (Tables 1 and 2). Kaiso bound the core KBS in proximal TGFβR2 oligonucleotides (TβR2-KBS-2, 3, 4) but not the distal TβR2-KBS1 probe (Figures 5a and b). However, Kaiso binding was abolished upon introduction of a point mutation in the core KBS in these probes (Figure 5b; Supplementary Figure 4). Surprisingly, despite the strong correlation between Kaiso and TGFβR1 expression in the TCGA BCa dataset, no binding was observed between Kaiso and any of the TβR1-KBS-1–4 probes even after methylation of the CpG sequences found in the *TGFβR1* (KBS2-4) probes (data not shown).

Chromatin immunoprecipitation (ChIP) experiments subsequently revealed that Kaiso bound the endogenous *TGFβR2* promoter containing core KBS in MDA-231 and Hs578T cells (Figure 5c). Intriguingly, despite no direct interaction between Kaiso and the minimal *TGFβR1* promoter region *in vitro*, we found that Kaiso associated with the *TGFβR1* promoter endogenously (Figure 5d). As the amplified *TGFβR1* promoter region contained a CpG dinucleotide in addition to several core KBS (Table 1), we repeated the ChIP–PCR experiments using chromatin from MDA-231 and Hs578T cells treated with the demethylating agent 5′-aza-cytidine. Demethylation slightly abolished binding of Kaiso to the *TGFβR1* promoter in MDA-231 cells but had no effect on Kaiso binding in Hs578T cells (Figure 5d). The specificity of Kaiso binding to the *TGFβR1* and *TGFβR2* promoters was confirmed using primers designed against a distal region of both promoters lacking KBS or CpG sites (Supplementary Figure 5). Collectively, these results implicate both *TGFβR1* and *TGFβR2* as Kaiso target genes, and suggest that Kaiso may regulate *TGFβR1* expression indirectly, whereas it may directly regulate *TGFβR2* expression.

High Kaiso and TGFβR1 expression correlates with poor survival in BCa patients

As the TGFβ pathway is highly implicated in BCa metastasis, we utilized the TCGA BCa dataset and correlated the expression levels of Kaiso, TGFβR1 or TGFβR2 with BCa survival. Consistent with Chen *et al.*,[44] high TGFβR1 (Supplementary Figure 6) but not high

TGFβR2 expression (data not shown) correlated with poor prognosis in BCa patients, although not significantly. Remarkably, increased Kaiso and TGFβR1 expression, but not increased Kaiso and TGFβR2 expression, correlated significantly with poor overall survival in BCa patients (Figures 6a and b). Kaiso may thus drive metastasis through TGFβR1 but not TGFβR2.

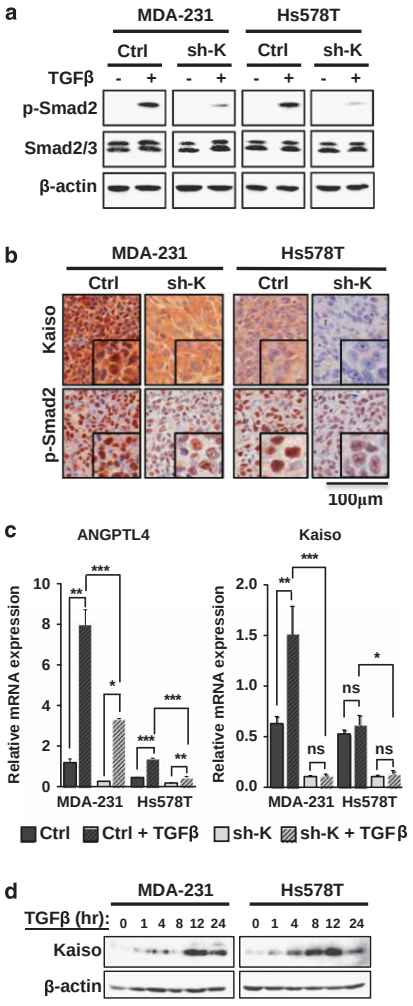

Figure 4. Kaiso-depletion attenuates TGFβ signaling and transcriptional responses. Cells were treated with 10 ng/ml of TGFβ for 1 h before assaying for TGFβ activity. (**a**) TGFβ treatment of control MDA-231 and Hs578T cells results in increased p-Smad2 levels. However, Kaiso-depleted MDA-231 and Hs578T cells treated with TGFβ display reduced p-Smad2 levels. (**b**) Kaiso-depleted MDA-231 and Hs578T xenografts exhibit decreased TGFβ signaling as evidenced by reduced p-Smad2 protein levels. (**c**) TGFβ-induced expression of *ANGPTL4* is attenuated in Kaiso-depleted cells treated with 10 ng/ml of TGFβ for 24 h. Interestingly, Kaiso expression is significantly increased by TGFβ treatment in MDA-231 cells. (**d**) Immunoblot analysis revealed a peak in Kaiso protein levels at 12 h in both MDA-231 and Hs578T cells in response to TGFβ treatment. All experiments were performed in triplicate. Representative images from all experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$, NS, not significant. β-Actin serves as a loading control.

Kinase-active TGFβR1 rescues TGFβ signaling but not the metastatic abilities of Kaiso-depleted MDA-231 cells

Based on the above findings, we questioned whether restoration of TGFβ signaling in Kaiso-depleted cells would restore their metastatic abilities. To address this, we overexpressed a constitutively kinase-active TGFβR1 (TRI^{204D}) in Kaiso-depleted MDA-231 and Hs578T cells. $T\beta RI^{204D}$ overexpression in Kaiso-depleted cells restored TGFβ signaling as evidenced by increased p-Smad2 and other non-Smad proteins (pAkt) compared with MDA-231-sh-K cells (Figure 7a). Remarkably, although TRI^{204D} overexpression restored TGFβ signaling, it was insufficient to restore the metastatic potential of the Kaiso-depleted cells (compare with metastatic foci generated by MDA-231-Ctrl cells in the lungs of injected mice) (Figure 7b). This suggested that Kaiso expression is important for TGFβ-mediated breast tumor metastasis.

DISCUSSION

Most cancer-related deaths are because of tumor metastasis to vital organs.[45] The recent association of Kaiso with EMT[19,20] coupled with its misexpression in several aggressive cancers (prostate, breast) implicates Kaiso in metastasis. In this study we report for the first time that Kaiso depletion attenuated the metastatic ability of highly invasive TNBC cells (MDA-231 and Hs578T) in mouse models of metastasis. As our *in vitro* studies showed that Kaiso-depleted cells underwent mesenchymal-to-epithelial transition and exhibited a more epithelial phenotype (that is, increased E-cadherin and ZO-1 but decreased Slug, ZEB1 and Vimentin expression), the effect of Kaiso depletion on the metastatic potential of breast tumor cells may be partially attributed to the attenuated EMT phenotype observed in these cells.

EMT is itself regulated by several distinct signaling pathways.[35] Thus, it was intriguing to find that Kaiso expression positively correlates with the expression of several members of the TGFβ signalsome. Importantly, Kaiso associates with proximal TGFβR1 and TGFβR2 promoter regions, and Kaiso depletion results in reduced TGFβR1 and TGFβR2 expression, and attenuated TGFβ signaling. Consequently, TGFβ-dependent activation of target genes like *ANGPTL4* and *ZEB1* that are known to promote tumor dissemination and invasiveness[18,46] was impaired by Kaiso silencing. As the TGFβ pathway is highly implicated in BCa metastasis, the effect of Kaiso depletion on the metastasis of MDA-231 and Hs578T cells may be due to attenuation of TGFβ signaling in these cells, that is, loss of Kaiso-dependent regulation of TGFβR1/2 expression.

Several studies suggest that expression levels of the TGFβ receptors (high vs low) may determine the biological specificity of the TGFβ signaling cascade and the differential activation of Smad vs non-Smad signaling pathways.[15–17] Our finding that Kaiso regulates expression of both TGFβR1 and TGFβR2 raises the possibility that Kaiso plays a central role in TGFβ-mediated tumorigenic effects. Consistent with this theory, our studies revealed that high Kaiso and TGFβR1 but not TGFβR2 expression is associated with poor overall survival in BCa patients. As metastasis accounts for poor overall survival in cancer patients, we surmise that Kaiso-dependent regulation of TGFβR1 but not TGFβR2 promotes TNBC metastasis.

Our unexpected finding that TGFβ treatment increased Kaiso expression in breast tumor cells suggests that TGFβ signaling may positively regulate Kaiso expression, and thus form a positive feedback loop that enhances TGFβ-mediated signaling and metastasis (Figure 8a). Intriguingly, Kaiso may itself be required for TGFβ signaling or participate in other pathways implicated in BCa metastasis as overexpression of a kinase-active TGFβR1 in Kaiso-depleted MDA-231 cells was insufficient to rescue their metastatic abilities. Such findings are consistent with our model (Figure 8b), and other studies that have implicated increased Kaiso

Table 1. Oligonucleotides representing different potential Kaiso-binding sites in the TGFβR1 promoter

TGFβR1 probe name	*Oligonucleotide sequence (5′–3′)*	*Location*
KBS-1 WT	CTGAT**TCCTGCTA**TCAAGGTTTA	−1212 to −1208
KBS-1 MUT	CTGAT**TCCT***T***CTA**TCAAGGTTTA	
KBS-2 WT	ATTTTGGCGTCG**CAG**AGGGAAGGTGGGTGGAGCGTCT**C**G**CAG**TAAATTAG	−1035 to −1002
KBS-2 MUT	ATTTTGGCGT**C***T***CAG**AGGGAAGGTGGGTGGAGCGTC**TC***T***CAG**TAAATTAG	
KBS-3 WT	GGTGCTGGGGC**TGGCAG**ACCCCGCC	−289 to −283
KBS-3 MUT	GGTGCTGGGGCG**GGCAG**ACCCCGCC	
KBS-4 WT	GCTGGGTCCCGCT**TGGCAG**CTCG	−117 to −111
KBS-4 MUT	GCTGGGTCCCGCT*G***GGCAG**CTCG	

Abbreviations: KBS, Kaiso-binding sequence; MUT, mutated; TGFβR1, transforming growth factor β receptor 1; WT, wild type. KBS—emboldened; CpG dinucleotides—underline; mutated nucleotides—italic.

Table 2. Oligonucleotides representing different potential Kaiso-binding sites in the TGFβR2 promoter

TGFβR2 probe name	*Oligonucleotide sequence (5′–3′)*	*Location*
KBS-1 WT	ATGGGCTGG**TGGCAG**AAGAGGGA	−1401 to −1395
KBS-1 MUT	ATGGGCTGG**TGG***A***AG**AAGAGGGA	
KBS-2 WT	CCCTTGCCT**CTGCAA**TCTTCCTC	−1081 to −1075
KBS-2 MUT	CCCTTGCCT**CT***T***CAA**TCTTCCTCT	
KBS-3 WT	TTACAGTTT**CTGCTA**TACTCTATA	−707 to −701
KBS-3 MUT	TTACAGTTT**CTG***A***TA**TACTCTATA	
KBS-4 WT	AAACATGAT**TGGCAG**CTACGAGA	−35 to −29
KBS-4 MUT	AAACATGAT**TGG***A***AG**CTACGAGA	

Abbreviations: KBS, Kaiso-binding sequence; MUT, mutated; TGFβR2, transforming growth factor β receptor 2; WT, wild type. KBS—emboldened; mutated nucleotides—italic.

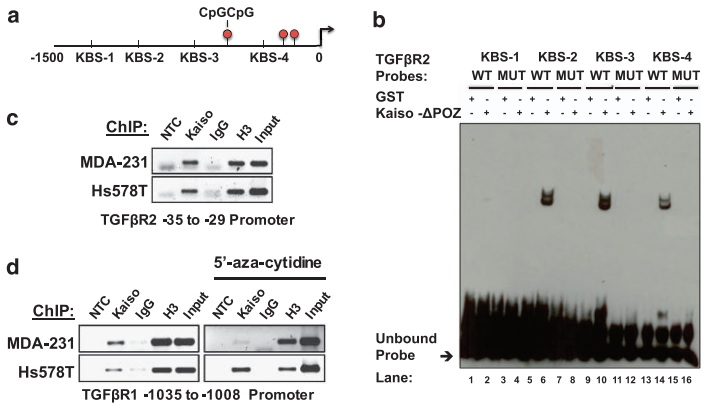

Figure 5. Kaiso associates with the endogenous TGFβR1 and TGFβR2 promoter in breast cancer cell lines. (**a**) Schematic illustration of the TGFβR2 promoter highlighting multiple KBS. Four double-stranded oligonucleotides were designed to contain core KBS from different regions of the promoter and utilized in electrophoretic mobility shift assay (EMSA) to determine Kaiso binding. (**b**) EMSA shows that Kaiso binds the proximal TGFβR2 promoter in a KBS-dependent manner (lanes 6, 10 and 14). This interaction was abolished (lanes 8, 12 and 16) upon introduction of a point mutation in the core KBS sequence of these probes or competition with the cold unlabeled wild-type probe. (**c**) ChIP of MDA-231 and Hs578T chromatin revealed that Kaiso binds the TGFβR2 promoter endogenously. (**d**) ChIP experiments of MDA-231 and Hs578T chromatin shows that Kaiso also interacts with the TGFβR1 promoter endogenously even after 5′-aza-cytidine treatment. Representative images are shown. All experiments were conducted in triplicate. H3, Histone 3 positive control; Input, 10% input. MUT, mutated; NTC, no template control; WT, wild type.

expression in the aggressiveness and overall survival of BCa patients.[20,29] However, it remains to be determined whether increased TGFβ signaling first induces high Kaiso expression or vice versa.

Collectively, these data implicate Kaiso as an important factor in TNBC aggressiveness and metastasis and suggest that it may be a relevant target for the development of therapies that will restrain the metastasis of aggressive breast cancers such as those of the TNBC subtype. Our finding that Kaiso can modulate TGFβ signaling further suggests that targeting Kaiso will alter the pro-metastatic phenotype associated with TGFβ signaling in advanced breast cancers.

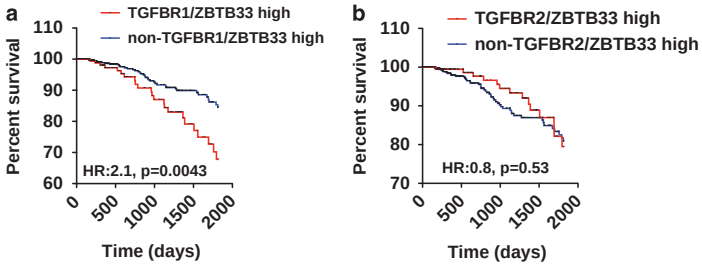

Figure 6. High Kaiso and TGFβR1 expression correlates with poor prognosis in breast cancer patients. (**a**) Kaplan–Meier survival curves show that high Kaiso and TGFβR1 expression correlates negatively with overall survival in the TCGA breast cancer data set. (**b**) High Kaiso and TGFβR2 expression does not correlate with overall survival in the TCGA breast cancer data set. Statistical significance was determined by log-rank test and *P*-values are indicated.

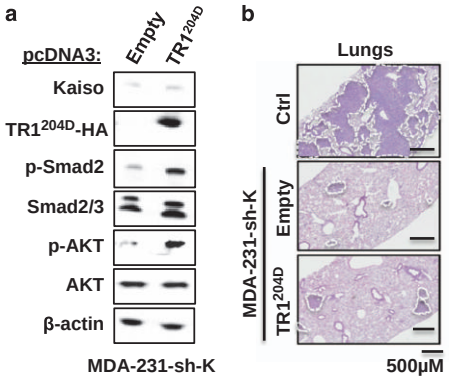

Figure 7. Re-expression of a constitutively active TGFβR1 in Kaiso-depleted cells is insufficient to restore breast cancer metastasis. (**a**) Overexpression of a constitutively active TGFβR1 ($TR1^{204D}$) in Kaiso-depleted cells restores TGFβ signaling as evidenced by increased levels of p-Smad2 and p-Akt. (**b**) Hematoxylin and eosin (H&E) staining shows that overexpression of kinase-active TGFβR1 in Kaiso-depleted cells did not restore the metastatic capabilities of the cells. Representative images are shown. β-Actin serves as a loading control.

MATERIALS AND METHODS

Cell culture

The human breast cancer cell lines MDA-231 and MCF-7 were obtained from ATCC (Manassas, VA, USA), and Hs578T were a generous gift from Dr John Hassell (McMaster University, Hamilton, Canada). All cell lines were cultured as previously described.[47] For all TGFβ treatments, 10 ng/ml of TGFβ1 (R&D Systems, Minneapolis, MN, USA) was used.

Generation of stable Kaiso-depleted cell lines

Kaiso depletion was achieved using a pRetroSuper (pRS) vector containing Kaiso-specific shRNAs (sh-Kaiso) that targeted the mRNA sequences, 5′-AAAAGATCATTGTTACCGATT-3′ referred to as sh-K1, or 5′-TTTTAACAT TCATTCTTGGGAGAAG-3′ referred to as sh-K2. Then, 6 μg of pRS-sh-Kaiso plasmid or control vector (pRS-Kaiso scrambled) were transfected into MDA-231 or Hs578T using the Turbofect transfection reagent (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. At 48 h post transfection, cells were treated with Puromycin (Invitrogen, Carlsbad, CA, USA) at 0.8 μg/ml (MDA-231) or 1.5 μg/ml (Hs578T) to select for stable Kaiso knockdown. Optimal Kaiso depletion was confirmed using immunoblot analysis of whole-cell lysates of individual clones, and clones exhibiting efficient Kaiso knockdown were selected for further studies.

Immunoblot analysis

Immunoblot analysis was performed as previously described.[48] Primary antibody incubations were performed overnight at 4 °C at the following dilutions: rabbit anti-Kaiso polyclonal (1:10 000); mouse anti-E-cadherin monoclonal (BD Biosciences, Mississauga, ON, Canada 610182; 1:1000); rabbit anti-ZO-1 polyclonal (Invitrogen 40-2200; 1:4000); rabbit anti-Vimentin polyclonal (Cell Signalling Technology (CST, Danvers, MA, USA) D21H3-XP); 1:1000); rabbit anti-ZEB1 polyclonal (Santa Cruz Technology, Dallas, TX, USA, H-102; 1:1000); rabbit anti-Slug polyclonal (CST-C19G7; 1:1000); rabbit anti-TGFβR1 monoclonal (CST-3712; 1:1000); rabbit anti-TGFβR2 polyclonal (Santa Cruz Technology sc-400; 1:2000); rabbit anti-phospho Smad2 monoclonal (Ser465/467) (CST-D43B4-XP; 1:800); rabbit anti-Smad2/3 monoclonal (CST-3102; 1:1000); mouse anti-β-actin monoclonal (Sigma Aldrich, Oakville, ON, Canada; 1:50 000). Primary antibody signals were amplified with horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in a 1:10 000 dilution before visualization using Clarity Western Enhanced Chemiluminescence Substrate and the Bio-Rad ChemiDoc MP imaging system (Bio-Rad Laboratories, Mississauga, ON, Canada). All immunoblot experiments were performed in triplicate.

Rescue experiments

A pCDNA3 vector expressing the sequence encoding the murine Kaiso cDNA (mKaiso) that is not targeted by the Kaiso-specific shRNA was utilized for Kaiso rescue experiments. Transient transfection of the pCDNA3 mKaiso vector into MDA-231-sh-K1 was achieved using Turbofect. At 3 weeks post transfection, whole-cell lysates obtained from the pCDNA3-mKaiso and pCDNA3-empty (control) transfected cells were subjected to immunoblot analysis of specified proteins after transient selection in media containing 0.8 μg Puromycin and 1000 μg Geneticin. For rescue of TGFβ signaling, constitutively active TGFβR1 ($TGF\beta R1^{T204D}$), hereafter $T\beta RI^{204D}$, was stably transfected into Kaiso-depleted MDA-231 cells using Turbofect. At 48 h post transfection, cells were treated with selection media containing 1000 μg Geneticin and Puromycin (Invitrogen) at 0.8 μg/ml to select for stable TRI^{204D}-overexpressing Kaiso-depleted clones. Total protein isolated from control and experimental ($T\beta RI^{204D}$) Kaiso-depleted cells was used for immunoblot analysis. Where applicable, all experiments were performed in triplicate.

Reverse transcription–PCR (RT–PCR)

RNA was isolated from control and Kaiso-depleted breast cancer cells using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA synthesis and RT–PCR analysis were performed using the Superscript One-Step RT–PCR with Platinum Taq kit (Invitrogen) and the primers are indicated in Table 3. RT–PCR reactions were performed using the Eppendorf–Thermal cycler (Eppendorf, Hauppauge, NY, USA) under the following conditions: reverse transcription at 50 °C for 30 min, followed by initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at the specified temperature as indicated in Table 3 for 30 s, extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Then, 10 μl of each RT–PCR reaction was electrophoresed on 1% agarose/ethidium bromide gels and images captured using the Bio-Rad ChemiDoc MP imaging system. All experiments were performed in triplicate.

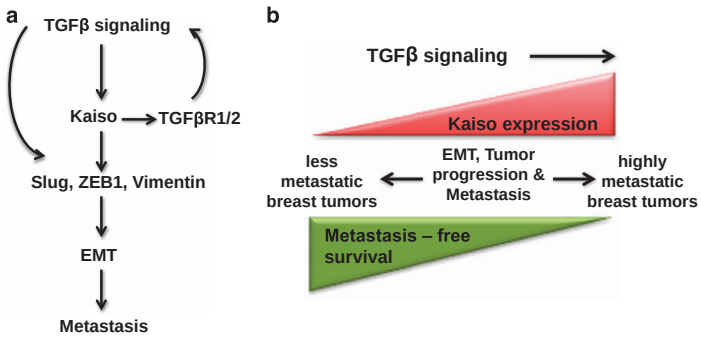

Figure 8. Potential model of the role of Kaiso in tumor progression and metastasis. (**a**) TGFβ signaling increases Kaiso expression that in turn promotes TGFβ signaling through increased expression of TGFβR1 and/or TGFβR2. TGFβ and Kaiso then promote EMT through increased expression of Slug, ZEB1 and/or Vimentin. (**b**) Less aggressive breast cancers exhibit low Kaiso expression, whereas highly metastatic breast tumors display high Kaiso expression, correlating with shorter metastasis-free survival. However, it remains to be determined whether high Kaiso expression occurs before tumor cells become highly aggressive or vice versa.

Table 3. List of primer sequences used for RT–PCR analysis with their annealing temperatures

Target		*Sequence (5′–3′)*	*Annealing temperature*
Kaiso	Forward	TGCCTATTATAACAGAGTCTTT	55 °C
	Reverse	AGTAGGTGTGATATTTGTTAAAG	
E-cadherin	Forward	CACCCTGGCTTTGACGCCGA	63 °C
	Reverse	AAACGGAGGCCTGATGGGGCG	
ZO-1	Forward	CGGGAAGTTACGTGGCGAA	60 °C
	Reverse	CTCCATTGCTGTGCTCTTGG	
Vimentin	Forward	TACGTGACTACGTCCACCCG	63 °C
	Reverse	ATCTCCTCCTGCAATTTCTCCC	
Slug	Forward	AGACCCCCATGCCATTGAAG	63 °C
	Reverse	CTTCTCCCCCGTGTGAGTTC	
ZEB-1	Forward	AGAATTCACAGTGGAGAGAAGCC	53 °C
	Reverse	CGTTTCTTGCAGTTTGGGCATT	
β-Actin	Forward	CTCTTCCAGCCTTCCTTCCT	55 °C

Abbreviation: RT–PCR, reverse transcription–PCR.

Table 4. List of primer sequences used for qRT–PCR analysis with their annealing temperatures

Target		*Sequence (5′–3′)*	*Annealing temperature*
Kaiso	Forward	AGAGGAAAGGGCATGGAGAGT	60.8 °C
	Reverse	GGCCACGTTGCTCATTCAAG	
TGFβR1	Forward	CCGTTTGTATGTGCACCCTC	60 °C
	Reverse	GCCAGGTGATGACTTTACAGTAGT	
TGFβR2	Forward	CTCGGTCTATGACGAGCAGC	60 °C
	Reverse	CCTCCATTTCCACATCCGACT	
ZEB-1	Forward	AGAATTCACAGTGGAGAGAAGCC	60 °C
	Reverse	CGTTTCTTGCAGTTTGGGCATT	
ANGPTL4	Forward	CAGCCTGCAGACACAACTCA	60 °C
	Reverse	ATTCGCAGGTGCTGCTTCTC	
β-Actin	Forward	CTCTTCCAGCCTTCCTTCCT	55 °C
	Reverse	AGCACTGTGTTGGCGTACAG	

Abbreviation: qRT–PCR, quantitative reverse transcription–PCR.

Quantitative RT–PCR

RNA (1 μg) isolated using the GeneJet RNA-plus isolation kit (Macherey-Negel) from control and TGFβ1-treated Kaiso-depleted cells was converted to cDNA using the qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD, USA) according to the manufacturer's protocols. For quantitative RT–PCR reactions, cDNA was amplified using the PerfeCta SYBR Green SuperMix ROX (Quanta BioSciences) as described in Pierre *et al.*,[43] with the primers indicated in Table 4. The expression of each target was determined using a standard curve and normalized to the expression levels of β-actin. Statistical significance (using *t*-test and one-way analysis of variance with Tukey's test where appropriate) was determined using data obtained from at least three trials.

Electrophoretic mobility shift assay

Double-stranded oligonucleotides corresponding to the specified KBS in the TGFβR1 and TGFβR2 promoters (see Table 1) were biotin-labeled using a Biotin 3′ End DNA Labeling kit (Pierce Biotechnology, Rockford, IL, USA) as per the manufacturer's protocol. TGFβR1 probes containing a CpG dinucleotide (KBS2–4) were also methylated with the CpG methyltransferase (M.SssI; New England Biolabs, Ipswich, MA, USA) as described in Pierre *et al.*[49] Following biotinylation, complementary oligonucleotides were annealed by heating to 90 °C for 1 min and then allowed to cool slowly to room temperature. The reaction was then frozen and stored at − 20 °C until use. Binding reactions were performed using 100 fmol of biotinylated double-stranded DNA probe and 200 ng of purified protein in 20 μl of binding buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 25% Glycerol, 1 mM dithiothreitol and Halt protease phosphatase inhibitor cocktail). To eliminate nonspecific binding, reaction mixtures were first incubated with 2 μg poly (deoxyinosinic-deoxycytidylic) acid (poly dI-dC) on ice for 1 h. Reaction mixtures containing biotinylated double-stranded DNA probe were incubated at room temperature for 30 min. For competition assays, a 100-fold excess (10 pmol) of unlabeled (cold) DNA was added. Reaction mixtures were loaded onto a 4.8% non-denaturing polyacrylamide gel and electrophoresed in 0.5× TBE at 100 V at 4 °C. Nucleic acids were

transferred onto a nylon membrane in 0.5× TBE and the membrane crosslinked using a 312 nm UV lamp for 10 min. Visualization was performed utilizing a horseradish peroxidase-conjugated streptavidin Chemiluminescent Nucleic Acid Detection Module kit (Pierce) and hyperfilm (GE Healthcare, Mississauga, ON, Canada, 28906839) according to the manufacturer's protocol. Where applicable, experiments were performed in triplicate.

ChIP and ChIP–PCR

MDA-231 and Hs578T cells were cultured to achieve ~80% confluency before chromatin isolation. Treatment with the demethylating agent, 5-azacytidine, ChIP and ChIP–PCR experiments were performed as previously described.[42,49] The following primers (−1035/−1008) KBS forward: 5′-AGGGCAAATTGGGACTGGAG-3′ and (−1035/−1008) KBS reverse: 5′-GAGGCCTGCAACTTGCTCTA-3′ at 65 °C, (−35/−29) KBS forward: 5′-CAGCTGAAAGTCGGCCAAAG-3′ and (−35/−29) KBS reverse: 5′-AGC CCCTAGCTCTCTCGTAG-3′ were used to amplify minimal TGFβR1 and TGFβR2 promoter regions respectively containing one or more core KBS (CTGCnA) and/or CpGs. The following primers were used as negative controls to confirm Kaiso binding specificity: (−2960/−2725) TGFβR1 negative-forward: 5′-GGAGCCTGGGAAATTGACAT-3′ and (−2960/−2725) TGFβR1 negative-reverse: 5′-CTCCAGTGCCTTGTACCCTG-3′, and (−2642/−2274) TGFβR2 negative-forward: 5′-TTGCCCAAGTTCCTCCAGAT-3′ and (−2642/−2274-) TGFβR2 negative-reverse: 5′-TTGCCCAAGTTCCTCCAGAT-3′.

Ethics statement and metastasis studies

All experiments with NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ or NOD SCID Gamma (NSG) mice were approved by the Animal Research Ethics Board at McMaster University (AUP Number 14-05-14) and performed in accordance with the guidelines of the Animal Research Ethics Board. Female and male NSG mice were purchased from Charles River (Wilmington, MA, USA). To study Kaiso depletion effects on *in vivo* breast tumor metastasis, we injected 4.5×10^6 Kaiso-shRNA or control-shRNA MDA-231 or Hs578T breast tumor cells in a Dulbecco's modified Eagle's medium/serum-free media/Matrigel mixture under the fourth mammary fat pad of the right abdominal mammary gland of ~5–8-week-old female NSG mice. No randomization was used in our studies as we used similar-aged pups obtained from the same breeding pair for each experiment. Most experiments were performed using at least five mice/treatment condition. Non-invasive monitoring of mice was performed weekly, and increased to 2–3 times weekly upon tumor appearance. Tumor growth was monitored externally with vernier calipers and tumor volume (in mm^3) measured using the following formula (length/2 × width^2) 2–3 times weekly. Mice were killed when tumor volume reached end point (~3300 mm^3), and necropsies performed blindly by a veterinary pathologist to detect macrometastases. Tissues were perfused and fixed in 10% formalin before harvest and histological examination.

Experimental metastasis studies

For experimental metastasis, 5×10^5 MDA-231 and 1×10^6 Hs578T control (Ctrl) and sh-Kaiso (sh-K) cells resuspended in 200 µl 1× phosphate-buffered saline (PBS) were injected into the tail veins of ~6-week-old female NSG mice, whereas 1×10^6 MDA-231 Ctrl, sh-Kaiso empty (sh-K-E) and TGFβR1^{204D} (sh-K-TR1^{204D}) cells were injected into ~6-week-old female NSG mice ($n=5$/cell line). Mice were killed 5–6 weeks post injection and harvested tissues embedded in paraffin before the preparation of 5 µm thick tissue sections on slides that were subsequently H&E stained.

Immunohistochemical staining of xenograft tissues

Harvested xenografts were embedded in paraffin before the preparation of 5 µm thick tissue sections on slides that were either stained with H&E, mouse anti-Kaiso 12H monoclonal (1:800)[50] or p-Smad2 (CST-138D4; 1:200 for MDA-231 xenografts and 1:50 for Hs578T xenografts) primary antibodies overnight at 4 °C. Briefly, xenograft tissues were dewaxed by warming on a slide warmer at 60 °C for 20 min followed by immersion in xylenes 3×5 min. All other steps were performed as previously described,[31] but we utilized PBS in place of TBS. Images were obtained using the Aperio Slide scanner (Leica Biosystems, Concord, ON, Canada).

Gene expression analysis of TCGA and GEO data sets

Level 3 IlluminaHiSeq_RNASeqV2 expression (Illumina, iNC., San Diego, CA, USA) and associated clinical data were downloaded for all available patients from the TCGA data portal[51] (19 March 2014; $n=977$). We used RSEM-quantified gene expression values to represent gene expression.[52] For consistency, we used transcript levels of the genes *ESR1* and *ERBB2* to assign estrogen receptor and HER2 status to each patient. Transcript profiling data from the GEO dataset, GSE20685 ($n=327$), was performed on Affymetrix U133 Plus 2.0 gene chips (Affymetrix, Santa Clara, CA, USA) and downloaded from the GEO website.[35] Robust Multi-Array was used to pre-process the dataset and gene expression values were calculated based on median expression of all probe sets mapping to a given gene based on Unigene ID. All genomic data processing was completed using R software.

Statistical analysis

All statistical tests were completed using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA), and $P<0.05$ indicated significance. Data are presented as means ± s.e.m. Unpaired Student's *t*-test was used for statistical analysis of two data sets, whereas one-way analysis of variance with Tukey/Newman–Keuls test was used for analysis of more than two data sets.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C *et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]*. International Agency for Research on Cancer: Lyon, France, 2013.
2 Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–752.
3 Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI *et al.* Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res* 2010; **12**: R68.
4 Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med* 2010; **363**: 1938–1948.
5 Cleere DW. Triple-negative breast cancer: a clinical update. *Commun Oncol* 2010; **7**: 203–211.
6 Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res* 2008; **68**: 989–997.
7 Karihtala P, Auvinen P, Kauppila S, Haapasaari KM, Jukkola-Vuorinen A, Soini Y. Vimentin, zeb1 and Sip1 are up-regulated in triple-negative and basal-like breast cancers: association with an aggressive tumour phenotype. *Breast Cancer Res Treat* 2013; **138**: 81–90.
8 Yamashita N, Tokunaga E, Kitao H, Hisamatsu Y, Taketani K, Akiyoshi S *et al.* Vimentin as a poor prognostic factor for triple-negative breast cancer. *J Cancer Res Clin Oncol* 2013; **139**: 739–746.
9 Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; **119**: 1420–1428.
10 Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* 2014; **16**: 488–494.
11 Akhurst RJ, Hata A. Targeting the TGFbeta signalling pathway in disease. *Nat Rev Drug Discov* 2012; **11**: 790–811.
12 Zhang YE. Non-Smad pathways in TGF-beta signaling. *Cell Res* 2009; **19**: 128–139.
13 Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997; **390**: 465–471.
14 Serra R, Crowley MR. TGF-beta in mammary gland development and breast cancer. *Breast Dis* 2003; **18**: 61–73.

15 Rojas A, Padidam M, Cress D, Grady WM. TGF-beta receptor levels regulate the specificity of signaling pathway activation and biological effects of TGF-beta. *Biochim Biophys Acta* 2009; **1793**: 1165–1173.
16 Pannu J, Gore-Hyer E, Yamanaka M, Smith EA, Rubinchik S, Dong JY *et al.* An increased transforming growth factor beta receptor type I:type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type II in scleroderma. *Arthritis Rheum* 2004; **50**: 1566–1577.
17 Wakefield LM, Roberts AB. TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* 2002; **12**: 22–29.
18 Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR *et al.* TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* 2008; **133**: 66–77.
19 Jones J, Wang H, Zhou J, Hardy S, Turner T, Austin D *et al.* Nuclear kaiso indicates aggressive prostate cancers and promotes migration and invasiveness of prostate cancer cells. *Am J Pathol* 2012; **181**: 1836–1846.
20 Jones J, Wang H, Karanam B, Theodore S, Dean-Colomb W, Welch DR *et al.* Nuclear localization of Kaiso promotes the poorly differentiated phenotype and EMT in infiltrating ductal carcinomas. *Clin Exp Metastasis* 2014; **31**: 497–510.
21 Daniel JM, Spring CM, Crawford HC, Reynolds AB, Baig A. The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. *Nucleic Acids Res* 2002; **30**: 2911–2919.
22 Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* 2011; **11**: 558–572.
23 Lloyd BH, Platt-Higgins A, Rudland PS, Barraclough R. Human S100A4 (p9Ka) induces the metastatic phenotype upon benign tumour cells. *Oncogene* 1998; **17**: 465–473.
24 Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K. Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. *Gut* 1999; **45**: 252–258.
25 Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 2008; **68**: 3645–3654.
26 Dai SD, Wang Y, Miao Y, Zhao Y, Zhang Y, Jiang GY *et al.* Cytoplasmic Kaiso is associated with poor prognosis in non-small cell lung cancer. *BMC Cancer* 2009; **9**: 178.
27 Lopes EC, Valls E, Figueroa ME, Mazur A, Meng FG, Chiosis G *et al.* Kaiso contributes to DNA methylation-dependent silencing of tumor suppressor genes in colon cancer cell lines. *Cancer Res* 2008; **68**: 7258–7263.
28 Prokhortchouk A, Sansom O, Selfridge J, Caballero IM, Salozhin S, Aithozhina D *et al.* Kaiso-deficient mice show resistance to intestinal cancer. *Mol Cell Biol* 2006; **26**: 199–208.
29 Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P *et al.* Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer. *PLoS ONE* 2012; **7**: e37864.
30 Zhou L, Zhong Y, Yang FH, Li ZB, Zhou J, Liu XH *et al.* Kaiso represses the expression of glucocorticoid receptor via a methylation-dependent mechanism and attenuates anti-apoptotic activity of glucocorticoids in breast cancer cells. *BMB Rep* (e-pub ahead of print 1 October 2015).
31 Chaudhary R, Pierre CC, Nanan K, Wojtal D, Morone S, Pinelli C *et al.* The POZ-ZF transcription factor Kaiso (ZBTB33) induces inflammation and progenitor cell differentiation in the murine intestine. *PLoS ONE* 2013; **8**: e74160.
32 Iorns E, Drews-Elger K, Ward TM, Dean S, Clarke J, Berry D *et al.* A new mouse model for the study of human breast cancer metastasis. *PLoS ONE* 2012; **7**: e47995.
33 Thompson EW, Paik S, Brünner N, Sommers CL, Zugmaier G, Clarke R *et al.* Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J Cell Physiol* 1992; **150**: 534–544.
34 Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med* 2006; **12**: 895–904.
35 Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* 2009; **19**: 156–172.
36 Ammanamanchi S, Brattain MG. Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells. *J Biol Chem* 2004; **279**: 32620–32625.
37 Portella G, Cumming SA, Liddell J, Cui W, Ireland H, Akhurst RJ *et al.* Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma in vivo: implications for tumor invasion. *Cell Growth Diff* 1998; **9**: 393–404.
38 Valcourt U, Kowanetz M, Niimi H, Heldin CH, Moustakas A. TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. *Mol Biol Cell* 2005; **16**: 1987–2002.
39 Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R *et al.* TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999; **103**: 197–206.
40 Ganapathy V, Ge R, Grazioli A, Xie W, Banach-Petrosky W, Kang Y *et al.* Targeting the Transforming Growth Factor-beta pathway inhibits human basal-like breast cancer metastasis. *Mol Cancer* 2010; **9**: 122.
41 Joseph JV, Conroy S, Tomar T, Eggens-Meijer E, Bhat K, Copray S *et al.* TGF-β is an inducer of ZEB1-dependent mesenchymal transdifferentiation in glioblastoma that is associated with tumor invasion. *Cell Death Dis* 2014; **5**: e1443.
42 Donaldson NS, Pierre CC, Anstey MI, Robinson SC, Weerawardane SM, Daniel JM. Kaiso represses the cell cycle gene cyclin D1 via sequence-specific and methyl-CpG-dependent mechanisms. *PLoS ONE* 2012; **7**: e50398.
43 Pierre CC, Longo J, Bassey-Archibong BI, Hallett RM, Milosavljevic S, Beatty L *et al.* Methylation-dependent regulation of hypoxia inducible factor-1 alpha gene expression by the transcription factor Kaiso. *Biochim Biophys Acta* 2015; **1849**: 1432–1441.
44 Chen C, Zhao K-N, Masci PP, Lakhani SR, Antonsson A, Simpson PT *et al.* TGFβ isoforms and receptors mRNA expression in breast tumours: prognostic value and clinical implications. *BMC Cancer* 2015; **15**: 1010.
45 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–674.
46 Drake JM, Strohbehn G, Bair TB, Moreland JG, Henry MD. ZEB1 enhances transendothelial migration and represses the epithelial phenotype of prostate cancer cells. *Mol Biol Cell* 2009; **20**: 2207–2217.
47 Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T *et al.* A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006; **10**: 515–527.
48 Donaldson NS, Nordgaard CL, Pierre CC, Kelly KF, Robinson SC, Swystun L *et al.* Kaiso regulates Znf131-mediated transcriptional activation. *Exp Cell Res* 2010; **316**: 1692–1705.
49 Pierre CC, Longo J, Bassey-Archibong BI, Hallett RM, Milosavljevic S, Beatty L *et al.* Methylation-dependent regulation of hypoxia inducible factor-1 alpha gene expression by the transcription factor Kaiso. *Biochim Biophys Acta* 2015; **1849**: 1432–1441.
50 Daniel JM, Ireton RC, Reynolds AB. Monoclonal antibodies to Kaiso: a novel transcription factor and p120ctn-binding protein. *Hybridoma* 2001; **20**: 159–166.
51 The Cancer Genome Atlas. Database: https://tcga-data.nci.nih.gov/tcga/, Retrieved 19 March 2014 (Internet).
52 Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 2011; **12**: 323.

Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/oncsis)

CHAPTER 5: KAISO & PULMONARY INTRAVASCULAR INVASION OF TNBC CELLS

Kaiso is highly expressed in TNBC tissues of women of African ancestry compared to Caucasian women.

Preface

This chapter describes the published manuscript titled: ***"Loss of Kaiso expression in breast cancer cells prevents intra-vascular invasion in the lung and secondary metastasis"*** by Kwiecien JM, Bassey-Archibong BI, Dabrowski W, Rayner LG, Lucas AR, and Daniel JM. ***(PLoS ONE, 2017; 12(9): e0183883)*** in its original form***.*** This is an open-access article distributed under the Creative Commons Attribution License, which allows unrestricted use, reproduction, and distribution in any medium, provided appropriate credit is given to the author and attribution parties, and sources cited.

This manuscript extensively describes the pathological findings of the effects of Kaiso depletion on the intra-vascular invasion and secondary metastasis of TNBC cells. We found that control TNBC cells formed macrometastases in the lungs and other distal organs including the heart and liver of xenografted mice. The control tumor masses in the lungs were further characterized by massive intravascular invasion of large blood vessels and formation of thrombi, which we postulate enhance the invasion of tumor cells into the blood vessel. In contrast, Kaiso depletion restricted the formation of large tumor masses in the lung

parenchyma, completely prevented the intravascular invasion of large blood vessels, and inhibited secondary metastases to distal organs. These findings led us to hypothesize that intravascular invasion of the lungs may promote secondary metastases to distant organs, and that the lungs may constitute a barrier for less invasive breast tumors such as the Kaiso-depleted TNBC cells.

Contributions:

Dr. J Kwiecien wrote the first draft of the manuscript and generated the Figures. BI Bassey-Archibong conducted all the experiments for the manuscript including the immunohistochemistry for E-cadherin, Vimentin, MMP-2 and MMP-9. LG Rayner conducted the immunohistochemistry for Kaiso. Dr. JM Daniel provided intellectual guidance during the study. Dr. J Kwiecien, BI Bassey-Archibong and Dr. JM Daniel co-wrote the final version of the manuscript. All authors contributed to the interpretation of the data and edited the manuscript text.

PLOS ONE

RESEARCH ARTICLE

Loss of Kaiso expression in breast cancer cells prevents intra-vascular invasion in the lung and secondary metastasis

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Abstract

The metastatic activity of breast carcinomas results from complex genetic changes in epithelial tumor cells and accounts for 90% of deaths in affected patients. Although the invasion of the local lymphatic vessels and veins by malignant breast tumor cells and their subsequent metastasis to the lung, has been recognized, the mechanisms behind the metastatic activity of breast tumor cells to other distal organs and the pathogenesis of metastatic cancer are not well understood. In this study, we utilized derivatives of the well-established and highly metastatic triple negative breast cancer (TNBC) cell line MDA-MB-231 (MDA-231) to study breast tumor metastasis in a mouse model. These MDA-231 derivatives had depleted expression of Kaiso, a POZ-ZF transcription factor that is highly expressed in malignant, triple negative breast cancers. We previously reported that Kaiso depletion attenuates the metastasis of xenografted MDA-231 cells. Herein, we describe the pathological features of the metastatic activity of parental (Kaiso^{positive}) versus Kaiso^{depleted} MDA-231 cells. Both Kaiso^{positive} and Kaiso^{depleted} MDA-231 cells metastasized from the original tumor in the mammary fat pad to the lung. However, while Kaiso^{positive} cells formed large masses in the lung parenchyma, invaded large pulmonary blood vessels and formed secondary metastases and large tumors in the distal organs, Kaiso^{depleted} cells metastasized only to the lung where they formed small metastatic lesions. Importantly, intravascular invasion and secondary metastases in distal organs were not observed in mice xenografted with Kaiso^{depleted} cells. It thus appears that the lung may constitute a barrier for less invasive breast tumors such as the Kaiso^{depleted} TNBC cells; this barrier may limit tumor growth and prevents Kaiso^{depleted} TNBC cells from invading the pulmonary blood vessels and forming secondary metastases in distal organs.

data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

An estimated 90% of medical fatalities in cancer patients are due to metastases [1]. Carcinomas of the mammary gland [2,3], prostate gland [4], liver [5–9], pancreas [10], endometrium [11], thyroid gland [12,13] and Merkel cell [14] have been shown to invade lymphoid vessels and blood vessels [2,6,8,9,11–14] resulting in metastases to distant organs, particularly the lung [7,9]. According to the systemic and pulmonary circulatory patterns, cancer cells that escape the primary tumor site and enter the bloodstream would first disseminate to the lung via the right ventricle before dispersing from the lung through the left heart to distal organs. Characterization of the mechanisms of malignant mammary carcinoma indicates that in the primary tumor, a small population of cells travel towards the blood vessels, and invade them in a complex process involving enhanced activity of genes regulating the dynamics of the actin cytoskeleton, e.g. Mena [2,15–17] and LIM kinase1 [18]. In a series of *in vivo* experiments in mouse and rat models utilising multiphoton microscopy, migrating tumor cells were seen to be assisted by perivascular macrophages in the process of vascular invasion and metastases to distant organs, which involved chemotaxis regulated by EGF and CSF-1 [1,15–17,19–23].

The progression of breast tumors towards an invasive behaviour and metastasis are postulated to involve several molecular factors associated with the complex epithelial-to-mesenchymal transformation (EMT) process that endows tumor cells with the ability to modulate their cell-cell adhesion and the extracellular matrix [24–26,47,48,50–52], apparently involved in the first line of defense against metastatic tumors. EMT is frequently accompanied by loss of the epithelial marker E-cadherin, concurrent with elevated expression of vimentin [41–45], an intermediate filament that participates in cell motility [46], as well as increased expression of matrix metalloproteases-2 and -9 (MMP-2, MMP-9) [27–32] that are often assessed in the determination of poor prognosis in breast cancer patients [33–36]. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) are known to activate pro-enzyme forms of MMP-2 and MMP-9 to active forms [37,38] and tPA and uPA have been proposed as markers for breast cancer progression [39,40].

Despite remarkable advancements in our understanding of cellular and molecular mechanisms involved in tumor growth and intravascular invasion at primary tumor sites, relatively little is known about how malignant tumors travel to distant organs. Since all lymph and venous blood from the body flows via the right heart ventricle to the lung, it is expected that most if not all primary metastases of carcinomatous tumors are trapped in small pulmonary blood vessels. However, the relevant questions regarding the pathogenesis of metastatic cancer remain; how do secondary metastases travel to other distant organs, and what are the mechanisms involved in the generation of these secondary metastases?

To gain insight into the mechanisms involved in breast tumor metastases to distant organs, we studied the pathogenesis of secondary metastases of parental, Kaiso^{positive} and experimental Kaiso^{depleted} MDA-231 TNBC cells in immunocompromised mice [53]. Kaiso is a dual-specificity transcription factor that is highly expressed in, and linked with the aggressive features of breast, prostate, colon and pancreatic carcinomas [54–57]. We previously reported that Kaiso depletion strongly inhibited the metastasis of TNBC cells to distal organs [53]. Herein, we describe histological analyses of the metastases generated by parental Kaiso^{positive} and Kaiso^{depleted} MDA-231 cells.

We found that Kaiso^{positive} MDA-231 breast cancer cells implanted into the mammary fad pad of immunocompromised mice [53] formed large tumor masses in the lung parenchyma, invaded large blood vessels and metastasised to other distant organs where it also formed large masses. In contrast, Kaiso^{depleted} tumor cells formed small clusters only in the lung parenchyma and did not invade blood vessels and did not metastasize to distant organs. While the

role of high Kaiso expression in the metastatic activity of human breast cancer cells was reported in our previous paper [53], we describe here the critical importance of high Kaiso expression in the propagation of breast tumors to distal organs beyond the lung, which we call "secondary metastases". We propose that the lung serves as the second line of defense against carcinomas with metastatic potential; tumors with less malignant characteristics such as Kaiso^{depleted} MDA-231 cells are trapped, do not progress and perhaps are eliminated. In contrast, malignant tumors such as Kaiso^{positive} MDA-231 cells thrive in the lung to form large masses that then, invade the large pulmonary blood vessels, travel via the left heart ventricle and lodge in small blood vessels of a variety of peripheral organs where they form secondary metastases.

Materials and methods

Generation of stable Kaiso-depleted MDA-231 cells

The MDA-231 TNBC cell line was purchased from the **A**merican **T**ype **C**ulture **C**ollection (ATCC) (Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza BioWhittaker®, Walkersville, MD, USA) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Logan, Utah, USA), 0.1 mg/mL penicillin/streptomycin and 0.25 g/mL Fungizone (Invitrogen, Grand Island, NY, USA). Cells were passaged every 2 or 3 days and grown in a 5% CO_2 humidified incubator at 37˚C. Stable Kaiso-depletion was achieved by using multiple Kaiso-specific **s**hort **h**airpin **RNA**s (shRNA) that target the Kaiso mRNA specifically as previously described [49]. A scrambled shRNA that does not target the Kaiso mRNA was used as a control. Stable control (Kaiso^{positive}) and Kaiso^{depleted} (sh-K) MDA-231 cells were maintained in DMEM-supplemented media treated with Puromycin (Invitrogen) at 0.8 μg/mL.

Western blot analysis

Stable control Kaiso^{positive} and Kaiso^{depleted} (sh-K1 and sh-K2) MDA-231 cells were cultured until ~80–90% confluent, washed twice with cold PBS, and then harvested by cell scraping into cold microfuge tubes. Control, sh-K1 and sh-K2 MDA-231 cells were then pelleted by centrifugation, lysed, and protein isolated as previously described [59]. Rabbit anti-Kaiso polyclonal (1:5,000 dilution; a generous gift from Dr. A. Reynolds), and mouse anti-β-actin monoclonal (1:50,000 dilution, Sigma Aldrich, Oakville, ON, Canada) primary antibody incubations were performed overnight at 4˚C. Secondary antibody incubations were performed with goat anti-rabbit- or donkey anti-mouse-horseradish peroxidase-conjugated secondary antibodies. (1;10,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Signals were then amplified with Clarity Western Enhanced Chemiluminescence substrate as previously described [53]. The sh-K2 MDA-231 cells, hereafter referred as sh-K or Kaiso^{depleted} MDA-231 cells, where chosen for all subsequent studies as these cells displayed the most efficient Kaiso knockdown.

Animal studies

All animal studies were approved and performed at McMaster University, Ontario, Canada according to the guidelines by the Canadian Council for Animal Care. Extensive description of animal studies has been outlined previously [53]. Briefly, 4.5 x 10^6 Kaiso^{positive} or Kaiso^{depleted} MDA-231 cells were injected subcutaneously into the mammary fat pad of 6–8 week old female **N**OD **S**CID **G**amma (NSG, Jackson Laboratories) mice (n = 5 each per condition), and allowed to form prominent subcutaneous masses up to 3,300 mm^3 in volume. Non-invasive

monitoring of mice was performed once a week, and increased to 2–3 times per week upon tumor appearance. Tumor growth was monitored externally with vernier calipers and tumor volume (in mm^3) measured using the following formula; length/2 × $width^2$, 2–3 times per week [53]. This tumor mass volume was achieved before the onset of serious clinical signs such as body weight loss, dehydration and lethargy that would require the application of the endpoint and euthanasia. The endpoint tumor volume of 3,300 mm^3 was determined in pilot experiments prior to the study. At endpoint, the mice were euthanized by overdosing with intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight), perfused when deeply anaesthetized, and fixed in 10% formalin and all routine tissues collected for histological examination.

Histology and Immunohistochemistry

Harvested and formalin-fixed tissues were processed, embedded in paraffin wax, and 5 μm thick tissue sections were then mounted on glass slides and stained with either hematoxylin & eosin (H&E) or Masson's trichrome. Immunohistochemical (IHC) analyses of tissue sections were performed as described previously [53]. Briefly, tissues were rehydrated in decreasing concentrations of alcohol, and deparaffinized in xylene before antigen retrieval by heating tissues in a sodium citrate solution (pH 6.0) in a microwave. Primary antibody incubations were performed overnight at 4˚C with the following antibodies: anti-Kaiso 6F mouse monoclonal (1:500), anti-Kaiso 12H mouse monoclonal (1:800) [58], anti-Vimentin rabbit monoclonal antibody (1:500; **C**ell **S**ignaling **T**echnology (CST), Danvers, MA, USA #5741), anti-E-cadherin mouse monoclonal antibody (1:50; BD Biosciences, Mississauga, ON, Canada 610182), anti-MMP-2 rabbit polyclonal antibody (1:1000; CST #4022BC), and anti-MMP-9 rabbit polyclonal antibody (1:1000; CST # 3852BC). Secondary antibody incubations were performed for 2 hours at room temperature with either biotinylated goat anti-mouse or donkey anti-rabbit antibody at a dilution of 1:1000. Negative controls were obtained by excluding primary antibody. Histological analysis of H&E, Masson's trichome and IHC-stained tissue sections were performed using a Nikon Eclipse 50 light microscope and representative phenotypes photographed.

Results and discussion

Protein expression

Expression of Kaiso in MDA-231 cells was abundant but remarkably reduced in sh-K1 (to 28%) and sh-K2 (to 6%) (see Fig 1) as the result of the stable transfection of the Kaiso-specific shRNA in these cells. Sh-K2 cells were utilized as $Kaiso^{depleted}$ in the xenograft studies.

Clinical observations and histological analyses

The subcutaneous masses in the mammary gland fat pad reached the endpoint volume of 3,300 mm^3 within 8 weeks in $Kaiso^{positive}$ tumors and 12 weeks in $Kaiso^{depleted}$ tumors [60]. Further characterization of the $Kaiso^{positive}$ and $Kaiso^{depleted}$ MDA-231 phenotypes revealed that the difference in tumor growth was due to Kaiso-depletion effects on cell proliferation, anchorage-independence and apoptosis [60].

The injection of the $Kaiso^{positive}$ and $Kaiso^{depleted}$ MDA-231 cells into the mammary fat pad of immunocompromised mice resulted in the formation of large subcutaneous masses (Fig 2Ai and 2Aiii) formed by large, pleomorphic cells with high mitotic index (Fig 2Aii and 2Aiv). Primary tumor masses formed by both types of mammary carcinoma cells were morphologically indistinguishable from each other (Fig 2A). Veins and lymphatic vessels in

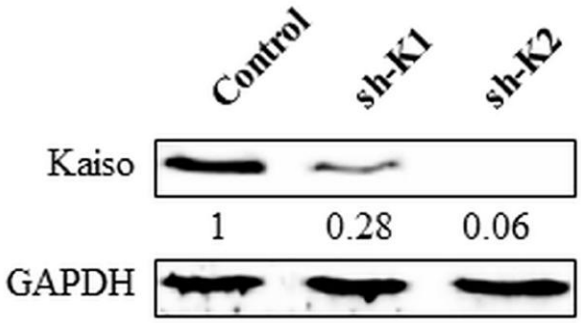

Fig 1. Kaiso^{depleted} MDA- 231 cells express negligible Kaiso compared to parental Kaiso^{positive} cells. Kaiso expression levels were determined using western blot. Both Kaiso^{depleted} clones (sh-K1 & sh-K2) expressed little Kaiso compared to the Kaiso^{positive} MDA-231 cells.

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vicinity to some subcutaneous masses were distended with clusters or single tumor cells scattered throughout the lumen (Fig 2B).

In the lung, Kaiso^{positive} cells formed large, often coalescing, non-circumscribed tumor masses with obliteration of the alveolar architecture (Fig 3A) formed by large, pleomorphic cells with a high mitotic index. Scattered neutrophils infiltrated the periphery of the tumor masses and the surrounding alveolar tissue. A proportion of large blood vessels encompassed by or

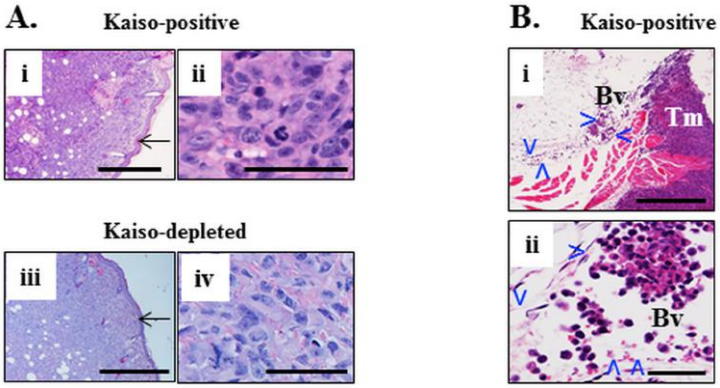

Fig 2. Primary subcutaneous tumors formed by Kaiso^{positive} and kaiso^{depleted} cells with invasion of the lumen of surrounding veins. Subcutaneous tumor mass of Kaiso^{positive} MDA-231 human mammary carcinoma cells (Ai, ii) and Kaiso^{depleted} tumor cells (Aiii, iv)) implanted into the fat pad of the mammary gland of female NRG mice. Tumor cells abut against the epidermis (arrow in Ai, iii) but do not invade it. Tumor cells are large, markedly pleomorphic, there is high mitotic index. (Bi) A vein (Bv, delineated by arrowheads) is adjacent to the subcutaneous tumor mass (Tm). It is distended by clumps and individual large pleomorphic cells (Bii) and also has scattered red blood cells. H&E. Size bars Ai, ii, Bi– 500 microns; Aii, iv, Bii– 50 microns.

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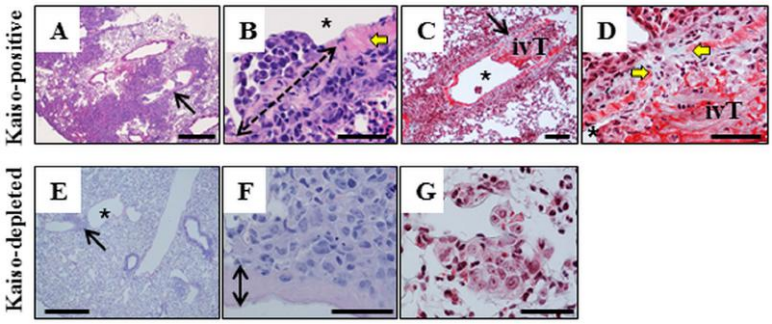

Fig 3. Metastasis of Kaiso^{positive} and Kaiso^{depleted} cells to the lung results in dramatically different tumor behavior. (A-D) Kaiso^{positive} tumors are numerous, large, obliterate the architecture of the pulmonary tissue and invade the lumen of large blood vessels (arrow in A). A segment of the vascular wall indicated by yellow arrows (B, D) is obliterated by tumor cells (double-headed interrupted arrow in B) that provide continuity between a perivascular mass and intravascular tumors (ivT) in the lumen (asterix in B, C). Apparent vascular invasion is associated with formation of intravascular thrombus (C, D). Intravascular surface of tumors or tumor thrombi is typically lined by endothelial cells (B). Tumor cells in intravascular masses or thrombi are large and pleomorphic (B, D). Kaiso^{depleted} cells form small interstitial aggregations (arrow in E) of large pleomorphic cells (F, G) that do not invade the wall or the lumen (F) of adjacent blood vessels. H&E–A, B, E, F; Masson's trichrome (C, D, G). Size bars; A, E– 500 microns, B-D, F, G– 50 microns.

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adjacent to tumors (Fig 3A) had masses of tumor cells protruding into the lumen, with the segmental concurrent obliteration of the vascular wall by tumor cells forming a continuity of perivascular and intravascular tumors (Fig 3B–3D). The apparent vascular invasion of Kaiso^{positive} tumors was often associated by formation of thrombi infiltrated by tumor cells (Fig 3D). In contrast, Kaiso^{depleted} tumor cells formed small aggregations scattered in the alveolar parenchyma (Fig 3E and 3G), sometimes adjacent to large blood vessels but with no invasion of the vascular wall or the lumen (Fig 3F). Kaiso^{depleted} tumor aggregations were often infiltrated by scattered neutrophils (Fig 3G).

Although Kaiso^{positive} and Kaiso^{depleted} cancer cells both formed subcutaneous masses and invaded nearby veins and lymphatics resulting in pulmonary metastases, Kaiso-depletion markedly supressed the ability of cancer masses to grow expansively in the lung, and eliminated their ability to invade blood vessels thereby restricting their spread to other organs. Indeed, tumors were not observed in any other organ except in the lung of mice injected with Kaiso^{depleted} cells. In contrast, in mice injected with Kaiso^{positive} cells, large tumors were observed in the liver (Fig 4A and 4B), kidney (Fig 4C and 4D), myocardium (Fig 4E and 4F), and infrequently in the adrenal gland and leptomeninges of the brain (not shown). Thus, Kaiso appear to play a regulatory role to in the; (i) expansive growth of metastatic tumors in the lung and (ii) invasion of the pulmonary blood vessels to spread to other organs supports the notion of Kaiso as a crucial factor in highly aggressive subtypes of breast cancer [53,54,60]. Our findings suggest that Kaiso could be a target for therapeutic strategies in the treatment of aggressive breast cancers. Some large blood vessels within a tumor mass or adjacent to it, such as in the liver (Fig 4A), or in kidney (Fig 4C) had intraluminal invasion of tumor cells with formation of thrombus and obliteration of the adjacent segment of the wall of the blood vessel (Fig 4B and 4D). In the myocardium, protrusions of the tumor cells into the lumen of ventricles (Fig 4E) resulted in the formation of fibrinous thrombus (Fig 4F) infiltrated by tumor

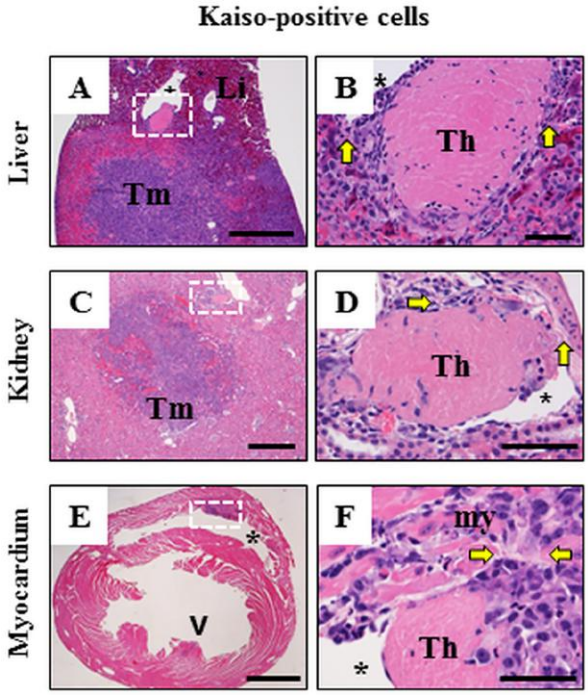

Fig 4. Intravascular invasion of secondary metastatic Kaiso^{positive} tumors. Low magnification images (A, C, E) and high magnification images (B, D, F) of tissue regions outlined by white dotted lines in A, C and E. Kaiso^{positive} cells metastatic to the liver (A, B) and kidney (C, D) formed large tumors and invaded adjacent blood vessels with formation of thrombi (Th in B, D) delineated from the surrounding tissue by yellow arrows. Tumor masses in the myocardium (white box and arrowhead in E) often resulted in invasion of the ventricle (asterix) with formation of a mass (white box in E) and thrombus (Th in F). Thrombus is delineated from myocardium (my) by yellow arrows in F. There is continuity between the masses of tumor cells in the myocardium and in the intraventricular thrombus (F). H&E. Size bars; A, C, E– 1,000 microns, B, D, F– 50 microns.

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cells. Large, sometimes coalescing tumor masses obliterated the organ architecture and were composed of large pleomorphic cells similar morphologically and immunohistochemically to those described in the subcutaneous mass and in the lung of mice injected with Kaiso^{positive} cells.

Intravascular (Fig 5A) or cardiac intraventricular (Fig 5B) invasion by the Kaiso^{positive} tumor cells often resulted in formation of a thrombus whose surface in some cases was apparently covered by endothelium (Fig 5Aii and 5Bii), delineating the remaining lumen of the blood vessel or the ventricle. Other thrombi however, where not delineated by endothelium but often by a layer of neutrophils (Fig 5Aiii and 5Biv). In some blood vessels adjacent areas of thrombi were or were not endothelialized while in others apparently endothelium-free small clusters or individual cancer cells were present in the lumen (Fig 5Aiii).

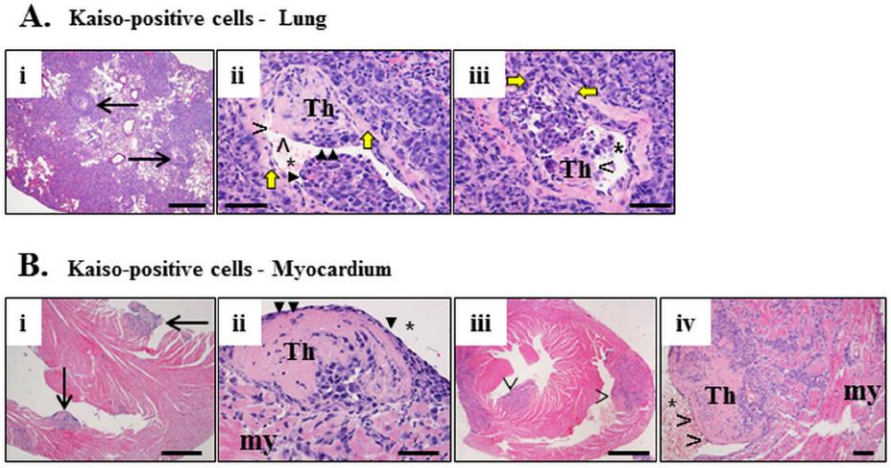

Fig 5. Thrombosis caused by Kaiso^{positive} tumors invading the blood vessels and heart ventricles. In the lung (A), a number of large blood vessels (two indicated by arrows) have intravascular thrombi delineated from the vascular wall by yellow arrows and protruding in the vascular lumen (Th in Aii, iii). The thrombi are infiltrated by neoplastic cells and are lined by endothelium (solid arrowheads in Aii) or not (open arrowhead in Aiii). In the myocardium (my, B) thrombi protruding into the ventricular lumen (Bi, iii) are also infiltrated by neoplastic cells (Th in Bii, iv) and either lined by endothelium (solid arrowheads in Bii) or not (open arrowheads in Biv). H&E. Size bars; Biii– 1,000 microns, Ai, Bi– 500 microns, Aii, iii, Bii, iv B– 50 microns.

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The above data indicate that the subcutaneously implanted Kaiso^{positive} MDA-231 cells efficiently penetrated the vascular wall and invaded the lumen of large blood vessels in the lung and other organs (Figs 4 and 5). This is in line with other studies that have also demonstrated vascular taxis and intravascular invasion of breast tumors implanted subcutaneously into mice [1,15–17,19–23]. The active penetration of the vascular wall by Kaiso^{positive} cells lead to their accessing of the lumen with frequent formation of thrombus. This pathogenesis implies two potential mechanisms; (1) tumor cells breached the endothelium of the tumor–invaded blood vessel, which may have led to thrombosis; (2) the tumor cells then invaded the thrombus as the convenient substrate, which lead to the increase of the intravascular load of cancer cells destined to metastasize to other organs. We also observed endothelium lining of the intravascular tumor masses with or without thrombosis. We consider that neo-endothelialization of the intravascular tumor masses and tumor thrombi may serve as a defense mechanism preserving the patency of the blood flow. We thus postulate that this putative defense mechanism may actually be subverted by the invading tumor cells allowing for the increase of their intravascular load and presumably leading to a greater chance of success of secondary metastases.

Immunohistochemical analyses

Kaiso^{positive} primary tumor tissues, as expected, stained positive for Kaiso (Fig 6A), which localized to both the nucleus and cytoplasm. Further analysis of the Kaiso^{positive} tumor tissues for other molecular markers implicated in tumor metastasis revealed that similar to our previous observations *in vitro* [53], Kaiso^{positive} MDA-231 primary tumor tissues stained moderately for Vimentin (Fig 6B) and negative for E-cadherin (Fig 6C). We also examined the Kaiso^{depleted}

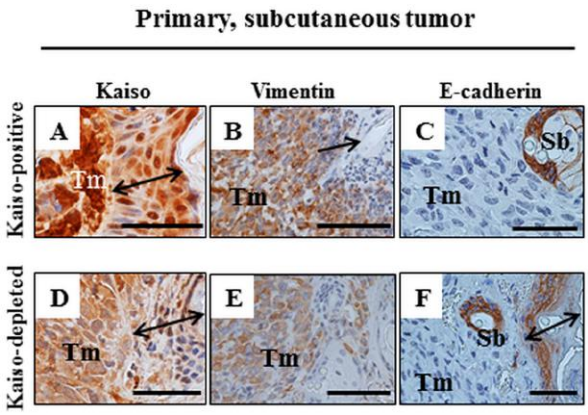

Fig 6. IHC of primary subcutaneous Kaiso^{positive} and Kaiso^{depleted} MDA-231 tumors. Tumor cells (Tm) of Kaiso^{positive} (A-C) and Kaiso^{depleted} (D-F) masses do not invade the epidermis (double-headed arrow in A, D, F, arrow in B). Kaiso^{positive} tumor cells are labeled strongly positive for Kaiso (A) and vimentin (B) while the Kaiso^{depleted} cells are labelled considerably less (D, E). The labeling with anti-E-cadherin antibody is negative for both types of tumor cells in contrast to the positive labelling of the mouse epithelium in sebaceous glands (Sb in C, F) and in epidermis (F). Size bars A-F– 50 microns.

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primary tumor tissues for Kaiso, Vimentin and E-cadherin expression. While we expected little to no Kaiso staining in the Kaiso^{depleted} MDA-231 tumors as per our western blot results (Fig 1), we were surprised to observe weak Kaiso staining in the primary tumors which was predominantly cytoplasmic (Fig 6D). The weak Kaiso staining could be due to deselection of the Kaiso^{depleted} MDA-231 cells as the mice were not subjected to constant treatment with Puromycin that would ensure selection and maintenance of shRNA plasmid in the stable cells *in vitro*. Nonetheless, the Kaiso staining observed in Kaiso^{depleted} tumor tissues was still remarkably reduced compared to the staining observed in the Kaiso^{positive} tumor tissues (Fig 6A and 6D). Kaiso^{depleted} MDA-231 tumor tissues also stained weakly for Vimentin (Fig 6E) as expected from *in vitro* findings in our previous report [53]. In contrast, while we had observed increased E-cadherin expression in the Kaiso^{depleted} MDA-231 cells *in vitro* [53], the Kaiso^{depleted} MDA-231 tumor tissues stained negative for E-cadherin (Fig 6F). This lack of E-cadherin staining could be due to the weak levels of Kaiso expression observed in the Kaiso^{depleted} MDA-231 primary tumors (Fig 6D) or due to other *in vivo* factors in the tumor microenvironment that are independent of Kaiso's expression or Kaiso's effect on E-cadherin expression.

Analysis of the tumor masses observed in the lung (Fig 7A) of mice injected with the Kaiso^{positive} and Kaiso^{depleted} MDA-231 cells also revealed that the Kaiso^{positive} tumor cells that metastasized to the lung exhibited strong Kaiso (Fig 7Ai) and Vimentin (Fig 7Aii) staining but no E-cadherin staining (Fig 7Aiii), while Kaiso^{depleted} tumor metastases in the lung displayed weak Kaiso (Fig 7Aiv) and Vimentin (Fig 7Av) staining, but no E-cadherin staining (Fig 7vi). These findings suggest a persistence of the molecular phenotype of both Kaiso^{positive} and Kaiso^{depleted} MDA-231 cells as they metastasized to the lung. We further analyzed the Kaiso^{positive} and Kaiso^{negative} MDA-231 lung metastases for the expression of MMP-2 and MMP-9.

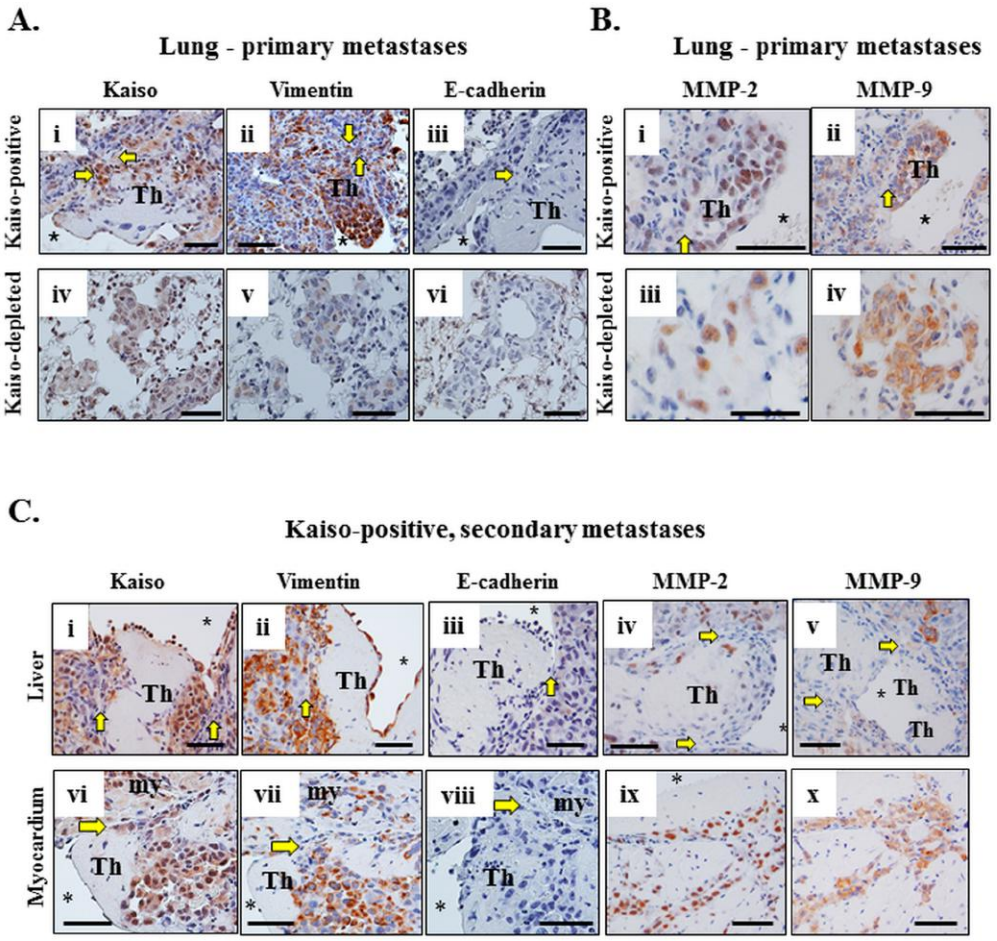

Fig 7. The molecular phenotype of the Kaiso^{positive} MDA-231 cells persist as they metastasize to other distal organs (liver and myocardium). (A) Neoplastic Kaiso^{positive} cells in lung metastases or thrombi are large, pleomorphic, and stain positive for Kaiso (i) and vimentin (ii), but negative for E-cadherin (iii). In contrast, Kaiso^{depleted} tumor cells are weakly stained for Kaiso (iv) and Vimentin (v) and negative for E-cadherin (vi). (B) Kaiso^{positive} tumor cells in lung metastases also stain strongly for MMP-2 (i), and MMP-9 (ii), while the Kaiso^{depleted} tumor cells stain weakly for MMP-2 (iii), but positive for MMP-9 (iv). The asterisk indicates the lumen of the blood vessel with the thrombus (Th) and the yellow arrows indicate the vascular wall and its obliteration by tumor cells in (A, B). (C) The thrombus (Th) formed in the large blood vessel of the liver (i-v) and in the ventricle of the heart (vi-viii) in mice injected with Kaiso^{positive} cells partially obliterated the vascular wall or endocardium indicated by the yellow arrows (i-viii). Neoplastic cells are stained positive for Kaiso (i, vi), vimentin (ii, vii), MMP-2 (iv, ix), and MMP-9 (v, x), and negative for E-cadherin (iii, viii). Scale bars; A-C– 50 microns.

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Although, MMP-2 and MMP-9 had not previously been examined in the Kaiso^{positive} and Kaiso^{negative} MDA-231 cells *in vitro*, they have been implicated in EMT and breast cancer progression [61–63]. Consistent with the EMT phenotype in the Kaiso^{positive} tumor cells, Kaiso^{positive} lung metastases displayed strong MMP-2 and MMP-9 expression (Fig 7Bi and 7Bii). In contrast, Kaiso^{depleted} lung metastases displayed reduced MMP-2 (Fig 7Biii) but not MMP-9 (Fig 7Biv) expression.

Examination of the Kaiso^{positive} tumor cells in secondary metastases (Fig 7C) also revealed strong Kaiso (Fig 7Ci) and Vimentin (Fig 7Cii) staining but no E-cadherin staining (Fig 7Ciii) in liver metastases. Similar to the lung metastases, the Kaiso^{positive} liver metastases also displayed strong MMP-2 (Fig 7Civ) and MMP-9 (Fig 7Cv) staining. Likewise, the Kaiso^{positive} tumor masses in the myocardium displayed strong Kaiso (Fig 7Cvi) and Vimentin (Fig 7Cvii) staining but no E-cadherin staining (Fig 7Cviii), as well as strong MMP-2 (Fig 7Cix) and MMP-9 (Fig 7Cx) staining. These findings also imply a persistence of the molecular phenotype of the Kaiso^{positive} MDA-231 cells as they metastasized to other distal organs.

In this study we analyzed the metastatic progression of Kaiso^{positive} and Kaiso^{negative} malignant mammary carcinomas using in vivo transplantation experiments in a mouse model. We found that Kaiso^{Positive} and Kaiso^{negative} MDA-231 cancer cells both formed subcutaneous masses and invaded nearby veins and lymphatics apparently leading to pulmonary metastases. However, Kaiso-depletion was associated with remarkable suppression of the growth of cancer cells in the lung. In contrast, high Kaiso-expressing tumor cells thrived in the lung, invaded large pulmonary blood vessels and metastasized to other organs. These findings suggest that Kaiso plays a key role in metastatic activity of MDA-231 cancer cells. The penetration of the vascular wall and invasion of the lumen of large blood vessels abundant in the lung and other organs appears to be the fundamental factor of malignancy of the Kaiso^{positive} tumor cells. This is in line with other studies that have also demonstrated vascular taxis and intravascular invasion of breast tumors implanted subcutaneously into mice [1,15–17,19–23]. Immunohistochemical characterization of Kaiso^{positive} and Kaiso^{depleted} tumors in this study revealed that Kaiso^{positive} tumor cells exhibit more features associated with malignancy (increased Kaiso, Vimentin, MMP-2 and MMP-9 expression) than the Kaiso^{depleted} tumor cells, which only displayed increased MMP-9 expression. Moreover, Kaiso expression seem to correlate positively with Vimentin and MMP-2 but not MMP-9 expression. Indeed Kaiso-depletion resulted in decreased Vimentin and MMP-2 but not MMP-9 expression. The similar staining of MMP-9 and lack of E-cadherin staining in both Kaiso^{positive} and Kaiso^{depleted} tumor cells, which is a marker of EMT, may explain the ability of both cell types to metastasize to the lungs. However, considering that only the Kaiso^{positive} tumor cells were capable of surviving in the lungs, invading blood vessels and forming macrometastases in other distal organs, the higher Kaiso expression in concert with the increased Vimentin and MMP-2 expression could be considered as the critical determinants that allowed the Kaiso^{positive} tumors to thrive after metastasis to the lung, and other distant organs.

We thus propose that the lung serves as the second line of defense against carcinomas with metastatic potential where tumors with less malignant characteristics, e.g. with reduced expression of Kaiso, are trapped, do not progress and perhaps are eliminated. Consequently, secondary metastases to the distant organs are prevented. We further postulate, that malignant tumors such as human breast tumors with high Kaiso expression can overcome this defensive mechanism, thrive in the lung and form large masses whose cells invade the blood vessels, travel via the left heart ventricle to lodge in small blood vessels of a variety of peripheral organs, and initiate multiple secondary metastatic tumors leading to accelerated demise (Fig 8). If this hypothesis is proven to be correct, potential cancer-suppressive tissue mechanisms in the lung should be considered in the pathogenesis of cancer metastasis. Also, models used to evaluate

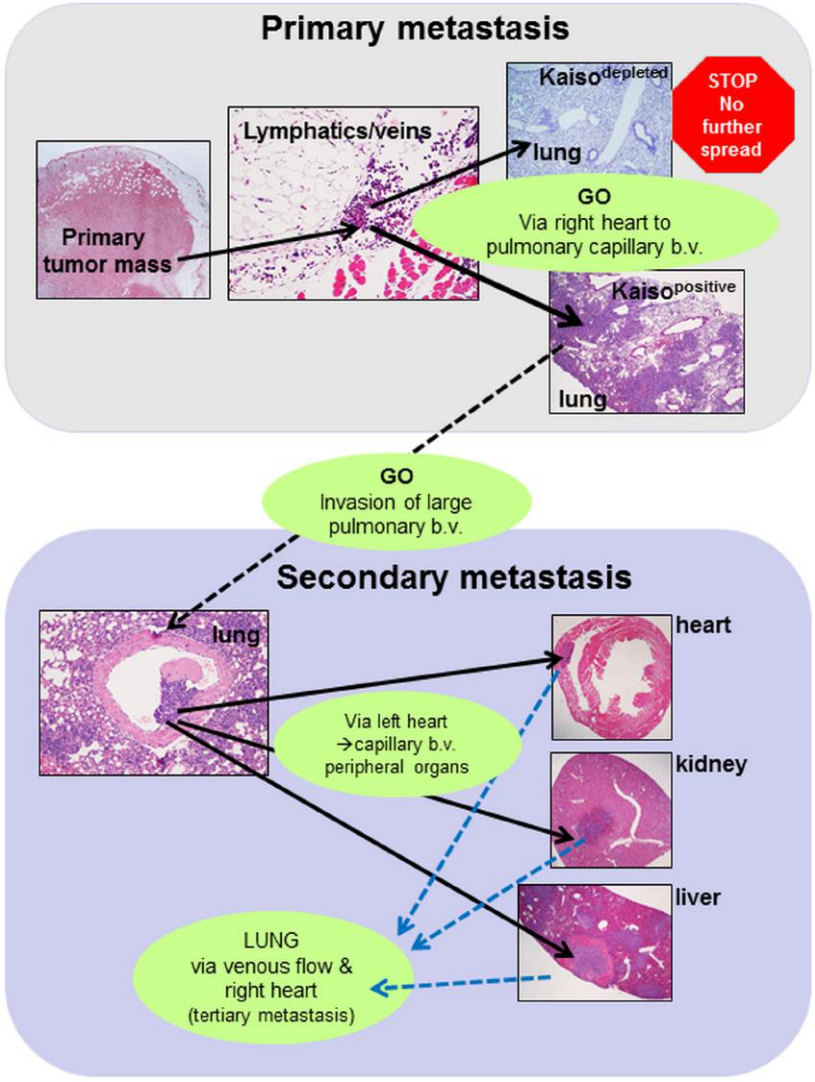

Fig 8. Conceptual pathogenesis of cancer metastasis. ***Primary metastasis***: Invasion of Kaiso^{positive} and Kaiso^{depleted} mammary carcinoma cells of the local veins and lymphatics allows the cells to migrate via the right heart ventricle to the lung where they are trapped in the capillary blood vessels (b.v.) and form pulmonary metastases. While in the lung, Kaiso^{positive} cells proliferate successfully and form large, coalescing masses that send the cells to actively cross the wall of adjacent blood vessels and invade their lumen. Kaiso^{depleted} tumor cells form small aggregations that do not invade blood vessels therefore

the secondary metastases do not occur. ***Secondary metastasis***: The intravascular invasion by the Kaiso^{positive} tumor cells in the lung presumably leads to its migration in the blood via the left heart to a variety of organs notably heart, liver and kidney, where they form metastases and tumors with the invasion of local blood vessels or heart ventricles in a fashion similar to that observed in the lung. This may lead to tertiary metastases; via the venous flow to the right heart and ultimately to the lung.

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the effectiveness of anti-cancer therapies should specifically include the analysis of the primary metastases in the lung and secondary metastases from the lung to other organs. The possibility of the lung as the second line of defence, with potential anti-cancer mechanisms sufficient to stall Kaiso^{depleted} but not Kaiso^{positive} breast cancer cells should be addressed in further studies.

Conclusions

In this report, we analyzed the metastatic progression of Kaiso^{positive} and Kaiso^{negative} malignant mammary carcinomas using *in vivo* transplantation experiments in a mouse model. Although this study utilizes the end point metastasis analysis of disseminated breast tumor cells, it highlights potential novel mechanisms involved in secondary metastases and provides detailed histological evidence of different behaviour of MDA-231 malignant breast cancer cells depending on the expression level of Kaiso. Both Kaiso^{positive} and Kaiso^{depleted} tumor types; (1) formed subcutaneous masses of cells with morphological features of malignancy; (2) invaded adjacent veins and lymphatic vessels; and (3) metastasized to the lung. However, while Kaiso^{positive} cells; (i) formed large pulmonary tumors; (ii) actively invaded pulmonary blood vessels apparently leading to (iii) secondary metastases and tumors in a variety of distal organs, Kaiso^{depleted} tumors formed only small aggregates in the lungs, did not invade pulmonary blood vessels and did not form secondary metastases. Thus, Kaiso may be a potent factor enabling breast cancer cells to overcome apparent inhibitory mechanisms in the lung and to send secondary metastases throughout distant organs.

Supporting information

S1 Checklist. ARRIVE.Guidelines.
(PDF)

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Writing – review & editing: Jacek M. Kwiecien, Blessing I. Bassey-Archibong, Wojciech Dabrowski, Alexandra R. Lucas, Juliet M. Daniel.

References

1. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331:1559–1564. https://doi.org/10.1126/science.1203543 PMID: 21436443
2. Di Modugno F, Mottolese M, Di Benedetto A, Conidi A, Novelli F, Perracchio L, et al. The cytoskeleton regulatory protein hMena (ENAH) is overexpressed in human benign breast lesions with high risk of transformation and human epidermal growth factor receptor-2-positive/hormonal receptor-negative tumors. Clin Cancer Res 2006; 12:1470–1478. https://doi.org/10.1158/1078-0432.CCR-05-2027 PMID: 16533770
3. Robinson BD, Sica GL, Liu YF, Rohan TE, Gertler FB, Condeelis JS, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. Clin Cancer Res 2009; 15:2433–2441. https://doi.org/10.1158/1078-0432.CCR-08-2179 PMID: 19318480
4. Dasgupta S, Wasson LM, Raunyar N, Prokai L, Borejdo J, Vishvanatha JK. Novel gene *C17orf37* in 17q12 amplicon promotes migration and invasion of prostate cancer cells. Oncogene 2009; 28: 2860–2872. https://doi.org/10.1038/onc.2009.145 PMID: 19503095
5. Chung JW, Park JH, Han JK, Choi BI, Han MC. Hepatocellular carcinoma and portal vein invasion: results of treatment with transcatheter oily chemoembolization. Am J Roentgenol 1995; 165:315–321.
6. Lee IJ, Chung JW, Kim H-C, Kim MU, Kim JD, Kim GM, et al. Extrahepatic collateral artery supply to the tumor thrombi of hepatocellular carcinoma invading inferior vena cava: the prevalence and determinant factors. J Vasc Interv Radiol 2009; 20:22–29. https://doi.org/10.1016/j.jvir.2008.09.030 PMID: 19026566
7. Nakashima T, Okuda K, Kojiro M, Jimi A, Yamaguchi R, Sakamoto K, et al. Pathology of hepatocellular carcinoma in Japan. 232 Consecutive cases autopsied in ten years. Cancer 1983; 51:863–877. PMID: 6295617
8. Quaglia A, Etessami N, Sim R, Difford J, Dhillon AP. Vascular invasion and herniation by hepatocellular carcinoma in cirrhosis: A wolf in sheep's clothing? Arch Pathol Lab Med 2005; 129:639–644. https://doi.org/10.1043/1543-2165(2005)129<0639:VIAHBH>2.0.CO;2 PMID: 15859635
9. Sugino T, Yamaguchi T, Hoshi N, Kusakabe T, Oguza G, Goodison S, et al. Sinusoidal tumor angiogenesis is a key component in hepatocellular carcinoma metastasis. Clin Exp Metastasis 2008; 25: 835–841. https://doi.org/10.1007/s10585-008-9199-6 PMID: 18712609
10. Kaneko T, Nakao A, Inoue S, Harada A, Nonami T, Itoh s, et al, Intraportal endovascular ultrasonography in the diagnosis of portal vein invasion by pancreatobiliary carcinoma. Ann Surg 1995; 222: 711–718. PMID: 8526577
11. Folkins AK, Nevadunsky NS, Sleemudin A, Jarboe EA, Muto MG, Feltmate CM, et al. Evaluation of vascular space involvement in endometrial adenocarcinomas: laparoscopic vs abdominal hysterectomies. Mod Pathol 2010; 23:1073–1079. https://doi.org/10.1038/modpathol.2010.91 PMID: 20473276
12. Al-Jarrah Q, Abou-Foul Ak, Heis H. Intravascular extension of papillary thyroid carcinoma to the internal jugular vein: A case report. Intl J Surg Case Rep 2014; 5:551–553.
13. Mete O, Asa SL. Pathological definition and clinical significance of vascular invasion in thyroid carcinomas of follicular epithelial derivation. Mod Pathol 2011; 24:1545–1552. https://doi.org/10.1038/modpathol.2011.119 PMID: 21804527
14. Kukko HM, Koljonen VSK, Tukiainen EJ, Haglund CH, Bohling TO. Vascular invasion is an early event in pathogenesis of Merkel cell carcinoma. Mod Pathol 2010; 23:1151–1156. https://doi.org/10.1038/modpathol.2010.100 PMID: 20473275

15. Philippar U, Roussos ET, Oser M, Yamaguchi H, Kim HD, Giampieri S, et al. A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. Dev Cell 2008; 15:813–828. https://doi.org/10.1016/j.devcel.2008.09.003 PMID: 19081071

16. Gertler F, Condeelis J. Metastasis: tumor cells becoming MENAcing. Trends Cell Biol 2011; 21:81–90. https://doi.org/10.1016/j.tcb.2010.10.001 PMID: 21071226

17. Roussos ET, Goswami S, Balsamo M, Wang Y, Stobezki R, Adler E, et al. Mena invasive (Mena(INV)) and Mena11a isoforms play distinct roles in breast cancer cell cohesion and association with TMEM. Clin Exp Metastasis 2011; 28:515–527. https://doi.org/10.1007/s10585-011-9388-6 PMID: 21484349

18. Wang W, Mouneinme G, Sidani M, Wyckoff J, Chen X, Makris A, et al. The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. J Cell Biol 2006; 173:395–404. https://doi.org/10.1083/jcb.200510115 PMID: 16651380

19. Condeelis J, Singer RH, Segall JE. The great escape: when cancer cells hijack the genes for chemotaxis and motility. Annu Rev Cell Dev Biol 2005; 21:695–718. https://doi.org/10.1146/annurev.cellbio.21.122303.120306 PMID: 16212512

20. Pignatelli J, Goswami S, Jones JG, Rohan TE, Pieri E, Chen X, et al. Invasive breast carcinoma cells from patients exhibit MenaINV—and macrophage-dependent transendothelial migration. Sci Signal 2014; 7:1–13.

21. Patsialou A, Bravo-Cordero JJ, Wang Y, Entenberg D, Liu H, Clarke M, et al. Intravital multiphoton imaging reveals multicellular streaming as a crucial component of in vivo cell migration in human breast tumors. IntraVital 2013; 2:e25295 1–15.

22. Wang W, Wyckoff JB, Frohlich VC, Oleynikov Y, Huttelmaier S, Zavadil J, et al. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. Cancer Res 2002; 62:6278–6288. PMID: 12414658

23. Wyckoff JB, Wang Y, Lin EY, Goswami S, Syanley ER, Segall JE, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res 2007; 67:2649–2656. https://doi.org/10.1158/0008-5472.CAN-06-1823 PMID: 17363585

24. Baker EA, Stephenson TJ, Reed MW, Brown NJ. Expression of proteinases and inhibitors in human breast cancer progression and survival. Mol Pathol 2002; 55:300–304. PMID: 12354933

25. Koshikawa N, Gianelli G, Cirulli V, Miyazaki K, Quaranta V. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol 2000; 148:615–624. PMID: 10662785

26. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003; 92:827–839. https://doi.org/10.1161/01.RES.0000070112.80711.3D PMID: 12730128

27. Asrani K, Keri RA, Galisteo R, Brown SA, Morgan SJ, Ghosh A, et al. The HER2- and heregulin β1 (HRG)-inducible TNFR superfamily member Fn14 promotes HRG-driven breast cancer cell migration, invasion, and MMP9 expression. Mol Cancer Res 2013; 11:393–404. https://doi.org/10.1158/1541-7786.MCR-12-0542 PMID: 23378579

28. Ding Z, Joy M, Bhargava R, Gunsaulus M, Lakshman N, Miron-Mendoza m, et al. Profilin-1 downregulation has contrasting effects on early vs late steps of breast cancer metastasis. Oncogene 2014; 33:2065–2074. https://doi.org/10.1038/onc.2013.166 PMID: 23686314

29. Jin H, Eun SY, Lee JS, Park SW, Lee JH, Chang KC, et al. P2Y2 receptor activation by nucleotides released from highly metastatic breast cancer cells increases tumor growth and invasion via crosstalk with endothelial cells. Breast Cancer Res 2014; 16:R77. https://doi.org/10.1186/bcr3694 PMID: 25156554

30. Leung HW, Wang Z, Yue GG, Zhao SM, Lee JK, Fung KP, et al. Cyclopeptide RA-V inhibits cell adhesion and invasion in both estrogen receptor positive and negative breast cancer cells via PI3K/AKT and NF-κB signaling pathways. Biochim Biophys Acta 2015; 1853:1827–1840. https://doi.org/10.1016/j.bbamcr.2015.04.020 PMID: 25953046

31. Perentes JY, Kirkpatrick ND, Nagano S, Smith EY, Shaver CM, Sgroi D, et al. Cancer cell-associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors. Cancer Res 2011; 71:4527–4538. https://doi.org/10.1158/0008-5472.CAN-10-4376 PMID: 21571860

32. Rizwani W, Schaal C, Kunigal S, Coppola D, Chellappan S. Mammalian lysine histone demethylase KDM2A regulates E2F1-mediated gene transcription in breast cancer cells. PLoS ONE 2014; 9: e100888. https://doi.org/10.1371/journal.pone.0100888 PMID: 25029110

33. Puzovic V, Brcic I, Ranogaiec I, Rakic-Razumovic J. Prognostic values of ETS-1, MMP-2 and MMP-9 expression and co-expression in breast cancer patients. Neoplasma 2014; 61:439–446. https://doi.org/10.4149/neo_2014_054 PMID: 24645837

34. Rabi ZA, Todorovic-Rakovicz N, Vujasinovic T, Milovanovic J, Nikolic-Vukosavljevic D. Markers of progression and invasion in short term follow up of untreated breast cancer patients. Cancer Biomark 2015; 15:45–54.

35. Van't Veer LJ, He YD, van de Viiver MJ, Dai H, Hart AA, Voskuil DW, et al. Gene expression profiling predicts clinical outcome of breast cancer. N Engl J Med 2002; 347:1999–2009.

36. Vucemilo T, Skoko M, Sarcevic B, Puljiz M, Alvir I, Turudic TP, et al. The level of serum pro-matrix metalloproteinase-2 as a prognostic factor in patients with invasive ductal breast cancer. Coll Antropol 2014; 38:135–140. PMID: 24851607

37. Hsu HH, Hu WS, Lin YM, Kuo WW, Chen LM, Chen WK, et al. JNK suppression is essential for 17β-Estradiol inhibits prostaglandin E2-Induced uPA and MMP-9 expressions and cell migration in human LoVo colon cancer cells. J Biomed Sci 2011; 18:61. https://doi.org/10.1186/1423-0127-18-61 PMID: 21859479

38. Hsu HH, Liu CJ, Shen CY, Chen YJ, Chen LM, Kuo WH, et al. p38α MAPK mediates 17β-estradiol inhibition of MMP-2 and -9 expression and cell migration in human lovo colon cancer cells. J Cell Physiol 2012; 227:3648–3660. https://doi.org/10.1002/jcp.24072 PMID: 22377968

39. Bouris P, Skandalis SS, Piperigkou Z, Afratis N, Karamanou K, Aletras AJ, et al. Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells. Matrix Biol 2015; 43:42–60. https://doi.org/10.1016/j.matbio.2015.02.008 PMID: 25728938

40. Maynadier M, Farnoud R, Lamy PJ, Laurent-Matha V, Garcia M, Rochefort H. Cathepsin D stimulates the activities of secreted plasminogen activators in the breast cancer acidic environment. Int J Oncol 2013; 43:1683–1690. https://doi.org/10.3892/ijo.2013.2095 PMID: 24026424

41. Han M, Liu M, Wang Y, Chen X, Xu J, Sun Y, et al. Antagonism of miR-21 reverses epithelial-mesenchymal transition and cancer stem cell phenotype through AKT/ERK1/2 inactivation by targeting PTEN. PLoS ONE 2012; 7:e39520. https://doi.org/10.1371/journal.pone.0039520 PMID: 22761812

42. Rhodes LV, Tate CR, Hoang VT, Burks HE, Gilliam D, Martin EC, et al. Regulation of triple-negative breast cancer cell metastasis by the tumor-suppressor liver kinase B1. Oncogenesis 2015; 4:e168. https://doi.org/10.1038/oncsis.2015.27 PMID: 26436950

43. Al Saleh S, Sharaf LH, Lugmani YA. Signalling pathways involved in endocrine resistance in breast cancer and associations with epithelial to mesenchymal transition (Review). Int J Oncol 2011; 38:1197–1217. https://doi.org/10.3892/ijo.2011.942 PMID: 21318221

44. Xu Y, Qin L, Sun T, Wu H, He T, Yang Z, et al. Twist1 promotes breast cancer invasion and metastasis by silencing Foxa1 expression. Oncogene 2016; https://doi.org/10.1038/onc.2016.286 PMID: 27524420

45. Yang E, Cisowski J, Nguyen N, O'Callaghan K, Xu J, Agarwal A, et al. Dysregulated protease activated receptor 1 (PAR1) promotes metastatic phenotype in breast cancer through HMGA2. Oncogene 2016; 35:1529–1540. https://doi.org/10.1038/onc.2015.217 PMID: 26165842

46. Thaiparambil JT, Bender L, Ganesh T, Kline E, Patel P, Liu Y, et al. Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. Int J Cancer 2011; 129:2744–2755. https://doi.org/10.1002/ijc.25938 PMID: 21538350

47. Li F, Zou Z, Suo N, Zhang Z, Wan F, Zhong G, et al. CCL21/CCR7 axis activating chemotaxis accompanied with epithelial-mesenchymal transition in human breast carcinoma. Med Oncol 2014; 31:180. https://doi.org/10.1007/s12032-014-0180-8 PMID: 25142946

48. Su P, Hu J, Zhang H, Li W, Jia M, Zhang X, et al. IMP3 expression is associated with epithelial-mesenchymal transition in breast cancer. Int J Clin Exp Pathol 2014; 7:3008–3017. PMID: 25031719

49. Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD, et al. Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Mol Cancer Res 2011; 9:997–1007. https://doi.org/10.1158/1541-7786.MCR-10-0490 PMID: 21665936

50. Cao YW, Wan GX, Sun JP, Cui XB, Hu JM, Liang WH, et al. Implications of the Notch1-Snail/Slug-epithelial to mesenchymal transition axis for lymph node metastasis in infiltrating ductal carcinoma. Kaohsiung J Med Sci 2015; 31:70–76. https://doi.org/10.1016/j.kjms.2014.11.008 PMID: 25645984

51. Fei F, Zhang D, Yang Z, Wang S, Wang X, Wu Z, et al. The number of polyploid giant cancer cells and epithelial-mesenchymal transition-related proteins are associated with invasion and metastasis in human breast cancer. J Exp Clin Cancer Res 2015; 34:158. https://doi.org/10.1186/s13046-015-0277-8 PMID: 26702618

52. Murakami E, Nakanishi Y, Hirotani Y, Ohni S, Tang X, Masuda S, et al. Roles of Ras homolog A in invasive ductal breast carcinoma. Acta Histochem Cytochem 2016; 49:131–140. https://doi.org/10.1267/ahc.16020 PMID: 27917007

53. Bassey-Archibong BI, Kwiecien JM, Milosavljevic SB, Hallett RM, Rayner LG, Erb MJ, et al. Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells. Oncogenesis 2016; 21: e208 1–10.

54. Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P, et al. Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer. PLoS ONE 2012; 7:e37864 1–9.

55. Jones J, Wang H, Karanam B, Theodore S, Dean-Colomb W, Welch DR, et al. Nuclear localization of Kaiso promotes the poorly differentiated phenotype and EMT in infiltrating ductal carcinomas. Clin Exp Metastasis 2014; 31:497–510. https://doi.org/10.1007/s10585-014-9644-7 PMID: 24570268

56. Pierre CC, Longo J, Mavor M, Milosavljevic SB, Chaudhary R, Gilbreath E, et al. Kaiso overexpression promotes intestinal inflammation and potentiates intestinal tumorigenesis in Apc(Min/+) mice. Biochim Biophys Acta 2015; 1852:1846–1855. https://doi.org/10.1016/j.bbadis.2015.06.011 PMID: 26073433

57. Jones J, Mukherjee A, Karanam B, Davis M, Jaynes J, Reams RR, et al. African Americans with pancreatic ductal adenocarcinoma exhibit gender differences in Kaiso expression. Cancer Lett 2016; 380:513–522. https://doi.org/10.1016/j.canlet.2016.06.025 PMID: 27424525

58. Daniel JM, Ireton RC, Reynolds AB. Monoclonal antibodies to Kaiso: a novel transcription factor and p120ctn-binding protein. Hybridoma 2001; 20:159–166. https://doi.org/10.1089/027245701750293484 PMID: 11461664

59. Donaldson NS, Norgaard CL, Pierre CC, Kelly KF, Robinson SC, Swystun L, et al. Kaiso regulates Znf131-mediated transcriptional activation. Exp Cell Res 2010; 316:1692–1705. https://doi.org/10.1016/j.yexcr.2010.03.011 PMID: 20303951

60. Bassey-Archibong BI, Rayner LGA, Hercules SM, Aarts CW, Dvorkin-Gheva A, Bramson JL, et al. Kaiso depletion attenuates the growth and survival of triple negative breast cancer cells. Cell Death Dis 2017; 8:e2689. https://doi.org/10.1038/cddis.2017.92 PMID: 28333150

61. Gialeli C, Theocharis AD, Karamanos NK. Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 2011; 278:16–27. https://doi.org/10.1111/j.1742-4658.2010.07919.x PMID: 21087457

62. Jezierska A, Motyl T. Matrix metalloproteinase-2 involvement in breast cancer progression: a mini-review. Med Sci Monit 2009; 15:RA32–40. PMID: 19182722

63. Mehner C, Hockla A, Miller E, Ran S, Radisky DC, Radisky ES. Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget 2014; 5:2736–2749. https://doi.org/10.18632/oncotarget.1932 PMID: 24811362

CHAPTER 6: HIGH KAISO EXPRESSION & TNBC IN WAA

Kaiso is highly expressed in TNBC tissues of women of African ancestry compared to Caucasian women

Preface

This chapter describes the published manuscript entitled: ***"Kaiso is highly expressed in TNBC tissues of women of African ancestry compared to Caucasian women"*** by Bassey-Archibong BI, Hercules SM, Rayner LGA, Skeete DHA, Connell SPS, Brain I, Daramola A, Banjo AAF, Byun JS, Gardner K, Dushoff J and Daniel JM, which has been reproduced in its original form ***(Cancer Causes and Control, 2017; 8(3): e2689).*** This is an open-access article distributed under the Creative Commons CC-BY License, which permits unrestricted reproduction and dissemination in any medium, provided the authors, attribution parties and sources are acknowledged.

This retrospective study examined the clinical parameters associated with TNBC in **w**omen of **A**frican **a**ncestry (WAA) and Caucasian women, and Kaiso expression patterns in TNBC tissues obtained from WAA and Caucasian women. It was conceived out of an effort to characterize Kaiso expression in TNBC tumors from WAA, since Kaiso is more highly expressed in TNBCs, which are most prevalent in premenopausal WAA compared to Caucasian women. We found that WAA are diagnosed with TNBC at younger ages than Caucasian women, and have more high-grade and lymph node positive tumors. Importantly,

we found that nuclear Kaiso is more highly expressed in TNBC tissues of WAA compared to Caucasian TNBC tissues. The highest nuclear Kaiso expression was observed in TNBC tissues from WAA that have a higher degree of African ancestry (Nigerian and Barbadian), suggesting a possible role for Kaiso in the racial disparity associated with TNBC prevalence.

Contributions:

BI Bassey-Archibong executed the research trip to collect TNBC tissues from Nigerian women while SM Hercules undertook the research trip to collect TNBC tissues from Barbadian women. BI Bassey-Archibong performed the statistical analysis that generated the Figures in 1A-C, and Figures 4A-D, and generated the data for Figures 3A and B. LG Rayner generated the data for Figure 2A and B. Dr. Desiree Skeete and Dr. Ian Brain performed the scoring of the Kaiso immunostain represented in Figures 3B and Figures 4A-D. Dr. JM Daniel and BI Bassey-Archibong conceived the study and co-wrote the manuscript. Dr. JM Daniel also provided significant guidance and intellectual input throughout the course of the study. All other authors assisted with the recruitment of patient populations (Dr. A Daramola, Dr. A Banjo, Dr. DH Skeete and Dr. Smith Connell), interpretation of the data (Dr. J Byun, Dr. K Gardner and Dr. J Dushoff). All authors edited the manuscript text.

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ORIGINAL PAPER

Kaiso is highly expressed in TNBC tissues of women of African ancestry compared to Caucasian women

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Abstract
Purpose Triple-negative breast cancer (TNBC) is most prevalent in young women of African ancestry (WAA) compared to women of other ethnicities. Recent studies found a correlation between high expression of the transcription factor Kaiso, TNBC aggressiveness, and ethnicity. However, little is known about Kaiso expression and localization patterns in TNBC tissues of WAA. Herein, we analyze Kaiso expression patterns in TNBC tissues of African (Nigerian), Caribbean (Barbados), African American (AA), and Caucasian American (CA) women.
Methods Formalin-fixed and paraffin embedded (FFPE) TNBC tissue blocks from Nigeria and Barbados were utilized to construct a Nigerian/Barbadian tissue microarray (NB-TMA). This NB-TMA and a commercially available TMA comprising AA and CA TNBC tissues (AA-CA-YTMA) were subjected to immunohistochemistry to assess Kaiso expression and subcellular localization patterns, and correlate Kaiso expression with TNBC clinical features.
Results Nigerian and Barbadian women in our study were diagnosed with TNBC at a younger age than AA and CA women. Nuclear and cytoplasmic Kaiso expression was observed in all tissues analyzed. Analysis of Kaiso expression in the NB-TMA and AA-CA-YTMA revealed that nuclear Kaiso H scores were significantly higher in Nigerian, Barbadian, and AA women compared with CA women. However, there was no statistically significant difference in nuclear Kaiso expression between Nigerian versus Barbadian women, or Barbadian versus AA women.
Conclusions High levels of nuclear Kaiso expression were detected in patients with a higher degree of African heritage compared to their Caucasian counterparts, suggesting a role for Kaiso in TNBC racial disparity.

Keywords Kaiso · TNBC · Women of African ancestry · Breast cancer racial disparity

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Introduction

Breast cancer (BCa) is a complex disease that occurs mostly in females and is a leading cause of female deaths worldwide [1–3]. The triple-negative breast cancer (TNBC) subtype accounts for a disproportionate number of BCa deaths due to its highly aggressive nature and metastatic tendencies [4–6]. As the name implies, triple-negative tumors represent a subset of breast tumors that are negative for the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor

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receptor-2 (HER2) [7]. Most TNBC are classified as basal-like cancers and are generally characterized by high histologic/nuclear grade, increased rate of recurrence, and a greater frequency of epidermal growth factor receptor (EGFR) amplification, p53 mutations, and breast cancer type 1 (BRCA1) mutations [7, 8]. Due to their triple-negative status for ER, PR, and HER2, TNBCs lack targeted-treatment options, and cannot be treated with hormonal (Tamoxifen) or anti-HER2 therapies [7].

There is increasing evidence that TNBC occurs more frequently in young premenopausal African and AA women compared to Caucasian women [7, 9–14]. For example, Stark and colleagues reported that among Ghanaian BCa cases, there was a TNBC prevalence of ~82% compared to the USA where TNBC prevalence was ~33% and ~10% among AA and CA cases, respectively [11]. Similarly, Agboola et al. reported a high incidence of TNBC among BCa cases in Nigerian women (~48%) compared with British women (~14%) [14]. The trend of high TNBC prevalence in AA and African females strongly suggests an ancestral genetic predisposition to TNBC in women of African ancestry (WAA) [15–17]. More disturbing, however, is the poor survival rate of AA TNBC patients compared with Caucasian TNBC patients [10, 18], which underscores the urgency to identify potential prognostic or diagnostic TNBC biomarkers in WAA.

Recent studies have found a correlation between increased nuclear expression of the transcription factor Kaiso and poor overall survival of AA breast cancer and prostate cancer patients compared to their Caucasian counterparts [19, 20]. These data hint at a role for Kaiso in the racial disparity in outcomes associated with breast and prostate cancer. Kaiso was first identified as a binding partner of the E-cadherin catenin cofactor—p120-catenin [21]. Kaiso is a dual-specificity transcription factor and member of the POZ-ZF family of transcription factors [21–25] that are implicated in vertebrate development and tumorigenesis. Kaiso has been most often characterized as a transcriptional repressor [26], but some studies indicate that Kaiso can also function as a transcriptional activator [27, 28]. Notably, several Kaiso target genes identified to date (*cyclinD1, matrilysin, E-cadherin*) have been linked to tumor onset, invasion, and metastasis [29–31].

Since its discovery, Kaiso has been implicated in the poor prognostic outcomes of several cancers including colorectal, non-small cell lung cancer, prostate, pancreatic ductal adenocarcinoma, and TNBC [20, 32–35]. Studies from our lab and others indicates that Kaiso plays both pro-oncogenic and tumor suppressive roles in several human cancers [19, 20, 33, 34, 36–38]. Notably, in addition to being implicated in racial disparities in breast cancer outcomes, high Kaiso expression correlates significantly with ER-α negativity, and the aggressiveness of basal/TNBCs [35, 38]. To date however, no studies have specifically examined and compared Kaiso expression and subcellular localization in TNBC tissues from WAA, who have the highest prevalence and worst outcomes from TNBC compared to Caucasian women. In this retrospective study, we evaluated Kaiso expression in TNBC specimens from Nigerian, Barbadian, AA, and CA patients. We found that nuclear Kaiso expression was significantly increased in TNBC tissues of Nigerian, Barbadian, and AA patients compared with their Caucasian counterparts. While there was no significant difference in nuclear Kaiso expression in TNBC tissues of Nigerian versus Barbadian patients (who have a higher percentage of African ancestry compared to AA), we found significantly more nuclear Kaiso expression in Nigerian versus AA patients, and a trend towards higher nuclear Kaiso expression in Barbadian versus AA patients. Collectively, these findings suggest that Kaiso may play a role in the racial disparity associated with TNBC in WAA.

Methods

Study population and characteristics of tumor samples

FFPE TNBC tissue blocks of 28 Nigerian TNBC patients diagnosed between 2011 and 2013 at the Lagos University Teaching Hospital (LUTH), Nigeria, and 46 Barbadian TNBC patients diagnosed between 2002 and 2011 at the Queen Elizabeth Hospital (QEH), Barbados were obtained from the archives of the Department of Anatomic and Molecular Pathology at LUTH and the Department of Pathology at QEH after approval by LUTH and QEH Ethics committees, respectively. The FFPE specimens were then shipped to the Developmental Histology Lab at the Yale Pathological Tissue Services (YPTS), Yale University (Connecticut, New Haven, USA), where they were hematoxylin and eosin (H&E) stained for histopathological confirmation, before tumor areas from each FFPE tissue block were selected for the construction of a Nigerian and Barbadian TNBC tissue microarray (NB-TMA). ER, PR, and HER2 status of the Nigerian tissues were confirmed by immunohistochemistry (IHC) conducted at LUTH, while ER, PR, and HER2 status of the Barbadian tissues were confirmed by IHC conducted at QEH, Barbados, the Human Tissue Resource Center (Chicago, IL, USA) or the Immunohistochemistry Lab at the University of Miami, Miller School of Medicine (Clinical Research Building, Miami, FL, USA). Any sample with less than 1% staining for ER and PR was scored negative; likewise, 0 or +1 for HER2 was considered negative. Available clinico-pathological data (age, tumor pathology, lymph node involvement, and grade)

were retrieved from the hardcopy pathology reports at LUTH and QEH, and are summarized in Table 1.

For the AA and CA patient population, we utilized the Yale tissue microarray 347 (YTMA-347), which was generated at the Yale Developmental Histology Lab, and comprised of 20 AA and 43 CA usable TNBC specimens that were diagnosed at the Yale-New Haven Hospital, Connecticut, USA between 1996 and 2004. ER, PR, and HER2 status were determined by IHC at the Yale Developmental Histology Lab. The clinico-pathological features of the YTMA-347 cohort are summarized in Table 1.

Immunohistochemistry

5-μm tissue sections prepared from the NB-TMA tissue block and the purchased YTMA-347 tissue slides were deparaffinized by warming at 60 °C for 20 min, followed by immersion in xylenes for 10 min. Tissue sections were then rehydrated in descending ethanol dilutions before they were subjected to heat antigen retrieval in a low pH buffer (pH 6.0) solution (DAKO, Glostrup, Denmark). Endogenous biotin, biotin receptors, and avidin binding sites on tissues were subsequently blocked using the Avidin/Biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA, USA), while endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide. Tissue slides were stained with mouse anti-Kaiso 6F monoclonal (1:10,000; [39]) or mouse anti-human cytokeratin clones AE1/AE3 monoclonal (1:500; Dako North America, Inc., Carpinteria, CA, USA) primary antibodies overnight at 4 °C, followed by secondary antibody incubations at room temperature for 2 h with biotinylated donkey anti-mouse secondary antibody (Vector Labs; 1:1000). Tissues were subsequently incubated in Vectastain (Vector Labs) for 30 min, rinsed in 1X PBS, and then incubated in diaminobenzidine (DAB) (Vector Labs) for 10 min. Counterstaining was achieved by incubating tissues in Harris hematoxylin (Sigma) for 10–60 s, followed by rinsing in tap water or as described in [40]. Slides were then dehydrated in ascending alcohol dilutions, and cleared with two rounds of xylenes before being mounted using Polymount (Polysciences Inc., Warrington, PA, USA). Negative control staining data were achieved by slide incubation with secondary antibodies only. Images of stained slides were captured using the Aperio Slide scanner (Leica Biosystems, ON, Canada). Stained tissues were scored blindly by two Pathologists, and the scores averaged to give a final score value. The intensity of staining was scored as 0, 1, 2, or 3 representing no, mild, moderate, or high staining intensity. The modified histochemical score (H-score) system was then used to generate the total score for each tissue with values spanning 0–300 using the formula: 3 × (percentage of cells with high intensity staining (3+) + 2 × (percentage of cells with moderate intensity staining (2+) + 1 × (percentage of cells with mild intensity staining (1+) for each slide.

Table 1 Clinico-pathological characteristics and analysis of study participants

	Nigerian (%) $n = 28$	Barbadian (%) $n = 46$	African American (%) $n = 20$	Caucasian American (%) $n = 43$	χ^2 value	p value
Age (years)						
≤50	20 (71.4%)	21 (45.7%)	6 (30.0%)	10 (23.3%)	16.89	0.0007
>50	5 (17.9%)	25 (54.3%)	7 (35.0%)	27 (62.8%)		
Unknown[a]	3 (10.7%)	0 (0.0%)	7 (35.0%)	6 (13.9%)		
Grade						
1	5 (17.9%)	0 (0.0%)	2 (10.0%)	13 (30.2%)	63.59	<0.0001
2	8 (28.6%)	9 (19.6%)	11 (55.0%)	21 (48.9%)		
3	10 (35.7%)	35 (76.1%)	0 (0.0%)	1 (2.3%)		
Unknown[a]	5 (17.8%)	2 (4.3%)	7 (35.0%)	8 (18.6%)		
Stage T						
T1–T2	6 (21.4%)	18 (39.1%)	7 (35.0%)	23 (53.5%)	30.52	<0.0001
T3–T4	11 (39.3%)	1 (2.2%)	1 (5.0%)	0 (0.0%)		
Unknown[a]	11 (39.3%)	27 (58.7%)	12 (60.0%)	20 (46.5%)		
Stage N						
N0	4 (14.3%)	11 (23.9%)	7 (35.0%)	26 (60.5%)	10.23	0.02
N1-N3	11 (39.3%)	10 (21.7%)	13 (65.0%)	12 (27.9%)		
Unknown[a]	13 (46.4%)	25 (54.4%)	0 (0.0%)	5 (11.6%)		

[a]Unknown cases were exempted from analysis

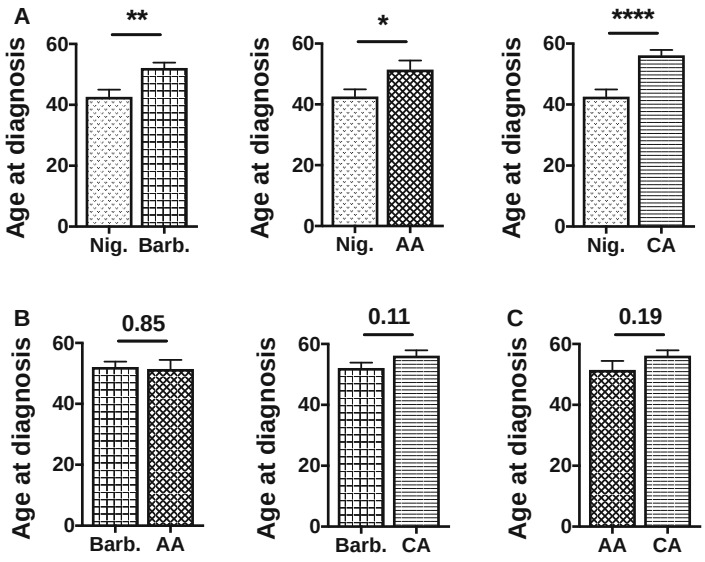

Fig. 1 Nigerian women are diagnosed with TNBC at younger ages than Barbadian, AA, and CA women. **a** The mean age at diagnosis for Nigerian TNBC patients was 42.6 years ($n = 25$) compared with 52.1 years for Barbadian women ($n = 46$), 51.5 years for AA women ($n = 13$), and 56.2 years for CA women ($n = 37$). No significant differences were observed between the mean age at diagnosis for Barbadian versus AA and CA TNBC patients (**b**) and for AA versus CA patients (**c**). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{****}p < 0.0001$

Statistical analysis

GraphPad Prism statistical software (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses. Standard unpaired Student's t test with Welch's correction was used for pairwise comparison of means. Chi square analysis was used to assess the difference in clinico-pathological features between the Nigerian, Barbadian, AA, and CA cohorts. Data are presented as mean ± SEM where applicable. For all statistical tests, p values <0.05 denote statistical significance.

Results

Clinico-pathological characteristics of study participants

This retrospective study involved a total of 28 Nigerian, 46 Barbadian, 20 African American (AA), and 43 Caucasian American (CA) TNBC patients. The mean age at time of diagnosis for Nigerian women was 42.6 years compared to 52.1 years for Barbadian women ($p = 0.002$), 51.5 years for AA women ($p = 0.03$), and 56.2 years for CA women ($p < 0.0001$; Fig. 1a). Comparison of the mean age at diagnosis between Barbadian, AA, and CA patients yielded no statistical significance (Fig. 1b, c). The percentage of younger women who presented with TNBC at time of diagnosis was significantly higher for the Nigerian cohort (71.4%; $n = 20$) compared with the Barbadian (45.7%; $n = 21$), AA (30.0%; $n = 6$), and CA (23.3%; $n = 10$) cohort ($p < 0.001$) (Table 1). Low-grade tumors were seldom observed in the Nigerian (17.9%; $n = 5$), Barbadian (0%; $n = 0$), and AA (10.0%; $n = 2$) cohorts compared to the CA (30.2%; $n = 13$) cohort ($p < 0.0001$; Table 1). Low-grade was defined as grade 1, medium-grade as grade 2, and high-grade as grade 3, respectively. Approximately 39.3% ($n = 11$) of Nigerian women presented with higher stage (T3–T4) tumors compared with 2.2% ($n = 1$) for Barbadian, 5.0% ($n = 1$) for AA, and 0% ($n = 0$) for CA women ($p < 0.0001$; Table 1). Finally, CA TNBC patients displayed a higher frequency of lymph node-negative tumors (60.5%; $n = 26$) compared with that observed in Nigerian (14.3%; $n = 4$), Barbadian (23.9%; $n = 11$), and AA (35.0%; $n = 7$) TNBC patients ($p = 0.02$; Table 1).

Kaiso is highly expressed in TNBC tissues of WAA compared to Caucasian women

Previously, we reported that Kaiso is highly expressed at the mRNA level in triple-negative tumors compared with

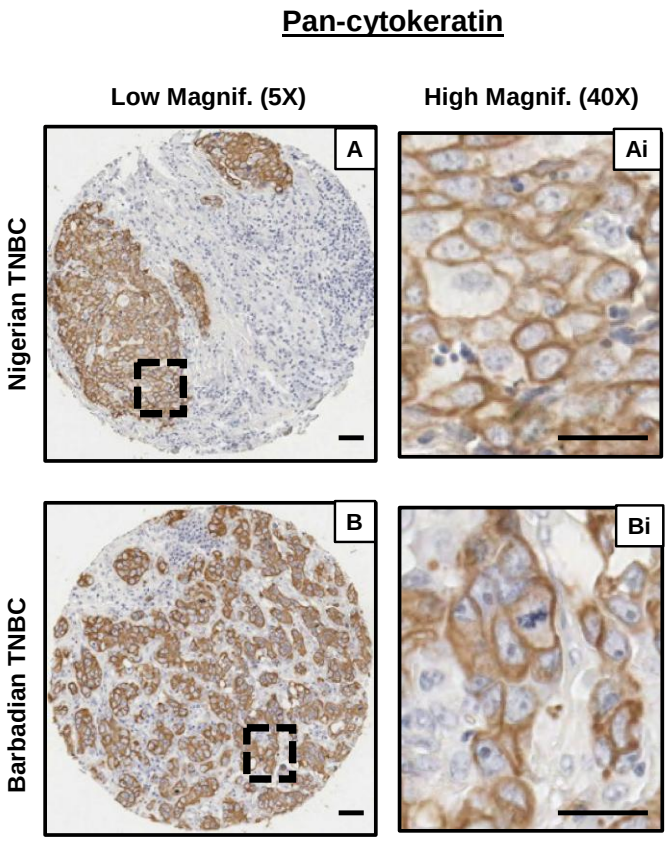

Fig. 2 Cytokeratin immunostaining of Nigerian and Barbadian TNBC tissues verifies tissue integrity. IHC images at low (5×) and high magnification (40×) show intact tissue cores (**a**, **b**) and membrane localization (**ai**, **bi**) of cytokeratin, which portrays good integrity of the Nigerian and Barbadian tissues. *Scale bar* 50 μm

hormone receptor-positive breast tumors in publicly available datasets downloaded from The Cancer Genome Atlas—TCGA website or the Gene Expression Omnibus—GEO website [35]. Thus, in this study, we utilized immunohistochemistry to specifically evaluate the expression and subcellular localization of Kaiso in TNBC tissues from Nigerian, Barbadian, AA, and CA patients. Tissue integrity of the Nigerian and Barbadian TNBC tissues was determined by immunostaining for pan-cytokeratin as described in the methods; Fig. 2a, b shows representative images of the tissue quality of the Nigerian and Barbadian TNBC tissues. As shown in Fig. 3a (representative images shown), Kaiso exhibited both nuclear and cytoplasmic localization in all TNBC tissues analyzed, with varying degrees of heterogeneity. Nuclear and cytoplasmic Kaiso staining intensity was scored as described in the methods, and Kaiso's relative expression in each TNBC cohort analyzed. As seen in Fig. 3b, we observed significantly higher cytoplasmic than nuclear Kaiso expression in the AA and CA TNBC cohorts ($p < 0.0001$), but did not find significant differences between nuclear and cytoplasmic Kaiso expression in the Nigerian and Barbadian TNBC cohorts.

Since nuclear but not cytoplasmic Kaiso expression is known to be associated with TNBC aggressiveness, and decreased survival of AA BCa patients [19, 38], we next performed comparative analysis of nuclear Kaiso expression between the Nigerian, Barbadian, AA, and CA cohorts. Interestingly, we observed a significantly higher level of nuclear Kaiso expression in TNBC tissues of patients of African ancestry (Nigerian, Barbadian, and AA) compared to their Caucasian counterparts (Fig. 4a). However, there was no significant difference between nuclear Kaiso expression in TNBC tissues of Nigerian and

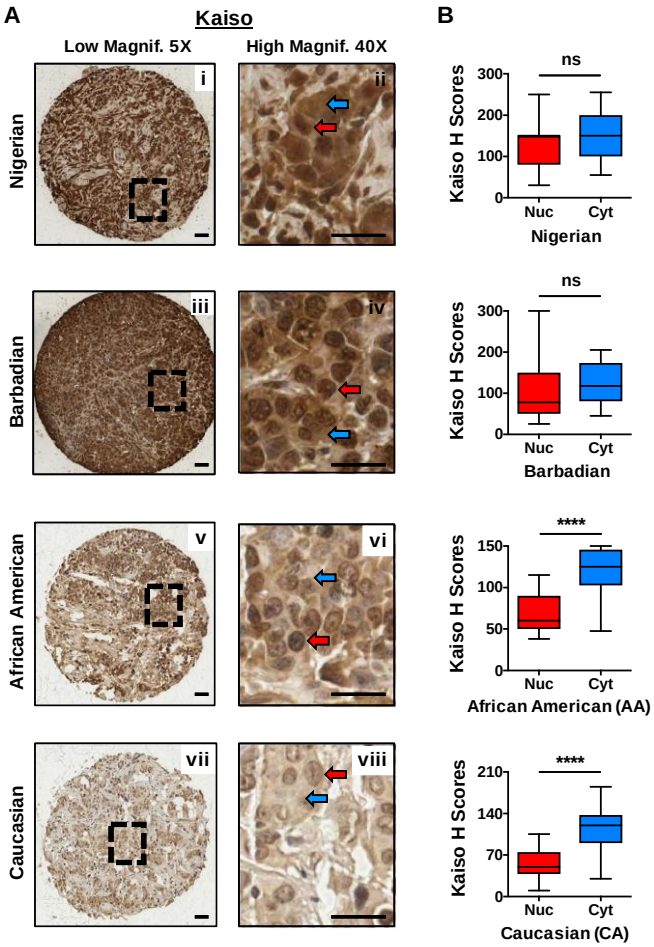

Fig. 3 Kaiso subcellular localization and expression in Nigerian, Barbadian, AA, and CA TNBC tissues. (**a**i–viii) IHC images showing Kaiso localization to both the nucleus and cytoplasm of Nigerian, Barbadian, AA, and CA TNBC tissues. (**b**) Graphical representation of nuclear and cytoplasmic Kaiso expression in Nigerian ($n = 19$), Barbadian ($n = 20$), AA ($n = 20$), and CA ($n = 39$) TNBC tissues. Cytoplasmic Kaiso expression was significantly higher than nuclear Kaiso expression in the AA and CA TNBC cohorts but not in the Nigerian and Barbadian TNBC cohorts. Red arrows indicate nuclear Kaiso staining, while blue arrows indicate cytoplasmic Kaiso staining. *Scale bar* 50 µm. *ns* not significant, $^{****}p < 0.0001$

Barbadian patients, who have ~99.8 and ~77.4% degree of African heritage, respectively [41, 42], or between TNBC tissues of Barbadian and AA patients, who have ~77.4 and ~72.5% degree of African heritage, respectively [42] (Fig. 4b). Remarkably however, there was significantly more nuclear Kaiso expression in TNBC tissues of Nigerian compared to AA patients (Fig. 4c), probably due to the higher degree of African heritage in Nigerian patients (~99.8%) compared to AA patients (~72.5%). Since TNBC is more prevalent in WAA compared to Caucasian women, these findings suggest a role for nuclear Kaiso expression levels in the racial disparity in TNBC prevalence.

Correlation between nuclear Kaiso expression and clinico-pathological features of study participants

Breast tumors of WAA are often associated with a higher histological grade and positive lymph node involvement compared to breast tumors of Caucasian women [11, 14]. Since previous studies from our lab and others have

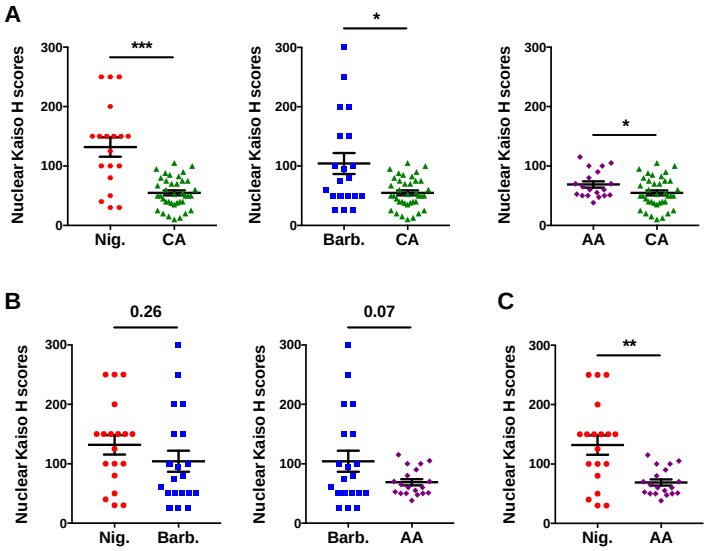

Fig. 4 Comparative analysis of nuclear Kaiso expression in Nigerian, Barbadian, AA, and CA TNBC tissues. Higher levels of nuclear Kaiso expression were detected in TNBC tissues of Nigerian, Barbadian, and AA compared with their Caucasian counterparts (**a**). Although no significant difference in nuclear Kaiso expression was observed between Nigerian versus Barbadian tissues, or between Barbadian versus AA tissues (**b**), there was a significant difference in nuclear Kaiso expression between Nigerian and AA TNBC tissues (**c**). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$

correlated increased Kaiso expression with advanced grade and metastasis of TNBC [35, 38], and lymph node involvement is an established prognostic marker for the metastatic potential of breast tumors [43], we next assessed the association of Kaiso expression with high-grade and lymph node involvement in Nigerian, Barbadian, AA, and CA patients. High-grade tumors were defined as grade 3 for Nigerian and Barbadian patients and grade 2 for AA and CA patients due to no analyzed grade 3 tumors in the AA and CA TNBC cohort (the only observed grade 3 CA patient could not be scored as a result of tissue loss). Low-grade tumors were thus defined as grades 1 and 2 for Nigerian and Barbadian patients, and grade 1 for AA and CA patients. Lymph node metastasis was considered positive if one or more lymph nodes were noted to contain cancer cells (n1–n3), and negative if there were no observed cancer cells in the lymph nodes (n0). Due to the small sample size used in the analysis, no significant correlation was found between high nuclear Kaiso expression and high-grade or lymph node-positive triple-negative tumors in any of the patient cohorts analyzed (Suppl. Figure 1).

Discussion

TNBC is most prevalent in WAA compared to Caucasian American/European females, but the reason for this disparity is currently unknown [11, 14, 16, 44]. Although poor socio-economic status has been linked to TNBC mortality in African and AA women, it does not fully explain the disproportionate prevalence and aggressiveness of TNBC in WAA compared to their Caucasian counterparts [17]. Thus, we and others have postulated that there may be an ancestral genetic predisposition to TNBC in WAA [17, 45].

Notably, a higher prevalence of TNBC has been reported in West-African women (Nigerians—65%, and Ghanaians—82.2%) compared with that reported in AA—$\sim$33% [9, 11, 46], thus supporting the idea of a relationship between percentage of African ancestry and TNBC prevalence. Since West-African countries such as Ghana and Nigeria are the founding ancestors of most WAA worldwide [41, 42, 47–49], we posit that there is a higher probability of identifying a founder mutation, if one exists, in Nigerian and Ghanaian populations, and also in more

homogeneous populations of the African Diaspora such as the Caribbean (e.g., Barbados).

Recent studies have linked high nuclear expression of the transcription factor Kaiso with increased TNBC aggressiveness [20, 38], and decreased survival of AA breast cancer patients compared with their Caucasian counterparts [19]. These reports suggest a link between increased nuclear Kaiso, TNBC aggressiveness/metastasis, and the racial disparity in prevalence/outcomes associated with breast cancer. Remarkably, our findings lend some credence to this hypothesis as we observed elevated expression of nuclear Kaiso in TNBC tissues from patients of African ancestry (Nigerians, Barbadians, and African Americans) compared to their Caucasian/European ancestry counterparts (CA) (see Fig. 4a). Thus, our previous findings in Kaiso-depleted mouse xenograft models [35, 51], where we demonstrated roles for Kaiso in TNBC cell growth, survival, and metastasis, may explain why high Kaiso-expressing triple-negative tumors in WAA are associated with a more aggressive phenotype and fatal outcomes than TNBC in Caucasian women.

Importantly, our findings highlight an interesting correlation between high nuclear Kaiso expression and percent African ancestry, which may be linked to the predisposition of young WAA to TNBC. However, this study is limited by the small sample size, the semi-quantitative method of analysis used, and lack of complete clinico-pathological information, which did not allow proper assessment of the correlation between Kaiso expression and the high tumor grade observed in African/Caribbean women compared to African American or Caucasian women. Additional studies using larger cohort sizes of West-African (Nigeria and others), Caribbean (Barbados and others), AA, and CA TNBC cases, coupled with quantitative methods of immunostain analysis such as the automated quantitative analysis (AQUA) system established by Rimm and colleagues [50], will undoubtedly provide more insight into the clinical relevance of nuclear Kaiso expression in the etiology of TNBC in WAA.

In conclusion, this is the first study to suggest a potential link between increased Kaiso expression and the predisposition of young WAA to TNBC. This observation, in addition to the previous identified roles for Kaiso in TNBC aggressiveness, metastasis, and poor overall survival in affected patients [35, 38, 51], raises two exciting possibilities: i) Kaiso expression could be utilized as a biomarker for the diagnosis and prognosis of TNBC in WAA and ii) Kaiso could be a molecular target for the development of treatment options against TNBC not only in WAA but also TNBC patients worldwide.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this retrospective study were in accordance with the ethical standards of LUTH and QEH, respectively. For this type of study formal consent is not required.

References

1. Hortobagyi GN, de la Garza Salazar J, Pritchard K, Amadori D, Haidinger R, Hudis CA et al (2005) The global breast cancer burden: variations in epidemiology and survival. Clin Breast Cancer. 6(5):391–401
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90
3. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C et al (2013) GLOBOCAN 2012 v1.0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. International Agency for Research on Cancer, Lyon
4. Oakman C, Viale G, Di Leo A (2010) Management of triple negative breast cancer. Breast 19(5):312–321
5. Carey LA (2011) Directed therapy of subtypes of triple-negative breast cancer. Oncologist 16(Suppl 1):71–78
6. Andre F, Zielinski CC (2012) Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. Ann Oncol 23(Suppl 6):vi46–vi51
7. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple-negative breast cancer. N Engl J Med 363(20):1938–1948
8. Irshad S, Ellis P, Tutt A (2011) Molecular heterogeneity of triple-negative breast cancer and its clinical implications. Curr Opin Oncol 23(6):566–577
9. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K et al (2006) Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 295(21):2492–2502
10. Lund MJ, Trivers KF, Porter PL, Coates RJ, Leyland-Jones B, Brawley OW et al (2009) Race and triple negative threats to breast cancer survival: a population-based study in Atlanta, GA. Breast Cancer Res Treat 113(2):357–370
11. Stark A, Kleer CG, Martin I, Awuah B, Nsiah-Asare A, Takyi V et al (2010) African ancestry and higher prevalence of triple-

negative breast cancer: findings from an international study. Cancer 116(21):4926–4932
12. Bhikoo R, Srinivasa S, Yu TC, Moss D, Hill AG (2011) Systematic review of breast cancer biology in developing countries (part 1): Africa, the Middle East, Eastern Europe, Mexico, the Caribbean and South America. Cancers 3:2358–2381
13. Amirikia KC, Mills P, Bush J, Newman LA (2011) Higher population-based incidence rates of triple-negative breast cancer among young African-American women: implications for breast cancer screening recommendations. Cancer 117(12):2747–2753
14. Agboola AJ, Musa AA, Wanangwa N, Abdel-Fatah T, Nolan CC, Ayoade BA et al (2012) Molecular characteristics and prognostic features of breast cancer in Nigerian compared with UK women. Breast Cancer Res Treat 135(2):555–569
15. Amend K, Hicks D, Ambrosone CB (2006) Breast cancer in African-American women: differences in tumor biology from European-American women. Can Res 66(17):8327–8330
16. Boyle P (2012) Triple-negative breast cancer: epidemiological considerations and recommendations. Ann Oncol 23(Suppl 6):vi7–vi12
17. Dietze EC, Sistrunk C, Miranda-Carboni G, O'Regan R, Seewaldt VL (2015) Triple-negative breast cancer in African-American women: disparities versus biology. Nat Rev Cancer 15(4):248–254
18. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V (2007) Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. Cancer 109(9):1721–1728
19. Jones J, Wang H, Karanam B, Theodore S, Dean-Colomb W, Welch DR et al (2014) Nuclear localization of Kaiso promotes the poorly differentiated phenotype and EMT in infiltrating ductal carcinomas. Clin Exp Metasis 31(5):497–510
20. Jones J, Wang H, Zhou J, Hardy S, Turner T, Austin D et al (2012) Nuclear kaiso indicates aggressive prostate cancers and promotes migration and invasiveness of prostate cancer cells. Am J Pathol 181(5):1836–1846
21. Daniel JM, Reynolds AB (1999) The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol Cell Biol 19(5):3614–3623
22. Kelly KF, Daniel JM (2006) POZ for effect—POZ-ZF transcription factors in cancer and development. Trends Cell Biol 16(11):578–587
23. Daniel JM, Spring CM, Crawford HC, Reynolds AB, Baig A (2002) The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. Nucleic Acids Res 30(13):2911–2919
24. Prokhortchouk A, Hendrich B, Jorgensen H, Ruzov A, Wilm M, Georgiev G et al (2001) The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. Genes Dev 15(13):1613–1618
25. Donaldson NS, Pierre CC, Anstey MI, Robinson SC, Weerawardane SM, Daniel JM (2012) Kaiso represses the cell cycle gene cyclin D1 via sequence-specific and methyl-CpG-dependent mechanisms. PLoS ONE 7(11):e50398
26. Daniel JM (2007) Dancing in and out of the nucleus: p120(ctn) and the transcription factor Kaiso. Biochim Biophys Acta 1773(1):59–68
27. Rodova M, Kelly KF, VanSaun M, Daniel JM, Werle MJ (2004) Regulation of the rapsyn promoter by kaiso and delta-catenin. Mol Cell Biol 24(16):7188–7196
28. Blattler A, Yao L, Wang Y, Ye Z, Jin VX, Farnham PJ (2013) ZBTB33 binds unmethylated regions of the genome associated with actively expressed genes. Epigenetics Chromatin 6(1):13
29. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL (2011) Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 11(8):558–572
30. Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K (1999) Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut 45(2):252–258
31. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Can Res 68(10):3645–3654
32. Pierre CC, Longo J, Mavor M, Milosavljevic SB, Chaudhary R, Gilbreath E et al (2015) Kaiso overexpression promotes intestinal inflammation and potentiates intestinal tumorigenesis in Apc(-Min/+) mice. Biochim Biophys Acta 1852(9):1846–1855
33. Dai SD, Wang Y, Miao Y, Zhao Y, Zhang Y, Jiang GY et al (2009) Cytoplasmic Kaiso is associated with poor prognosis in non-small cell lung cancer. BMC Cancer 9:178
34. Jones J, Mukherjee A, Karanam B, Davis M, Jaynes J, Reams RR et al (2016) African Americans with pancreatic ductal adenocarcinoma exhibit gender differences in Kaiso expression. Cancer Lett 380(2):513–522
35. Bassey-Archibong BI, Kwiecien JM, Milosavljevic SB, Hallett RM, Rayner LG, Erb MJ et al (2016) Kaiso depletion attenuates transforming growth factor-β signaling and metastatic activity of triple-negative breast cancer cells. Oncogenesis 5:e208
36. Prokhortchouk A, Sansom O, Selfridge J, Caballero IM, Salozhin S, Aithozhina D et al (2006) Kaiso-deficient mice show resistance to intestinal cancer. Mol Cell Biol 26(1):199–208
37. Lopes EC, Valls E, Figueroa ME, Mazur A, Meng FG, Chiosis G et al (2008) Kaiso contributes to DNA methylation-dependent silencing of tumor suppressor genes in colon cancer cell lines. Can Res 68(18):7258–7263
38. Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P et al (2012) Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer. PLoS ONE 7(5):e37864
39. Daniel JM, Ireton RC, Reynolds AB (2001) Monoclonal antibodies to Kaiso: a novel transcription factor and p120ctn-binding protein. Hybridoma 20(3):159–166
40. Chaudhary R, Pierre CC, Nanan K, Wojtal D, Morone S, Pinelli C et al (2013) The POZ-ZF transcription factor Kaiso (ZBTB33) induces inflammation and progenitor cell differentiation in the murine intestine. PLoS ONE 8(9):e74160
41. Tracing African roots; exploring the ethnic origins of the Afro-Diaspora
42. Murray T, Beaty TH, Mathias RA, Rafaels N, Grant AV, Faruque MU et al (2010) African and non-African admixture components in African Americans and an African Caribbean population. Genet Epidemiol 34(6):561–568
43. Weigelt B, Peterse JL, van't Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5(8):591–602
44. Huo D, Ikpatt F, Khramtsov A, Dangou JM, Nanda R, Dignam J et al (2009) Population differences in breast cancer: survey in indigenous African women reveals over-representation of triple-negative breast cancer. J Clin Oncol 27(27):4515–4521
45. Sawe RT, Kerper M, Badve S, Li J, Sandoval-Cooper M, Xie J et al (2016) Aggressive breast cancer in western Kenya has early onset, high proliferation, and immune cell infiltration. BMC Cancer 16:204
46. Adisa CA, Eleweke N, Alfred AA, Campbell MJ, Sharma R, Nseyo O et al (2012) Biology of breast cancer in Nigerian women: a pilot study. Ann Afr Med 11(3):169–175
47. Fraizer M (2005) Continuity and change in Caribbean immigration. People's World
48. Jackson FL (2008) Ancestral links of Chesapeake Bay region African Americans to specific Bight of Bonny (West Africa)

microethnic groups and increased frequency of aggressive breast cancer in both regions. Am J Hum Biol 20(2):165–173
49. Bryc K, Auton A, Nelson MR, Oksenberg JR, Hauser SL, Williams S et al (2010) Genome-wide patterns of population structure and admixture in West Africans and African Americans. Proc Natl Acad Sci USA 107(2):786–791
50. McCabe A, Dolled-Filhart M, Camp RL, Rimm DL (2005) Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. J Natl Cancer Inst 97(24):1808–1815
51. Bassey-Archibong BI, Rayner LG, Hercules SM, Aarts CW, Dvorkin-Gheva A, Bramson JL et al (2017) Kaiso depletion attenuates the growth and survival of triple negative breast cancer cells. Cell Death Dis 8(3):e2689

CHAPTER 7: DISCUSSION

Since its identification over two decades ago, Kaiso has been implicated in the pathogenesis of various human cancers including breast cancer (Cofre et al. 2012; Jones et al. 2014; Jones et al. 2012; Pierre et al. 2015b). However, despite the mounting evidence linking Kaiso to tumor-related processes and signaling pathways – like the canonical Wnt signalling pathway whose malfunction contributes to colon cancer – Kaiso's mechanism of action specifically in breast carcinogenesis remains largely unknown. The molecular factors implicated in the growth of some BCa subtypes e.g. ER-positive and HER2-enriched, have been largely elucidated and led to effective targeted therapies – e.g. Tamoxifen, Trastuzumab – for these BCa subtypes (Lumachi et al. 2013; Vogel et al. 2002). Unfortunately, there are currently no established biomarkers and thus no targeted therapies for the TNBC subtype, which causes it to be a clinical dilemma. Additionally, triple-negative tumors are highly aggressive and metastatic (Dent et al. 2007), and are predominantly diagnosed in young women of African ancestry – who undergo a more aggressive clinical course of the disease compared with their Caucasian counterparts, reviewed in (Dietze et al. 2015).

Recent studies by Jones *et al.* suggested an important role for Kaiso in TNBC aggressiveness and the racial disparity in breast cancer outcomes (Jones et al. 2014). Specifically, increased Kaiso expression was found to correlate with the poor overall survival outcomes of African American BCa patients' relative to

Caucasian women, while loss of Kaiso expression was shown to attenuate the migratory and invasive capacities of TNBC cells *in vitro* (Jones et al. 2014). Despite these novel findings, the biological and clinical relevance of Kaiso expression in TNBC remained unknown and this laid the foundation for the key studies in this thesis. Some of the major unanswered questions were: (i) what is the significance of high Kaiso expression in the growth, survival and metastasis of TNBCs; (ii) what is Kaiso's mechanism of action in triple negative tumors; and (iii) what is the relationship between Kaiso expression and TNBC prevalence/outcomes in women of African heritage. This thesis addressed these questions and unveiled several significant findings that will lay the foundation for future exploration of Kaiso's roles in hormone-receptor negative breast tumors.

7.1 Kaiso's biological relevance in the tumorigenesis and metastasis of TNBC

7.1.1 Kaiso depletion attenuates the proliferation and promotes the apoptosis of TNBC cells

Kaiso depletion attenuated the proliferative abilities of the well-established TNBC cell lines – MDA-231 and Hs578T *in vitro* ***(Chapter 3, Figure 1)***, but surprisingly Kaiso depletion had different effects on their proliferation *in vivo*. Specifically, Kaiso-depleted MDA-231 cells continued to exhibit reduced proliferative abilities and had delayed tumor onset when xenografted into NSG mice whereas Hs578T cells did not ***(Chapter 3, Figure 2; Appendix, Figure A1)***. This observation was

intriguing because it suggested a crucial cell context-dependent role for Kaiso in TNBC cell proliferation under *in vivo* conditions. Several other studies have also demonstrated cell context-dependent roles for Kaiso in proliferation. For example, whereas Kaiso-depletion resulted in decreased proliferation of cervical (Pozner et al. 2016) and prostate cancer cells (Wang et al. 2016), loss of Kaiso expression resulted in the increased proliferation of HCT116 colon cancer cells (Donaldson et al. 2012), HEK293 human embryonic kidney cells (Pozner et al. 2016), and mouse embryonic fibroblast cells (Koh et al. 2014). The differential role of Kaiso in cell proliferation could be due to several factors including (i) the specific subset of genes that Kaiso regulates as well as its transcriptional activity (whether as an activator or repressor) on the promoter of such genes in each cell type and (ii) Kaiso's interacting protein partner, their specific functions and unique expression in each cellular context.

Consistent with the first possibility, Kaiso's differential regulation of *cyclin D1* – that is essential for cell cycle progression and consequently cell proliferation (Baldin et al. 1993; Stacey 2003) – has been implicated in its diverse effects on proliferation in different cell types. Previous studies from our group demonstrated that Kaiso represses *cyclin D1* expression in HCT116 cells (Donaldson et al. 2012), while Kaiso activates *cyclin D1* expression in HeLa cells in other reports (Pozner et al. 2016). Correspondingly, loss of Kaiso expression in HCT116 cells led to increased *cyclin D1* expression (Donaldson et al. 2012) concomitant with

increased cell proliferation, while Kaiso depletion in HeLa cells resulted in reduced *cyclin D1* expression and thus reduced cell proliferation (Pozner et al. 2016). Notably, we found that Kaiso depletion resulted in decreased Cyclin D1 expression in MDA-231 and Hs578T cells *in vitro* ***(Chapter 3; Figure 1)***. Based on the findings from the above reports, we postulated that the reduced Cyclin D1 expression could be responsible for the decreased cell proliferation observed in the Kaiso-depleted cells *in vitro* ***(Chapter 3; Figure 1)***.

In line with our second theory, Kaiso has been shown to interact with the p53-p300 complex in HEK293 cells. This interaction promoted the activation of the cell cycle arrest gene *CDKN1A* in a p53-dependent manner, led to the subsequent induction of cell cycle arrest, and attenuated proliferation in these cells upon Kaiso overexpression (Koh et al. 2014). It is therefore possible that Kaiso's effect on cell proliferation can be modulated under different cellular contexts by its associating protein partner(s). While this scenario has not been definitively demonstrated for Kaiso, it has been determined for other proteins such as the basic helix-loop-helix leucine zipper transcription factor Max. In highly proliferating cells, Max interacts with its family member, Myc, at consensus E-box (CANNTG) sites (where n = any nucleotide). This results in Max-mediated promotion of cell proliferation as a consequence of its association with Myc – a pro-proliferation transcription factor, reviewed in (Dang et al. 1999; Zhou and Hurlin 2001). Conversely, in differentiating cells Max interacts with another family

member, Mad, at E-box sites, which diverts Max functions to a suppressor of proliferation as a result of the anti-proliferative transcriptional activities of Mad, reviewed in (Ayer and Eisenman 1993; Zhou and Hurlin 2001).

Tumorigenesis is typically associated with reduced apoptotic and increased proliferative tendencies (Jäger et al. 1997; Nagane et al. 1996; Shibata et al. 1999; Strasser et al. 1990). Although, control TNBC cells exhibited these properties, loss of Kaiso expression stimulated the apoptosis of MDA-231 and Hs578T cells in both *in vitro* and *in vivo* systems ***(Chapter 3, Figure 3; Appendix, Figure A1)*** in addition to suppressing the proliferation of these cells *in vitro* ***(Chapter 3, Figure 1; Appendix, Figure A1)***. These findings suggest a prominent and crucial role for Kaiso expression in TNBC tumorigenesis and survival. A possibility that is further supported by the increased sensitivity to chemotherapy-induced cell death observed in colon cancer cell lines, as well as the MDA-231 and Hs578T TNBC cells upon Kaiso depletion (Bassey-Archibong et al. 2017; Lopes et al. 2008). The combined reduction in cell proliferation and increased apoptosis observed in the Kaiso-depleted MDA-231 cells *in vivo* provides an alternative explanation for the delayed tumor onset exhibited by these cells ***(Chapter 3, Figure 2).*** A notion that is reinforced by the lack of delayed tumor onset in Kaiso-depleted Hs578T cells that exhibited sustained proliferative abilities *in vivo* despite their increased apoptotic activity ***(Appendix, Figure A1)***.

Previous studies from our lab and others had demonstrated pro-apoptotic roles for Kaiso (Koh et al. 2014; Pierre et al. 2015b), which is in contrast to the anti-apoptotic effect of Kaiso described in our TNBC experiments. One likely explanation for this paradox could be that Kaiso's effects on apoptosis are context-dependent, and determined by the Kaiso-interacting protein partner(s) that are differentially expressed in the various cell-types. Accordingly, Kaiso has been reported to interact with various proteins with distinctive biological functions (Barrett et al. 2012; Raghav et al. 2012; Rodova et al. 2004; Yoon et al. 2003) including the wt-p53 tumor suppressor (Koh et al. 2014) that in addition to mediating cell cycle arrest, is also a strong inducer of apoptosis in a wide variety of cell types (Vousden and Lane 2007).

Notably, Kaiso's interaction with wt-p53 in wt-p53 expressing cells like HEK293, was instrumental in mediating the pro-apoptotic functions of Kaiso reported in these cells (Koh et al. 2014). Intriguingly, we found that Kaiso interacts with the **mut**ant (mut) form of p53 in MDA-231 and Hs578T cells ***(Chapter 3, Figure 4)***, which may account for the anti-apoptotic function of Kaiso in these cells since mut-p53 has been implicated in TNBC cell survival (Bae et al. 2014; Braicu et al. 2013; Lim et al. 2009b). However, it is important to note that Kaiso may also mediate its anti-apoptotic functions independent of mut-p53, as the Kaiso-depleted MDA-231 and Hs578T cells displayed increased apoptosis though the level of mut-p53 was unchanged in response to Kaiso depletion in these cells

(Chapter 3, Figure 4). Nonetheless, and more importantly, our data intimates that in the absence of the potent pro-apoptotic wt-p53 protein, Kaiso functions as an anti-apoptotic protein either independently or in coalition with mut-p53. However, when wt-p53 is present, Kaiso functions as a pro-apoptotic protein in cooperation with wt-p53.

7.1.2 Kaiso depletion suppresses the metastasis and pulmonary intravascular invasion of TNBC cells

Another remarkable discovery of our study was that Kaiso depletion strongly inhibited the metastatic abilities of TNBC cells ***(Chapter 4, Figure 2)***, a phenotype that indicated an important role for Kaiso in TNBC metastasis. Metastasis occurs when tumor cells acquire the ability to break away from the primary tumor site and migrate to secondary sites such as the lungs and liver. This process typically involves multiple steps including intravasation into the blood circulatory system, either directly or indirectly via the lymphatic system, survival within the circulatory system until arrest at distant sites, extravasation from the circulatory system into the secondary site, and efficient growth or metastatic colonization of the new tissue/organ (Chambers et al. 2002; Pantel and Brakenhoff 2004). Mounting evidence indicates that it is the growth capabilities of disseminated tumor cells in target organs that contribute to metastatic efficiency rather than the ability of primary tumor cells to survive in

circulation, arrest and extravasate into secondary target tissues, reviewed in (Chambers and Matrisian 1997).

Remarkably, in addition to suppressing the capacity of TNBC cells to spread to other organs, particularly the lungs, Kaiso depletion also attenuated the ability of TNBC cells that made it to the lungs to grow and expand in their new host environment ***(Chapter 4, Figure 2)***. Indeed, while control MDA-231 and Hs578T cells thrived and expanded in the lungs, the Kaiso-depleted MDA-231 and Hs578T cells formed very few small masses in the lungs that was almost non-existent in the case of the Kaiso-depleted Hs578T cells ***(Chapter 4, Figure 2)***. The ability of disseminated tumor cells to grow in a specific organ is greatly influenced by the microenvironment and molecular factors present within such organs (Chambers and Matrisian 1997). Several studies with intravital videomicroscopy have shown that both highly and poorly metastatic cells can extravasate successfully at distant organs. However, only highly metastatic cells are then able to survive and colonize such distant organs (Koop et al. 1996; Morris et al. 1994), further emphasizing the influence of different environmental factors on the growth of disseminated cells at distant organs. Considering that only the control MDA-231 and Hs578T cells with high Kaiso expression were able to efficiently grow and colonize the lungs ***(Chapter 4, Figure 2)***, we hypothesize that Kaiso plays a key role in the efficiency of TNBC cells to overcome the natural

defense mechanisms of the lungs, and grow extensively to form macro-metastases.

According to pulmonary and systemic circulatory patterns, all cancer cells that escape from the primary breast tumor site into the bloodstream would first disseminate directly to the lungs via the right heart ventricle, before dispersing via the lungs through the left heart ventricle to distant body organs (Chambers et al. 2002). Notably, as previously mentioned, we noticed that although MDA-231 and Hs578T cells formed large tumor masses in the lungs, only the control MDA-231 cells intravasated or invaded into the pulmonary (lung) blood vessels adjacent to the tumor masses and formed extensive macro-metastases in other distant organs including the liver, kidney and myocardium ***(Chapter 5, Figure 4)***. Control Hs578T cells – that did not invade the pulmonary blood vessels – were restricted to the lungs and did not spread to, or establish tumor masses in any other organs ***(Chapter 4, Figure 2)***. Based on these observations, we postulated that secondary metastases of TNBC cells to distant organs form mostly from a proportion of TNBC cells that first colonized the lungs before circulating further to other remote organs. These findings imply a strong molecular role for Kaiso in enabling TNBC cells to not only metastasize to the lungs and establish extensive macro-metastases, but also overcome inhibitory mechanisms in the lung that would normally prevent further metastatic spread and subsequent colonization of other organs.

7.2 Mechanistic roles of Kaiso in TNBC tumorigenesis and metastasis

7.2.1 Kaiso promotes TNBC cell proliferation in part via modulation of c-Myc expression

In addition to decreasing cyclin D1 expression as indicated in the previous section, Kaiso depletion also attenuated the expression of *c-Myc* in MDA-231 but not Hs578T cells ***(Chapter 3; Figure 1)***. This may explain the reduced proliferation of MDA-231 cells *in vivo* ***(Chapter 3, Figure 2)***, since c-Myc is a direct promoter of cell cycle progression and thus cell proliferation (Kreipe et al. 1993; Schmidt 1999). Notably, while aberrant expression of *cyclin D1* and *c-Myc* has been linked with the tumorigenesis of breast cancers (Liao and Dickson 2000; Roy and Thompson 2006), only *c-Myc* overexpression/amplification is associated with triple-negative breast tumors (Network 2012; Xu et al. 2010), and implicated in the high proliferative tendencies of breast tumors (Kreipe et al. 1993; Schmidt 1999).

The *c-Myc* gene is not amplified in MDA-231 and Hs578T cells (Hollestelle et al. 2010), suggesting that increased expression of c-Myc mRNA and/or increased stability of the protein could be the source of its aberrant overexpression in these cells. However, although we and others have shown that Kaiso directly binds to the *cyclin D1* promoter and transcriptionally modulates *cyclin D1* expression in mammalian cells (Donaldson et al. 2012; Pozner et al. 2016), Kaiso has thus far only been shown to indirectly regulate *c-MYC* expression in mammalian tissues (Koh et al. 2013). We show for the first time that Kaiso directly binds the *c-MYC*

promoter *in vivo* in a KBS-specific manner in both MDA-231 and Hs578T cells, although Kaiso's interaction with the *c-MYC* promoter was more apparent in MDA-231 than Hs578T cells ***(Appendix, Figure A2)***. We also observed a significant decrease in *c-MYC* transcript levels in the Kaiso-depleted MDA-231 cells ***(Appendix, Figure A2)***, which is consistent with the reduced c-Myc protein levels observed in these cells ***(Chapter 3, Figure 1 and 2)***. The consistent effect of Kaiso depletion on *c-Myc* expression in MDA-231 cells at the transcript and protein level ***(Chapter 3, Figure 1 and 2; Appendix, Figure A2)*** indicates that Kaiso may function to activate *c-MYC* expression in MDA-231 cells. However, the negligible effect of Kaiso depletion on *c-Myc* expression in Hs578T cells ***(Chapter 3, Figure 1)*** implies that Kaiso's positive regulation of *c-Myc* expression may be context dependent and unique to a subset of TNBC cells.

Kaiso's positive influence on c-Myc expression in MDA-231 cells may also be contingent upon yet unidentified interacting Kaiso-proteins or nuclear co-factors expressed in these cells. In support of this theory, we showed that Kaiso interacts with **mut**-p53, which activates c-Myc expression (Frazier et al. 1998), with more affinity in MDA-231 than Hs578T cells ***(Chapter 3, Figure 4)***. Remarkably, we observed that mut-p53 associated with the c-Myc promoter region containing the consensus Kaiso binding sequence – another novel finding from this study – with more affinity in MDA-231 than Hs578T cells (Appendix, Figure A2), signifying that Kaiso may recruit mut-p53 to the c-Myc promoter. However, it remains to be determined empirically whether Kaiso transcriptionally activates c-Myc

expression in MDA-231 cells independently or in concert with mut-p53. While outside the scope of this thesis, this possibility could be addressed by performing promoter-reporter luciferase assays using an endogenous *c-MYC* promoter sequence containing wildtype and mutated Kaiso binding sites, as previously done in the characterization of Kaiso's transcriptional regulation of *cyclin D1* (Donaldson et al. 2012).

7.2.2 Kaiso promotes TNBC cell survival via modulation of BRCA1 expression

As mentioned earlier, Kaiso depletion stimulated the apoptosis of TNBC cells, which correlated with increased PUMA transcripts and protein in these cells ***(Chapter 3, Figure 4)***. Since PUMA is a strong inducer of apoptosis in a variety of cancer cells including BCa cells (Hikisz and Kiliańska 2012; Nakano and Vousden 2001; Yu et al. 2001), our findings hint at a survival function for Kaiso in TNBC cells that is mediated via the suppression of PUMA expression. Indeed, Kaiso binds a minimal *PUMA* promoter region *in vivo*, and ectopic Kaiso expression results in decreased PUMA expression in TNBC cells ***(Chapter 3, Figure 4)***. However, Kaplan–Meier survival curves generated using survival information of TNBC patients or other BCa patients, revealed a decreased but insignificant effect of high Kaiso and low PUMA expression on the overall survival of TNBC and other BCa patients ***(Chapter 3, Figure 4)***. Considering that prolonged survival of triple negative tumors in affected patients predisposes to

metastasis and reduced survival (Carey et al. 2007; O'Reilly et al. 2015; Pareja Fresia et al. 2016), our data indicates that Kaiso's influence on TNBC survival may be mediated through its regulation of other gene(s) involved in cell death or survival.

An interesting finding from our analyses of transcript information available in public BCa datasets was that Kaiso expression positively correlated with the expression of multiple DNA repair genes including BRCA1 (our unpublished data, personal communication with Dr. Robin Hallett). This was unexpected but intriguing since although pathogenic inactivation of BRCA1 is observed in a large proportion of triple-negative breast tumors (Foulkes et al. 2010), a small fraction of TNBC express BRCA1, and this has been associated with the reduced survival rates of TNBC patients following neoadjuvant chemotherapy (Jiang et al. 2016). Remarkably, Kaiso-depleted MDA-231 and Hs578T cells displayed a reduction in BRCA1 mRNA and protein expression, which was restored upon ectopic Kaiso expression in these cells ***(Chapter 3, Figure 6)***. Our subsequent finding that Kaiso interacted with the endogenous BRCA1 promoter in these cells ***(Chapter 3, Figure 6)***, supported a role for Kaiso in the regulation of BRCA1 expression.

The BRCA1 protein plays a crucial role in the homologous recombination repair of DNA double-strand breaks and cross-links (Bhattacharyya et al. 2000; Bunting et al. 2012; Zhang 2013). Loss of BRCA1 protein expression is thus

accompanied by reduced homologous recombination frequency (Snouwaert et al. 1999), and unrepaired DNA damage (Brodie and Deng 2001), which leads to genomic instability and a higher risk of malignancy (Deng and Scott 2000). In fact, genetic alterations in BRCA1 is associated with approximately 45% of hereditary breast cancers (Ouchi et al. 1998) and a high proportion of sporadic breast tumors including those characterized as triple-negative (Foulkes et al. 2010; Gonzalez-Angulo et al. 2011; Wong-Brown et al. 2015). Despite the obvious tumor suppressive functions of BRCA1, some triple-negative BCa tumors retain BRCA1 expression as previously mentioned, and this is postulated to reduce their sensitivity to chemotherapeutic agents and consequently enhance their survival after chemotherapy (Jiang et al. 2016). Thus, we hypothesized that Kaiso promotes the survival of TNBC cells in part via positive regulation of BRCA1 expression. Consistent with this theory, Kaplan–Meier survival curves revealed that increased Kaiso and BRCA1 expression correlated significantly with reduced overall survival rates of TNBC patients ***(Chapter 3, Figure 7)***. We also observed reduced survival rates of patients with non-triple-negative breast tumors who exhibited high Kaiso and high BRCA1 expression ***(Chapter 3, Figure 7)***. This further underscored a role for high Kaiso and BRCA1 expression in breast tumor survival. However, it remains possible that Kaiso also influences the survival of triple-negative and other breast tumors via regulation of other yet unidentified genes.

7.2.3 Kaiso positively modulates TGFβ signaling and transcriptional responses

Mounting evidence indicates a crucial role for the EMT program in the metastasis of breast tumors (Wang and Zhou 2013). Phenotypically, EMT is associated with a spindle-like morphology, stemness, increased apoptotic resistance, as well as increased migratory and invasive characteristics (Thiery et al. 2009; Tomaskovic-Crook et al. 2009). At the molecular level, EMT is characterized by the loss of epithelial cytokeratins and cell adhesion proteins such as E-cadherin and ZO-1, which is accompanied by the concomitant upregulation of several mesenchymal proteins including the E-cadherin repressors Slug and ZEB1, the intermediate filament Vimentin, and the ECM protein-degrading enzymes **m**atrix **m**etallo**p**roteases (MMP)-2 and -9 (Lamouille et al. 2014; Tomaskovic-Crook et al. 2009). Most triple-negative breast tumors are characterized by an over-representation of genes/proteins associated with EMT (Karihtala et al. 2013; Lehmann et al. 2011), thus insinuating that EMT is partially responsible for the highly metastatic nature of these tumors. Indeed, several studies have shown that the metastasis of TNBC cells can be suppressed by the inhibition of the EMT phenotype exhibited by these cells (Leconet et al. 2017; Rhodes et al. 2015; Rhodes et al. 2014). Considering the link between Kaiso and EMT (Jones et al. 2014; Jones et al. 2012), it was not surprising that we observed an attenuation of the EMT phenotype and suppression of the metastatic ability of TNBC cells in

Kaiso-depleted cells ***(Chapter 4, Figures 1 and 2)***. Thus, Kaiso might promote TNBC metastasis in part via induction of the EMT phenotype in TNBC cells.

Our observation that Kaiso depletion affected multiple molecular changes associated with EMT – i.e. decreased expression of Vimentin, Slug, ZEB1 and MMP2, as well as increased expression of ZO-1 in MDA-231 and Hs578T cells, and increased E-cadherin in MDA-231 cells – ***Chapter 4, Figure 1; Chapter 5, Figure 7*** – led us to postulate that Kaiso may positively drive the activation of crucial signaling pathway(s) implicated in EMT induction. Consistent with our theory, we found a positive correlation between high Kaiso expression and increased expression of several signaling components associated with the TGFβ pathway, which is a well-known regulator of EMT (Gonzalez and Medici 2014; Talbot et al. 2012; Zavadil and Böttinger 2005) and highly implicated in BCa metastasis. Activation of the TGFβ signaling cascade is dependent in part on the expression of the TGFβ-receptors I and II (Ammanamanchi and Brattain 2004; Shi and Massagué 2003). Remarkably, we observed that Kaiso associates with a minimal TGFβRI and II promoter region *in vivo* in MDA-231 and Hs578T cells ***(Chapter 4, Figure 5)***. Furthermore, Kaiso inhibition led to the reduced expression of TGFβRI and II transcripts and protein in these cells, which was reversed upon ectopic expression of a sh-resistant Kaiso form in the Kaiso-depleted MDA-231 and Hs578T cells ***(Chapter 4, Figure 3)***. Notably, this is the first report implicating Kaiso in the regulation of TGFβRI and II expression.

The biological specificity of the TGFβ signaling cascade can be affected by differential expression (e.g. high vs low) of TGFβRI and II (Pannu et al. 2004; Rojas et al. 2009). We noticed a more elevated expression of TGFβRI transcripts compared to TGFβRII transcripts in control MDA-231 and Hs578T cells ***(Chapter 4, Figure 3)*** that displayed increased metastatic tendencies *in vivo*. This observation correlates with the data obtained from our analyses of transcript information deposited in the TCGA BCa database that showed a more positive relationship between Kaiso and TGFβRI compared to Kaiso and TGFβRII ***(Chapter 4, Figure 3)***. Based on these findings, we hypothesize that Kaiso promotes TNBC metastasis by facilitating a larger increase in TGFβRI than TGFβRII expression. In support of this theory, we demonstrated that increased Kaiso and TGFβRI but not TGFβRII expression correlated with reduced overall survival rates of BCa patients.

Given the importance of TGFβRI and TGFβRII expression in the activation of TGFβ signaling (Shi and Massagué 2003) and BCa metastasis (Oft et al. 1998; Tang et al. 2003; Yin et al. 1999), it was not surprising to observe that Kaiso depletion, which led to reduced TGFβRI and TGFβRII expression, also attenuated the activation of TGFβ signaling and key TGFβ pro-metastatic responses – e.g. induction of *ANGPTL4* (Padua et al. 2008) – in MDA-231 and Hs578T cells treated with TGFβ ***(Chapter 4, Figure 4)***. We were however

surprised to find that TGFβ treatment in control MDA-231 and Hs578T cells led to an increase in Kaiso expression at the transcript and protein level ***(Chapter 4, Figure 4 and Supp. Figure 3)***. This unexpected finding indicated for the first time that Kaiso expression may be positively regulated by the TGFβ signaling cascade. Since Kaiso also positively regulates the TGFβ signaling cascade ***(Chapter 4, Figure 4)***, a positive feedback loop may exist between Kaiso and active TGFβ signaling that in turn promotes TNBC metastasis. However, we do not discount the possibility that Kaiso may promote TNBC metastasis via regulation of other oncogenic pathways – a concept supported by our finding that overexpression of a constitutive active form of TGFβRI in the Kaiso-depleted MDA-231 cells, failed to fully rescue their metastatic capabilities ***(Chapter 4, Figure 7)***. This notion is also supported by our observation that the Kaiso-depleted MDA-231 cells exhibited reduced pERK1/2 ***(Appendix, Figure A3)*** – a key downstream effector of several growth factors and receptor tyrosine kinases (Katz et al. 2007) including HER2 and EGFR, which are implicated in breast tumorigenesis. Interestingly, EGFR is aberrantly overexpressed in ~45-70% of triple-negative tumors (Nielsen et al. 2004; Park et al. 2014).

7.3 A potential link between high Kaiso expression and TNBC prevalence in WAA

7.3.1 High nuclear Kaiso expression is correlated with increased percentage of African ancestry in TNBC patients

Epidemiological studies emphasize a high incidence of TNBC in premenopausal **w**omen of **A**frican **a**ncestry (WAA) compared to Caucasian American and British women (Agboola et al. 2012; Carey et al. 2006; Lund et al. 2009; Stark et al. 2010). However, the reason for this racial disparity remains unknown. Notably, a higher incidence of TNBC is observed in West African female populations (e.g. Ghanaians – 82.2%, Nigerians – 65%) compared to that seen in the **A**frican **A**merican (AA) female population (~33%) (Adisa et al. 2012; Carey et al. 2006; Stark et al. 2010), suggesting an inherited ancestral predisposition to TNBC in WAA, since West African countries like Ghana and Nigeria are the founder population of most WAA in the USA (Jackson 2008). Recent studies demonstrated a link between increased nuclear Kaiso expression and poor overall survival outcomes of AA BCa patients compared with their Caucasian American counterparts (Jones et al. 2014), which hint at a role for Kaiso in the racial disparity associated with BCa outcomes. However, it was unknown whether a correlation existed between high nuclear Kaiso expression and the racial disparity associated with TNBC prevalence. In this study, we report for the first time an increased expression of nuclear Kaiso in tumor tissues obtained from TNBC patients of African ancestry compared to Caucasian women ***(Chapter 6,***

Figure 4). Intriguingly, nuclear Kaiso was more highly expressed in TNBC tissues of patients with a higher degree of African ancestry (Nigerian) compared to those with a lower percentage of African heritage (Barbadian, AA) ***(Chapter 6, Figure 4)***. This hints at a correlation between high nuclear Kaiso expression, high African heritage and the predisposition of WAA to TNBC. To date, no established founder mutations or genetic susceptibility loci have been linked to the predisposition of WAA to TNBC. However, recent genomic studies identified a TNBC-specific single-nucleotide polymorphism in WAA (Palmer et al. 2013), and seven founder mutations in either BRCA1 or BRCA2 in a subset of Bahamian BCa patients (Akbari et al. 2014). It will therefore be interesting to determine if there is a genetic predisposition to increased Kaiso expression in WAA via a founder mutation in the Kaiso gene, and this is the subject of ongoing studies in the Daniel lab.

7.4 Outstanding research questions

This thesis has provided novel insights into the biological roles of Kaiso in the tumorigenesis and metastasis of triple-negative tumors, which may be conserved in other aggressive tumors that display increased Kaiso expression. However, many unanswered questions remain including: (i) the role of $p120^{ctn}$ if any in Kaiso-mediated tumorigenesis and metastasis; (ii) the expression pattern and levels of the Kaiso-like protein ZBTB4 in TNBC cells and (iii) the effect of ZBTB4 on the observed oncogenic functions of Kaiso in TNBC cells. The following

section will focus on some of these pertinent questions that remained unanswered after the completion.

7.4.1 Does Kaiso regulate p120^{ctn} expression?

While p120^{ctn} has been implicated in the subcellular localization of Kaiso (van Hengel et al. 1999), nothing is known about Kaiso's effect on p120^{ctn} expression levels or patterns. Previous studies by our group demonstrated increased nuclear localization of p120^{ctn} in the intestines following intestinal-specific Kaiso overexpression (Chaudhary et al. 2013), suggesting a role for Kaiso in p120^{ctn} subcellular localization. In this work, we found that Kaiso-depletion led to an increase in p120^{ctn} expression in MDA-231 cells but a small decrease in p120^{ctn} expression in Hs578T and MDA-157 cells ***(Chapter 3, Figure 4 and Supp. Figure 4),*** which hint at a possible cell context-dependent regulatory role for Kaiso in p120^{ctn} expression. Decreased or altered expression patterns of p120^{ctn} is linked with the metastasis and reduced survival outcomes of breast and other cancers (Bellovin et al. 2005; Chung et al. 2007; Fei et al. 2009; Talvinen et al. 2010; Wang et al. 2006). Thus, it will be worthwhile to determine the role of Kaiso in the regulation of p120^{ctn} expression levels and patterns, and if this has any effect on the oncogenic roles of Kaiso in triple-negative tumors especially those that lack E-cadherin expression.

7.4.2 Elucidating the downstream intracellular pathways/signaling molecules involved in TGFβ regulation of Kaiso expression

Most Kaiso-related studies to date have focused mainly on characterizing the functions downstream of Kaiso, but not upstream (Chaudhary et al. 2013; Pierre et al. 2015b; Prokhortchouk et al. 2006; Robinson et al. 2017; Spring et al. 2005). As such, not much is known about the molecular factors or pathways that regulate Kaiso expression or function, except for the recent implication of the EGFR signaling cascade in the regulation of Kaiso expression (Jones et al. 2012). In this work, we report for the first time that Kaiso expression is increased at the mRNA and protein level in response to TGFβ treatment – and thus activated TGFβ signaling – in MDA-231 and Hs578T cells ***(Chapter 4, Figure 4 and Supp. Figure 3)***. TGFβ is known to activate multiple intracellular signaling pathways (e.g. PI3/AKT and ERK1/2) in addition to the canonical Smad signaling cascade to carry out its cellular/biological functions. These non-Smad pathways function to either strengthen, mitigate or modulate various downstream TGFβ cellular responses, reviewed in (Zhang 2009). How and which signaling cascade TGFβ utilizes to regulate Kaiso expression remains a mystery, as addressing these questions was beyond the scope of this study. However, future studies utilizing minimal Kaiso promoter-reporter constructs are warranted to, at a minimum, delineate which upstream TGFβ pathway regulates Kaiso expression. These studies can also be expanded to identify/determine which transcription factors bind and regulate the Kaiso promoter.

7.4.3 Is the EGFR-ERK signaling pathway involved in the regulation of Kaiso expression in TNBC cells?

EGFR like TGFβ-receptors can activate multiple intracellular signaling pathways including the Ras-Raf-MEK-ERK signaling cascade, reviewed in (Arteaga and Engelman 2014). The downstream effector component of the Ras-ERK signaling pathway is pERK1/2 (Downward 2003), and both MDA-231 and Hs578T cells express high levels of pERK1/2 (Eckert et al. 2004; Lev et al. 2004). The EGFR signaling pathway is implicated in the regulation of Kaiso expression in PCa cells (Jones et al. 2012). However, it has not been determined whether the EGF signaling cascade regulates Kaiso expression in TNBC cells, or what signaling components downstream of EGFR promotes Kaiso expression. EGF treatment studies and future knockdown or overexpression studies of principal EGFR downstream signaling molecules should be conducted to delineate if, and which EGFR signaling cascade regulates Kaiso expression in TNBC cells. Notably, loss of Kaiso expression resulted in decreased pERK1/2 levels – which is synonymous with reduced Ras-ERK signaling – in MDA-231 cells ***(Appendix, Figure A3)***. This raises the question of whether there is a positive feedback loop between Kaiso expression and active Ras-ERK signaling in a subset of TNBC cells, which also warrants further investigation. Addressing these questions will help to elucidate the specific signaling pathways/components involved in the regulation of Kaiso expression in TNBC cells. It will also throw some light on whether the EGFR-Ras-ERK and TGFβ signaling pathways cooperate to regulate

Kaiso expression in triple-negative tumors, and if this is essential for Kaiso-mediated metastasis of TNBC – since increasing evidence from cell culture and animal studies indicates the relevance of combined Ras and TGFβ signaling in cancer metastasis, reviewed in (Grusch et al. 2010).

7.4.4 Is Kaiso involved in facilitating the pro-metastatic functions of TGFβ?

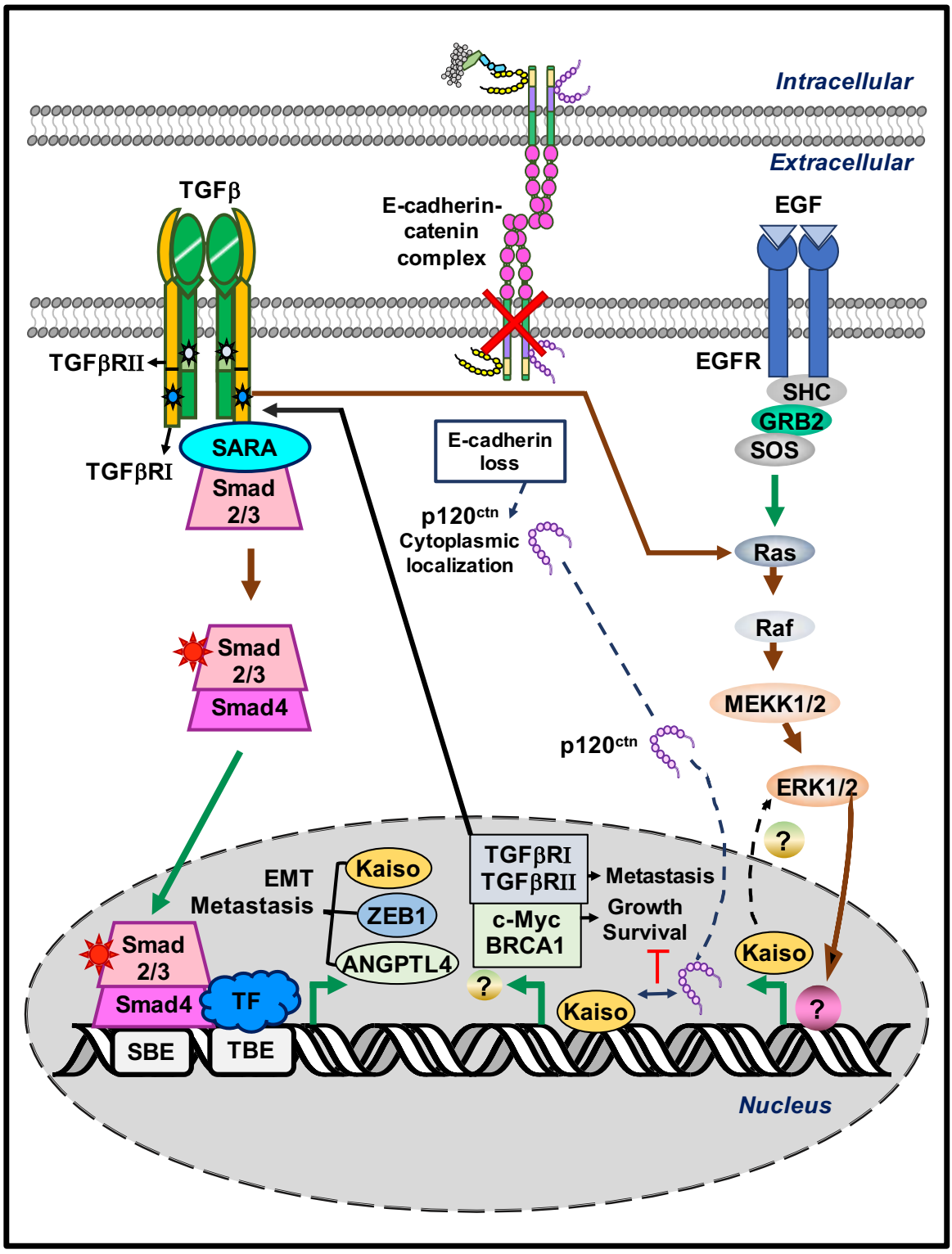
The TGFβ signaling pathway exhibit differential functions at the early and late stages of breast tumorigenesis. During the early stages of breast cancer, TGFβ functions as a potent suppressor of uncontrolled cell proliferation. However, in late-stage/advanced breast tumors, TGFβ becomes a powerful driver of invasion and metastasis, as the tumors are insensitive to TGFβ growth-inhibitory functions (Serra and Crowley 2003; Zarzynska 2014). The mechanism behind the shift in TGFβ functions is still been elucidated, but loss of C/EBPβ expression have been implicated in the abolition of TGFβ growth-inhibitory effects (Gomis et al. 2006). Our preliminary findings reveal that Kaiso binds the C/EBPβ promoter *in vivo*, and Kaiso depletion results in an increased expression of C/EBPβ mRNA and protein in MDA-231 cells ***(Appendix, Figure A4)***. This finding hint that Kaiso may negatively regulate C/EBPβ expression in MDA-231 cells, which in turn may contribute to the abrogation of TGFβ growth-inhibitory responses in these cells. However, whether Kaiso transcriptionally inhibits C/EBPβ expression remains to be determined empirically and should be addressed in future studies. Such information will have profound implications in the overall understanding of if, and

how Kaiso promotes the “switch” in TGFβ functions from a growth-suppressor to a driver of metastasis in TNBC cells.

7.5 Closing remarks

The past five years have seen a boost in studies that correlate increased Kaiso expression with the poor prognostic features of multiple human cancers including aggressive breast cancers. However, prior to this work, not much was known regarding the biological relevance of Kaiso in triple-negative breast tumors. The findings reported here shed significant light on the importance of Kaiso expression in triple-negative breast tumors, and offer novel insights into the molecular functions of Kaiso in the survival and metastasis of these tumors. Importantly, we demonstrated that Kaiso interacts with the BRCA1 promoter and regulates BRCA1 expression, which is associated with the chemo-resistance of a subset of TNBC cells. We also showed that Kaiso positively regulates the TGFβ signaling pathway and pro-metastatic transcriptional responses involved in TNBC metastasis. Collectively, these findings led us to generate a model of Kaiso’s role in the survival and metastasis of triple-negative tumors (Figure 7.1). While there are still many unanswered questions as highlighted above, our observations indicate an oncogenic role for Kaiso in the tumorigenesis and metastasis of triple-negative breast tumors.

Figure 7.1: Conceptual model of Kaiso's role in the tumorigenesis and metastasis of triple-negative tumors. The TGFβ signaling cascade activates the expression of Kaiso and other pro-metastatic proteins in triple-negative tumors. The increase in Kaiso expression by TGFβ could be via the Smad transcription complex or via phospho(p)-ERK1/2. Kaiso expression could also be activated by the EGFR-pERK1/2 signaling pathway in triple-negative tumors independently of TGFβ. The increased Kaiso expression then promotes the expression of c-Myc, BRCA1, TGFβRI and II, as well as other yet unidentified proteins independently or in collaboration with other cofactors, which then functions to drive the growth, survival and metastasis of triple-negative tumors. Kaiso might also function in a positive feedback loop to increase TGFβ signaling (via increased TGFβRI and II expression) and the EGFR-pERK1/2 pathway (via promoting increased p-ERK1/2 levels) through yet unidentifed mechanism(s). Kaiso's functions can however be modulated by Kaiso interaction with other proteins like nuclear $p120^{ctn}$.


Intracellular
Extracellular
TGFβ
E-cadherin-
catenin
complex
EGF
TGFβRII
EGFR
SHC
GRB2
SOS
SARA
TGFβRI
Smad
2/3
E-cadherin
loss
p120ctn
Cytoplasmic
localization
Ras
Raf
MEKK1/2
ERK1/2
Smad
2/3
Smad4
p120ctn
?
EMT
Metastasis
Kaiso
ZEB1
ANGPTL4
TGFβRI
TGFβRII
Metastasis
c-Myc
BRCA1
Growth
Survival
Kaiso
Smad
2/3
Smad4
TF
?
Kaiso
?
SBE
TBE
Nucleus

APPENDIX

Figure A1.1: Kaiso depletion did not delay the growth of Hs578T cells *in vivo* but increased their apoptosis. (A) Kaiso-depleted (sh-K) Hs578T xenografts displayed no delay in tumor onset and development when compared to control (Ctrl) Hs578T xenografted tumors as seen by time-course analysis of Ctrl and sh-K Hs578T tumor volumes. **(B)** IHC-stained images of Ctrl and sh-K Hs578T xenograft tissues with Ki-67 and PCNA antibodies show no marked difference in Ctrl and sh-K Hs578T cell proliferation. **(C)** IHC images of c-Caspase3 stained Ctrl and sh-K Hs578T xenograft tissues show that Kaiso depletion results in increased cell death of Hs578T tumor cells. Representative images shown.

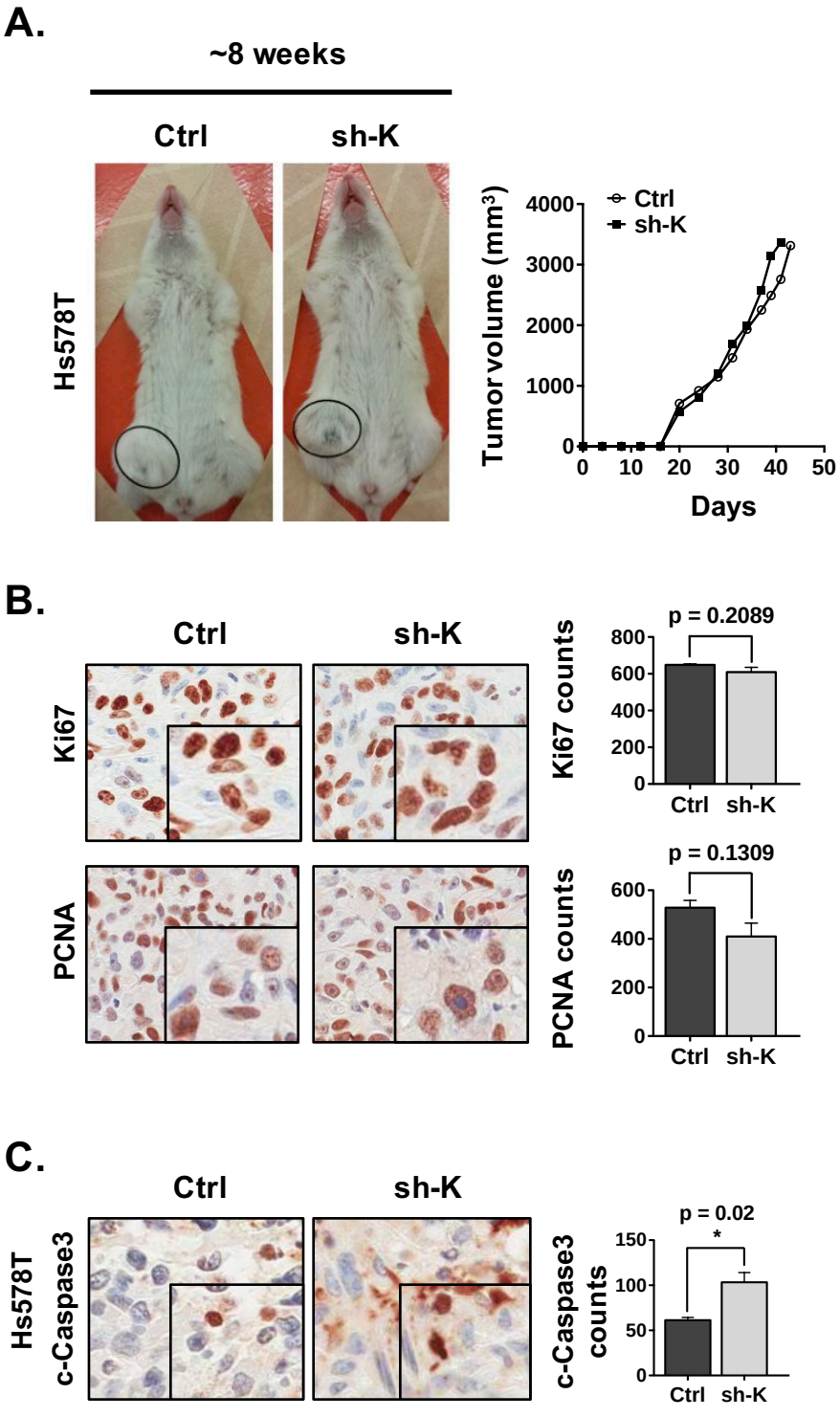

A.
~8 weeks
Ctrl
sh-K
Hs578T
Tumor volume (mm3)
Ctrl
sh-K
Days
B.
Ctrl
sh-K
Ki67
PCNA
Ki67 counts
PCNA counts
p = 0.2089
p = 0.1309
C.
Hs578T
c-Caspase3
c-Caspase3 counts
p = 0.02
*

Figure A2.1: Kaiso depletion results in decreased c-Myc transcripts in MDA-231 cells (A) Schematic depiction of the minimal *c-Myc* promoter region analyzed, which shows the position of the core KBS (cKBS) amplified by ChIP-PCR in MDA-231 cells. Both Kaiso and **m**utant(m)-p53 bound to KBS sites in the indicated *c-Myc* promoter region. **(B)** Data obtained from quantitative RT-PCR analysis showed that Kaiso depletion attenuates *c-Myc* expression in MDA-231 cells. Representative data shown from three independent experiments.

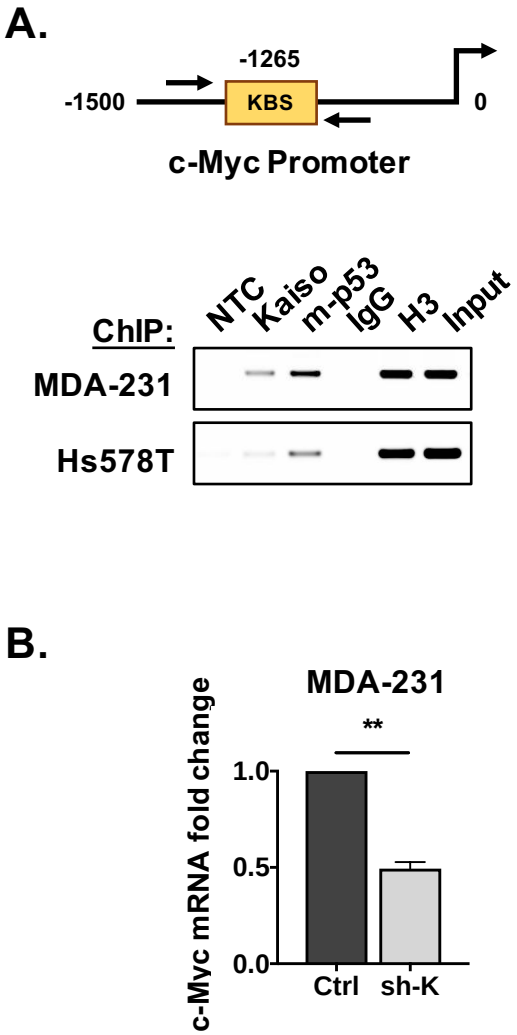

A.
-1265
-1500
KBS
0
c-Myc Promoter
ChIP:
NTC
Kaiso
m-p53
IgG
H3
Input
MDA-231
Hs578T
B.
MDA-231
**
c-Myc mRNA fold change
1.0
0.5
0.0
Ctrl
sh-K

Figure A3.1: Kaiso depletion results in reduced pERK1/2 expression Immunoblot analysis showing that Kaiso depletion results in reduced levels of phospho(p)-ERK1/2 in MDA-231 cells.

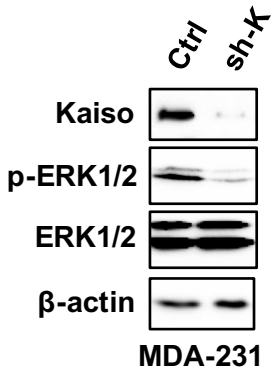
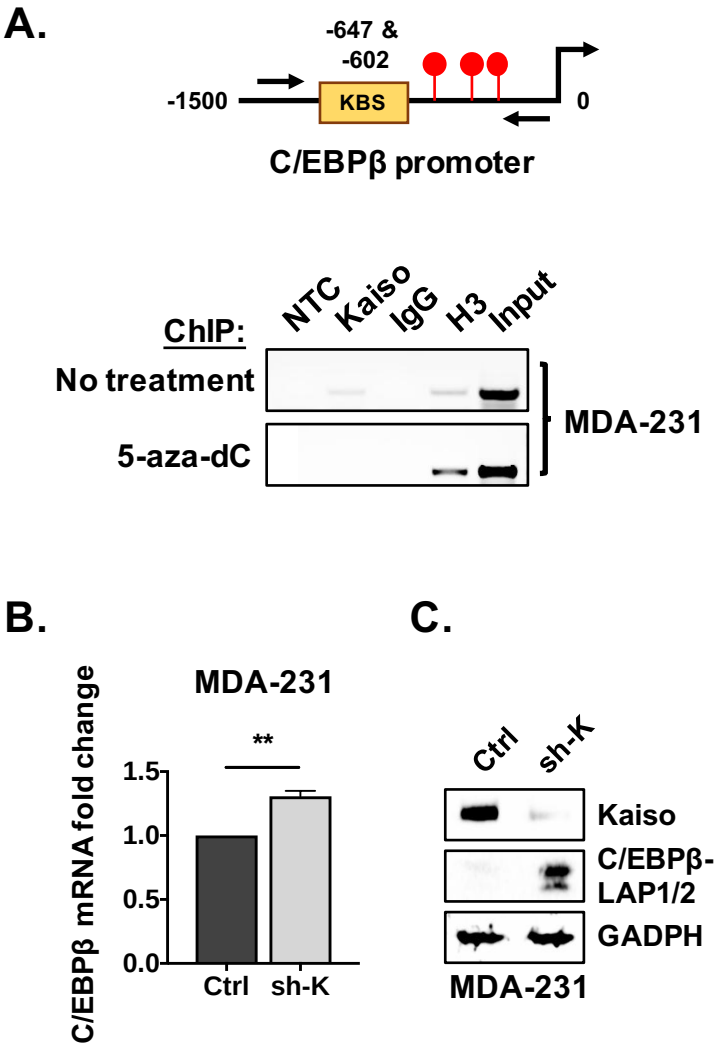

Ctrl
sh-K
Kaiso
p-ERK1/2
ERK1/2
β-actin
MDA-231

Figure A4.1: Kaiso depletion attenuates C/EBPβ expression in MDA-231 cells (A) Schematic illustration of the minimal C/EBPβ promoter region containing core KBS (cKBS) and CpG-dinucleotides that was amplified by ChIP-PCR in MDA-231 cells. Kaiso binds the C/EBPβ promoter region *in vivo* in a methyl-CpG dependent manner as Kaiso binding was lost upon 5-aza-dC treatment. Kaiso depletion resulted in an increased expression of C/EBPβ mRNA **(B)** and protein **(C)** in MDA-231 cells. Data representative of three independent experiments.


A.
-647 &
-602
-1500
KBS
0
C/EBPβ promoter
ChIP:
NTC
Kaiso
IgG
H3
Input
No treatment
5-aza-dC
MDA-231
B.
C.
MDA-231
**
C/EBPβ mRNA fold change
1.5
1.0
0.5
0.0
Ctrl
sh-K
Ctrl
sh-K
Kaiso
C/EBPβ-
LAP1/2
GADPH
MDA-231

References

Abe K, Takeichi M. 2008. Eplin mediates linkage of the cadherin catenin complex to f-actin and stabilizes the circumferential actin belt. Proc Natl Acad Sci U S A. 105(1):13-19.

Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K. 1999. Contribution of matrilysin (mmp-7) to the metastatic pathway of human colorectal cancers. Gut. 45(2):252-258.

Adisa CA, Eleweke N, Alfred AA, Campbell MJ, Sharma R, Nseyo O, Tandon V, Mukhtar R, Greninger A, Risi JD et al. 2012. Biology of breast cancer in nigerian women: A pilot study. Annals of African medicine. 11(3):169-175.

Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, Solari A, Bobisse S, Rondina MB, Guzzardo V et al. 2009. A mutant-p53/smad complex opposes p63 to empower tgfbeta-induced metastasis. Cell. 137(1):87-98.

Agboola AJ, Musa AA, Wanangwa N, Abdel-Fatah T, Nolan CC, Ayoade BA, Oyebadejo TY, Banjo AA, Deji-Agboola AM, Rakha EA et al. 2012. Molecular characteristics and prognostic features of breast cancer in nigerian compared with uk women. Breast cancer research and treatment. 135(2):555-569.

Aho S, Levänsuo L, Montonen O, Kari C, Rodeck U, Uitto J. 2002. Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells. J Cell Sci. 115(Pt 7):1391-1402.

Akbari MR, Donenberg T, Lunn J, Curling D, Turnquest T, Krill-Jackson E, Zhang S, Narod SA, Hurley J. 2014. The spectrum of brca1 and brca2 mutations in breast cancer patients in the bahamas. Clin Genet. 85(1):64-67.

Akhurst RJ, Hata A. 2012. Targeting the tgfbeta signalling pathway in disease. Nature reviewsDrug discovery. 11(10):790-811.

Ammanamanchi S, Brattain MG. 2004. Restoration of transforming growth factor-beta signaling through receptor ri induction by histone deacetylase activity inhibition in breast cancer cells. J Biol Chem. 279(31):32620-32625.

Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, Reynolds AB. 2000. Inhibition of rhoa by p120 catenin. Nat Cell Biol. 2(9):637-644.

Anders C, Carey LA. 2008. Understanding and treating triple-negative breast cancer. Oncology (Williston Park). 22(11):1233-1239; discussion 1239-1240, 1243.

Arteaga CL, Engelman JA. 2014. Erbb receptors: From oncogene discovery to basic science to mechanism-based cancer therapeutics. Cancer Cell. 25(3):282-303.

Ayer DE, Eisenman RN. 1993. A switch from myc:Max to mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. Genes Dev. 7(11):2110-2119.

Bae Y-H, Shin J-M, Park H-J, Jang H-O, Bae M-K, Bae S-K. 2014. Gain-of-function mutant p53-r280k mediates survival of breast cancer cells. Genes & Genomics. 36(2):171-178.

Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. 1993. Cyclin d1 is a nuclear protein required for cell cycle progression in g1. Genes Dev. 7(5):812-821.

Bandyopadhyay A, Agyin JK, Wang L, Tang Y, Lei X, Story BM, Cornell JE, Pollock BH, Mundy GR, Sun LZ. 2006. Inhibition of pulmonary and skeletal metastasis by a transforming growth factor-beta type i receptor kinase inhibitor. Cancer Res. 66(13):6714-6721.

Barcellos-Hoff MH, Ewan KB. 2000. Transforming growth factor-beta and breast cancer: Mammary gland development. Breast Cancer Res. 2(2):92-99.

Barrett CW, Smith JJ, Lu LC, Markham N, Stengel KR, Short SP, Zhang B, Hunt AA, Fingleton BM, Carnahan RH et al. 2012. Kaiso directs the transcriptional corepressor mtg16 to the kaiso binding site in target promoters. PLoS One. 7(12):e51205.

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

Beavon IR. 2000. The e-cadherin-catenin complex in tumour metastasis: Structure, function and regulation. Eur J Cancer. 36(13 Spec No):1607-1620.

Bellovin DI, Bates RC, Muzikansky A, Rimm DL, Mercurio AM. 2005. Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease. Cancer Res. 65(23):10938-10945.

Benito M, Parker J, Du Q, Wu J, Xiang D, Perou CM, Marron JS. 2004. Adjustment of systematic microarray data biases. Bioinformatics. 20(1):105-114.

Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. 2000. The breast cancer susceptibility gene brca1 is required for subnuclear assembly of rad51 and survival following treatment with the dna cross-linking agent cisplatin. J Biol Chem. 275(31):23899-23903.

Bhola NE, Balko JM, Dugger TC, Kuba MG, Sánchez V, Sanders M, Stanford J, Cook RS, Arteaga CL. 2013. Tgf-β inhibition enhances chemotherapy action against triple-negative breast cancer. J Clin Invest. 123(3):1348-1358.

Bienz M, Clevers H. 2000. Linking colorectal cancer to wnt signaling. Cell. 103(2):311-320.

Blattler A, Yao L, Wang Y, Ye Z, Jin VX, Farnham PJ. 2013. Zbtb33 binds unmethylated regions of the genome associated with actively expressed genes. Epigenetics & chromatin. 6(1):13-8935-8936-8913.

Borellini F, Oka T. 1989. Growth control and differentiation in mammary epithelial cells. Environ Health Perspect. 80:85-99.

Braicu C, Pileczki V, Irimie A, Berindan-Neagoe I. 2013. P53sirna therapy reduces cell proliferation, migration and induces apoptosis in triple negative breast cancer cells. Mol Cell Biochem. 381(1-2):61-68.

Bray F, McCarron P, Parkin DM. 2004. The changing global patterns of female breast cancer incidence and mortality. Breast Cancer Res. 6(6):229-239.

Brodie SG, Deng CX. 2001. Brca1-associated tumorigenesis: What have we learned from knockout mice? Trends Genet. 17(10):S18-22.

Buck-Koehntop BA, Martinez-Yamout MA, Dyson HJ, Wright PE. 2012. Kaiso uses all three zinc fingers and adjacent sequence motifs for high affinity binding to sequence-specific and methyl-cpg dna targets. FEBS Lett. 586(6):734-739.

Bunting SF, Callén E, Kozak ML, Kim JM, Wong N, López-Contreras AJ, Ludwig T, Baer R, Faryabi RB, Malhowski A et al. 2012. Brca1 functions independently of homologous recombination in dna interstrand crosslink repair. Mol Cell. 46(2):125-135.

Burness ML, Grushko TA, Olopade OI. 2010. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: Promising clinical target or only a marker? Cancer J. 16(1):23-32.

Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML, Perou CM. 2007. The triple negative paradox: Primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res. 13(8):2329-2334.

Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S et al. 2006. Race, breast cancer subtypes, and survival in the carolina breast cancer study. JAMA. 295(21):2492-2502.

Chambers AF, Groom AC, MacDonald IC. 2002. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2(8):563-572.

Chambers AF, Matrisian LM. 1997. Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst. 89(17):1260-1270.

Chaudhary R, Pierre CC, Nanan K, Wojtal D, Morone S, Pinelli C, Wood GA, Robine S, Daniel JM. 2013. The poz-zf transcription factor kaiso (zbtb33) induces inflammation and progenitor cell differentiation in the murine intestine. PloS one. 8(9):e74160.

Chen CR, Kang Y, Massagué J. 2001. Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. Proc Natl Acad Sci U S A. 98(3):992-999.

Chen CR, Kang Y, Siegel PM, Massagué J. 2002. E2f4/5 and p107 as smad cofactors linking the tgfbeta receptor to c-myc repression. Cell. 110(1):19-32.

Chen H, Paradies NE, Fedor-Chaiken M, Brackenbury R. 1997a. E-cadherin mediates adhesion and suppresses cell motility via distinct mechanisms. J Cell Sci. 110 (Pt 3):345-356.

Chen YG, Liu F, Massague J. 1997b. Mechanism of tgfbeta receptor inhibition by fkbp12. EMBO J. 16(13):3866-3876.

Christiansen JJ, Rajasekaran AK. 2006. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. Cancer research. 66(17):8319-8326.

Christofori G. 2006. New signals from the invasive front. Nature. 441(7092):444-450.

Chung Y, Lam AK, Luk JM, Law S, Chan KW, Lee PY, Wong J. 2007. Altered e-cadherin expression and p120 catenin localization in esophageal squamous cell carcinoma. Ann Surg Oncol. 14(11):3260-3267.

Claassen GF, Hann SR. 2000. A role for transcriptional repression of p21cip1 by c-myc in overcoming transforming growth factor beta -induced cell-cycle arrest. Proc Natl Acad Sci U S A. 97(17):9498-9503.

Cofre J, Menezes JR, Pizzatti L, Abdelhay E. 2012. Knock-down of kaiso induces proliferation and blocks granulocytic differentiation in blast crisis of chronic myeloid leukemia. Cancer cell international. 12(1):28-2867-2812-2828.

Collina F, Di Bonito M, Li Bergolis V, De Laurentiis M, Vitagliano C, Cerrone M, Nuzzo F, Cantile M, Botti G. 2015. Prognostic value of cancer stem cells markers in triple-negative breast cancer. Biomed Res Int. 2015:158682.

Coughlin SS, Ekwueme DU. 2009. Breast cancer as a global health concern. Cancer Epidemiol. 33(5):315-318.

Coussens LM, Werb Z. 2002. Inflammation and cancer. Nature. 420(6917):860-867.

Covington KR, Fuqua SA. 2014. Role of mta2 in human cancer. Cancer Metastasis Rev. 33(4):921-928.

Dai SD, Wang Y, Miao Y, Zhao Y, Zhang Y, Jiang GY, Zhang PX, Yang ZQ, Wang EH. 2009. Cytoplasmic kaiso is associated with poor prognosis in non-small cell lung cancer. BMC cancer. 9:178.

Dai SD, Wang Y, Zhang JY, Zhang D, Zhang PX, Jiang GY, Han Y, Zhang S, Cui QZ, Wang EH. 2011. Upregulation of δ-catenin is associated with poor prognosis and enhances transcriptional activity through kaiso in non-small-cell lung cancer. Cancer Sci. 102(1):95-103.

Dai Y, Wilson G, Huang B, Peng M, Teng G, Zhang D, Zhang R, Ebert MP, Chen J, Wong BC et al. 2014. Silencing of jagged1 inhibits cell growth and invasion in colorectal cancer. Cell Death Dis. 5:e1170.

Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE, Wonsey D, Zeller K. 1999. Function of the c-myc oncogenic transcription factor. Exp Cell Res. 253(1):63-77.

Daniel CW, Silberstein GB, Van Horn K, Strickland P, Robinson S. 1989. Tgf-beta 1-induced inhibition of mouse mammary ductal growth: Developmental specificity and characterization. Dev Biol. 135(1):20-30.

Daniel JM. 2007. Dancing in and out of the nucleus: P120(ctn) and the transcription factor kaiso. Biochimica et biophysica acta. 1773(1):59-68.

Daniel JM, Ireton RC, Reynolds AB. 2001. Monoclonal antibodies to kaiso: A novel transcription factor and p120ctn-binding protein. Hybridoma. 20(3):159-166.

Daniel JM, Reynolds AB. 1995. The tyrosine kinase substrate p120cas binds directly to e-cadherin but not to the adenomatous polyposis coli protein or alpha-catenin. Mol Cell Biol. 15(9):4819-4824.

Daniel JM, Reynolds AB. 1999. The catenin p120(ctn) interacts with kaiso, a novel btb/poz domain zinc finger transcription factor. Molecular and cellular biology. 19(5):3614-3623.

Daniel JM, Spring CM, Crawford HC, Reynolds AB, Baig A. 2002. The p120(ctn)-binding partner kaiso is a bi-modal dna-binding protein that recognizes both a sequence-specific consensus and methylated cpg dinucleotides. Nucleic acids research. 30(13):2911-2919.

Davis JN, McGhee L, Meyers S. 2003a. The eto (mtg8) gene family. Gene. 303:1-10.

Davis MA, Ireton RC, Reynolds AB. 2003b. A core function for p120-catenin in cadherin turnover. J Cell Biol. 163(3):525-534.

Defossez PA, Kelly KF, Filion GJ, Perez-Torrado R, Magdinier F, Menoni H, Nordgaard CL, Daniel JM, Gilson E. 2005. The human enhancer blocker ctc-binding factor interacts with the transcription factor kaiso. The Journal of biological chemistry. 280(52):43017-43023.

Della Ragione F, Tiunova A, Vacca M, Strazzullo M, González E, Armstrong J, Valero R, Campanile C, Pineda M, Hulten M et al. 2006. The x-linked methyl binding protein gene kaiso is highly expressed in brain but is not mutated in rett syndrome patients. Gene. 373:83-89.

Deng CX, Scott F. 2000. Role of the tumor suppressor gene brca1 in genetic stability and mammary gland tumor formation. Oncogene. 19(8):1059-1064.

Dent R, Hanna WM, Trudeau M, Rawlinson E, Sun P, Narod SA. 2009. Pattern of metastatic spread in triple-negative breast cancer. Breast Cancer Res Treat. 115(2):423-428.

Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA. 2007. Triple-negative breast cancer: Clinical features and patterns of recurrence. Clin Cancer Res. 13(15 Pt 1):4429-4434.

DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA, Jemal A. 2016. Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. CA Cancer J Clin. 66(1):31-42.

Desmedt C, Di Leo A, de Azambuja E, Larsimont D, Haibe-Kains B, Selleslags J, Delaloge S, Duhem C, Kains JP, Carly B et al. 2011. Multifactorial approach to predicting resistance to anthracyclines. J Clin Oncol. 29(12):1578-1586.

Dietze EC, Sistrunk C, Miranda-Carboni G, O'Regan R, Seewaldt VL. 2015. Triple-negative breast cancer in african-american women: Disparities versus biology. Nat Rev Cancer. 15(4):248-254.

Ding MJ, Su KE, Cui GZ, Yang WH, Chen L, Yang M, Liu YQ, Dai DL. 2016. Association between transforming growth factor-β1 expression and the clinical features of triple negative breast cancer. Oncol Lett. 11(6):4040-4044.

Donaldson NS, Nordgaard CL, Pierre CC, Kelly KF, Robinson SC, Swystun L, Henriquez R, Graham M, Daniel JM. 2010. Kaiso regulates znf131-mediated transcriptional activation. Experimental cell research. 316(10):1692-1705.

Donaldson NS, Pierre CC, Anstey MI, Robinson SC, Weerawardane SM, Daniel JM. 2012. Kaiso represses the cell cycle gene cyclin d1 via sequence-specific and methyl-cpg-dependent mechanisms. PloS one. 7(11):e50398.

Downward J. 2003. Targeting ras signalling pathways in cancer therapy. Nat Rev Cancer. 3(1):11-22.

Eckert LB, Repasky GA, Ulkü AS, McFall A, Zhou H, Sartor CI, Der CJ. 2004. Involvement of ras activation in human breast cancer cell signaling, invasion, and anoikis. Cancer Res. 64(13):4585-4592.

Ewan KB, Shyamala G, Ravani SA, Tang Y, Akhurst R, Wakefield L, Barcellos-Hoff MH. 2002. Latent transforming growth factor-beta activation in mammary gland: Regulation by ovarian hormones affects ductal and alveolar proliferation. Am J Pathol. 160(6):2081-2093.

Fei Y, Cheng Z, Liu S, Liu X, Ge Z, Wang F, Zong G, Wang W. 2009. Expression and clinical significance of p120 catenin mrna and protein in pancreatic carcinoma. Bosn J Basic Med Sci. 9(3):191-197.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. 2015. Cancer incidence and mortality worldwide: Sources, methods and major patterns in globocan 2012. Int J Cancer. 136(5):E359-386.

Filion GJ, Zhenilo S, Salozhin S, Yamada D, Prokhortchouk E, Defossez PA. 2006. A family of human zinc finger proteins that bind methylated dna and repress transcription. Mol Cell Biol. 26(1):169-181.

Forbes JF. 1997. The incidence of breast cancer: The global burden, public health considerations. Semin Oncol. 24(1 Suppl 1):S1-20-S21-35.

Foulkes WD, Smith IE, Reis-Filho JS. 2010. Triple-negative breast cancer. The New England journal of medicine. 363(20):1938-1948.

Frazier MW, He X, Wang J, Gu Z, Cleveland JL, Zambetti GP. 1998. Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete c-terminal domain. Mol Cell Biol. 18(7):3735-3743.

Frederick JP, Liberati NT, Waddell DS, Shi Y, Wang XF. 2004. Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of smad3 to a novel repressive smad binding element. Mol Cell Biol. 24(6):2546-2559.

Gabay M, Li Y, Felsher DW. 2014. Myc activation is a hallmark of cancer initiation and maintenance. Cold Spring Harb Perspect Med. 4(6).

Galiatsatos P, Foulkes WD. 2006. Familial adenomatous polyposis. Am J Gastroenterol. 101(2):385-398.

Gall TM, Frampton AE. 2013. Gene of the month: E-cadherin (cdh1). J Clin Pathol. 66(11):928-932.

Ganapathy V, Ge R, Grazioli A, Xie W, Banach-Petrosky W, Kang Y, Lonning S, McPherson J, Yingling JM, Biswas S et al. 2010. Targeting the transforming growth factor-beta pathway inhibits human basal-like breast cancer metastasis. Mol Cancer. 9:122.

Giehl K, Menke A. 2006. Moving on: Molecular mechanisms in tgf b -induced epithelial cell migration. Signal Transduction. 6(5):355 - 364.

Giepmans BN, van Ijzendoorn SC. 2009. Epithelial cell-cell junctions and plasma membrane domains. Biochim Biophys Acta. 1788(4):820-831.

Gjorevski N, Nelson CM. 2011. Integrated morphodynamic signalling of the mammary gland. Nat Rev Mol Cell Biol. 12(9):581-593.

Gomis RR, Alarcón C, Nadal C, Van Poznak C, Massagué J. 2006. C/ebpbeta at the core of the tgfbeta cytostatic response and its evasion in metastatic breast cancer cells. Cancer Cell. 10(3):203-214.

Gonzalez DM, Medici D. 2014. Signaling mechanisms of the epithelial-mesenchymal transition. Sci Signal. 7(344):re8.

Gonzalez-Angulo AM, Timms KM, Liu S, Chen H, Litton JK, Potter J, Lanchbury JS, Stemke-Hale K, Hennessy BT, Arun BK et al. 2011. Incidence and outcome of brca mutations in unselected patients with triple receptor-negative breast cancer. Clin Cancer Res. 17(5):1082-1089.

Gottardi CJ, Wong E, Gumbiner BM. 2001. E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. J Cell Biol. 153(5):1049-1060.

Grosheva I, Shtutman M, Elbaum M, Bershadsky AD. 2001. P120 catenin affects cell motility via modulation of activity of rho-family gtpases: A link between cell-cell contact formation and regulation of cell locomotion. J Cell Sci. 114(Pt 4):695-707.

Grusch M, Petz M, Metzner T, Oztürk D, Schneller D, Mikulits W. 2010. The crosstalk of ras with the tgf-β family during carcinoma progression and its implications for targeted cancer therapy. Curr Cancer Drug Targets. 10(8):849-857.

Guinebretière JM, Menet E, Tardivon A, Cherel P, Vanel D. 2005. Normal and pathological breast, the histological basis. Eur J Radiol. 54(1):6-14.

Halbleib JM, Nelson WJ. 2006. Cadherins in development: Cell adhesion, sorting, and tissue morphogenesis. Genes Dev. 20(23):3199-3214.

Heldin CH, Miyazono K, ten Dijke P. 1997. Tgf-beta signalling from cell membrane to nucleus through smad proteins. Nature. 390(6659):465-471.

Hikisz P, Kiliańska ZM. 2012. Puma, a critical mediator of cell death--one decade on from its discovery. Cell Mol Biol Lett. 17(4):646-669.

Hollestelle A, Nagel JH, Smid M, Lam S, Elstrodt F, Wasielewski M, Ng SS, French PJ, Peeters JK, Rozendaal MJ et al. 2010. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. Breast Cancer Res Treat. 121(1):53-64.

Hong JY, Oh IH, McCrea PD. 2016. Phosphorylation and isoform use in p120-catenin during development and tumorigenesis. Biochim Biophys Acta. 1863(1):102-114.

Hosking CR, Ulloa F, Hogan C, Ferber EC, Figueroa A, Gevaert K, Birchmeier W, Briscoe J, Fujita Y. 2007. The transcriptional repressor glis2 is a novel binding partner for p120 catenin. Mol Biol Cell. 18(5):1918-1927.

Huang RY, Guilford P, Thiery JP. 2012. Early events in cell adhesion and polarity during epithelial-mesenchymal transition. J Cell Sci. 125(Pt 19):4417-4422.

Huang Y, Lin L, Shanker A, Malhotra A, Yang L, Dikov MM, Carbone DP. 2011. Resuscitating cancer immunosurveillance: Selective stimulation of dll1-notch signaling in t cells rescues t-cell function and inhibits tumor growth. Cancer Res. 71(19):6122-6131.

Iioka H, Doerner SK, Tamai K. 2009. Kaiso is a bimodal modulator for wnt/beta-catenin signaling. FEBS letters. 583(4):627-632.

Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L et al. 2002. A novel role for p120 catenin in e-cadherin function. The Journal of cell biology. 159(3):465-476.

Irshad S, Ellis P, Tutt A. 2011. Molecular heterogeneity of triple-negative breast cancer and its clinical implications. Current opinion in oncology. 23(6):566-577.

Ishiyama N, Lee SH, Liu S, Li GY, Smith MJ, Reichardt LF, Ikura M. 2010. Dynamic and static interactions between p120 catenin and e-cadherin regulate the stability of cell-cell adhesion. Cell. 141(1):117-128.

Ismail-Khan R, Bui MM. 2010. A review of triple-negative breast cancer. Cancer control : journal of the Moffitt Cancer Center. 17(3):173-176.

Ivanov DB, Philippova MP, Tkachuk VA. 2001. Structure and functions of classical cadherins. Biochemistry (Mosc). 66(10):1174-1186.

Jackson FL. 2008. Ancestral links of chesapeake bay region african americans to specific bight of bonny (west africa) microethnic groups and increased frequency of aggressive breast cancer in both regions. Am J Hum Biol. 20(2):165-173.

Jiang G, Wang Y, Dai S, Liu Y, Stoecker M, Wang E. 2012. P120-catenin isoforms 1 and 3 regulate proliferation and cell cycle of lung cancer cells via beta-catenin and kaiso respectively. PloS one. 7(1):e30303.

Jiang T, Shi W, Wali VB, Pongor LS, Li C, Lau R, Győrffy B, Lifton RP, Symmans WF, Pusztai L et al. 2016. Predictors of chemosensitivity in triple negative breast cancer: An integrated genomic analysis. PLoS Med. 13(12):e1002193.

Johansson J, Berg T, Kurzejamska E, Pang MF, Tabor V, Jansson M, Roswall P, Pietras K, Sund M, Religa P et al. 2013. Mir-155-mediated loss of c/ebpβ shifts the tgf-β response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer. Oncogene. 32(50):5614-5624.

Jones J, Mukherjee A, Karanam B, Davis M, Jaynes J, Reams RR, Dean-Colomb W, Yates C. 2016. African americans with pancreatic ductal adenocarcinoma exhibit gender differences in kaiso expression. Cancer Lett. 380(2):513-522.

Jones J, Wang H, Karanam B, Theodore S, Dean-Colomb W, Welch DR, Grizzle W, Yates C. 2014. Nuclear localization of kaiso promotes the poorly differentiated phenotype and emt in infiltrating ductal carcinomas. Clinical & experimental metastasis. 31(5):497-510.

Jones J, Wang H, Zhou J, Hardy S, Turner T, Austin D, He Q, Wells A, Grizzle WE, Yates C. 2012. Nuclear kaiso indicates aggressive prostate cancers and promotes migration and invasiveness of prostate cancer cells. The American journal of pathology. 181(5):1836-1846.

Jung P, Hermeking H. 2009. The c-myc-ap4-p21 cascade. Cell Cycle. 8(7):982-989.

Jäger R, Herzer U, Schenkel J, Weiher H. 1997. Overexpression of bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. Oncogene. 15(15):1787-1795.

Kalluri R, Weinberg RA. 2009. The basics of epithelial-mesenchymal transition. J Clin Invest. 119(6):1420-1428.

Kang Y, He W, Tulley S, Gupta GP, Serganova I, Chen CR, Manova-Todorova K, Blasberg R, Gerald WL, Massagué J. 2005. Breast cancer bone metastasis mediated by the smad tumor suppressor pathway. Proc Natl Acad Sci U S A. 102(39):13909-13914.

Kao KJ, Chang KM, Hsu HC, Huang AT. 2011. Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: Implications for treatment optimization. BMC Cancer. 11:143.

Karihtala P, Auvinen P, Kauppila S, Haapasaari KM, Jukkola-Vuorinen A, Soini Y. 2013. Vimentin, zeb1 and sip1 are up-regulated in triple-negative and basal-like breast cancers: Association with an aggressive tumour phenotype. Breast cancer research and treatment. 138(1):81-90.

Katz M, Amit I, Yarden Y. 2007. Regulation of mapks by growth factors and receptor tyrosine kinases. Biochim Biophys Acta. 1773(8):1161-1176.

Kelly KF, Daniel JM. 2006. Poz for effect--poz-zf transcription factors in cancer and development. Trends in cell biology. 16(11):578-587.

Kelly KF, Otchere AA, Graham M, Daniel JM. 2004a. Nuclear import of the btb/poz transcriptional regulator kaiso. Journal of cell science. 117(Pt 25):6143-6152.

Kelly KF, Spring CM, Otchere AA, Daniel JM. 2004b. Nls-dependent nuclear localization of p120ctn is necessary to relieve kaiso-mediated transcriptional repression. Journal of cell science. 117(Pt 13):2675-2686.

Kim SW, Fang X, Ji H, Paulson AF, Daniel JM, Ciesiolka M, van Roy F, McCrea PD. 2002. Isolation and characterization of xkaiso, a transcriptional repressor that associates with the catenin xp120(ctn) in xenopus laevis. J Biol Chem. 277(10):8202-8208.

Kim SW, Park JI, Spring CM, Sater AK, Ji H, Otchere AA, Daniel JM, McCrea PD. 2004. Non-canonical wnt signals are modulated by the kaiso transcriptional repressor and p120-catenin. Nature cell biology. 6(12):1212-1220.

Kimelman D, Xu W. 2006. Beta-catenin destruction complex: Insights and questions from a structural perspective. Oncogene. 25(57):7482-7491.

Koh DI, An H, Kim MY, Jeon BN, Choi SH, Hur SS, Hur MW. 2015. Transcriptional activation of apaf1 by kaiso (zbtb33) and p53 is attenuated by rela/p65. Biochim Biophys Acta. 1849(9):1170-1178.

Koh DI, Han D, Ryu H, Choi WI, Jeon BN, Kim MK, Kim Y, Kim JY, Parry L, Clarke AR et al. 2014. Kaiso, a critical regulator of p53-mediated transcription of cdkn1a and apoptotic genes. Proc Natl Acad Sci U S A. 111(42):15078-15083.

Koh DI, Yoon JH, Kim MK, An H, Kim MY, Hur MW. 2013. Kaiso is a key regulator of spleen germinal center formation by repressing bcl6 expression in splenocytes. Biochem Biophys Res Commun. 442(3-4):177-182.

Koop S, Schmidt EE, MacDonald IC, Morris VL, Khokha R, Grattan M, Leone J, Chambers AF, Groom AC. 1996. Independence of metastatic ability and extravasation: Metastatic ras-transformed and control fibroblasts extravasate equally well. Proc Natl Acad Sci U S A. 93(20):11080-11084.

Kreipe H, Feist H, Fischer L, Felgner J, Heidorn K, Mettler L, Parwaresch R. 1993. Amplification of c-myc but not of c-erbb-2 is associated with high proliferative capacity in breast cancer. Cancer Res. 53(8):1956-1961.

Kretzschmar M. 2000. Transforming growth factor-beta and breast cancer: Transforming growth factor-beta/smad signaling defects and cancer. Breast Cancer Res. 2(2):107-115.

Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. 2012. Tgf-β - an excellent servant but a bad master. J Transl Med. 10:183.

Lakhtakia R. 2014. A brief history of breast cancer: Part i: Surgical domination reinvented. Sultan Qaboos Univ Med J. 14(2):e166-169.

Lamouille S, Xu J, Derynck R. 2014. Molecular mechanisms of epithelial-mesenchymal transition. Nature reviewsMolecular cell biology. 15(3):178-196.

Leconet W, Chentouf M, du Manoir S, Chevalier C, Sirvent A, Aït-Arsa I, Busson M, Jarlier M, Radosevic-Robin N, Theillet C et al. 2017. Therapeutic

activity of anti-axl antibody against triple-negative breast cancer patient-derived xenografts and metastasis. Clin Cancer Res. 23(11):2806-2816.

Lee M, Ji H, Furuta Y, Park JI, McCrea PD. 2014. P120-catenin regulates rest and corest, and modulates mouse embryonic stem cell differentiation. J Cell Sci. 127(Pt 18):4037-4051.

Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, Pietenpol JA. 2011. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 121(7):2750-2767.

Lev DC, Kim LS, Melnikova V, Ruiz M, Ananthaswamy HN, Price JE. 2004. Dual blockade of egfr and erk1/2 phosphorylation potentiates growth inhibition of breast cancer cells. Br J Cancer. 91(4):795-802.

Li B, Dewey CN. 2011. Rsem: Accurate transcript quantification from rna-seq data with or without a reference genome. BMC Bioinformatics. 12:323.

Li Y, Zou L, Li Q, Haibe-Kains B, Tian R, Desmedt C, Sotiriou C, Szallasi Z, Iglehart JD, Richardson AL et al. 2010. Amplification of laptm4b and ywhaz contributes to chemotherapy resistance and recurrence of breast cancer. Nat Med. 16(2):214-218.

Liao DJ, Dickson RB. 2000. C-myc in breast cancer. Endocr Relat Cancer. 7(3):143-164.

Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M et al. 2008. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol. 26(8):1275-1281.

Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A et al. 2009a. Aberrant luminal progenitors as the candidate target population for basal tumor development in brca1 mutation carriers. Nat Med. 15(8):907-913.

Lim LY, Vidnovic N, Ellisen LW, Leong CO. 2009b. Mutant p53 mediates survival of breast cancer cells. Br J Cancer. 101(9):1606-1612.

Liu Y, Dong QZ, Wang S, Xu HT, Miao Y, Wang L, Wang EH. 2014. Kaiso interacts with p120-catenin to regulate β-catenin expression at the transcriptional level. PLoS One. 9(2):e87537.

Lloyd BH, Platt-Higgins A, Rudland PS, Barraclough R. 1998. Human s100a4 (p9ka) induces the metastatic phenotype upon benign tumour cells. Oncogene. 17(4):465-473.

Loi S, Haibe-Kains B, Majjaj S, Lallemand F, Durbecq V, Larsimont D, Gonzalez-Angulo AM, Pusztai L, Symmans WF, Bardelli A et al. 2010. Pik3ca mutations associated with gene signature of low mtorc1 signaling and better outcomes in estrogen receptor-positive breast cancer. Proc Natl Acad Sci U S A. 107(22):10208-10213.

Lopes EC, Valls E, Figueroa ME, Mazur A, Meng FG, Chiosis G, Laird PW, Schreiber-Agus N, Greally JM, Prokhortchouk E et al. 2008. Kaiso

contributes to dna methylation-dependent silencing of tumor suppressor genes in colon cancer cell lines. Cancer research. 68(18):7258-7263.
Lumachi F, Brunello A, Maruzzo M, Basso U, Basso SM. 2013. Treatment of estrogen receptor-positive breast cancer. Curr Med Chem. 20(5):596-604.
Lund MJ, Trivers KF, Porter PL, Coates RJ, Leyland-Jones B, Brawley OW, Flagg EW, O'Regan RM, Gabram SG, Eley JW. 2009. Race and triple negative threats to breast cancer survival: A population-based study in atlanta, ga. Breast Cancer Res Treat. 113(2):357-370.
Ma F, Li H, Li Y, Ding X, Wang H, Fan Y, Lin C, Qian H, Xu B. 2017. Aldehyde dehydrogenase 1 (aldh1) expression is an independent prognostic factor in triple negative breast cancer (tnbc). Medicine (Baltimore). 96(14):e6561.
Macias H, Hinck L. 2012. Mammary gland development. Wiley Interdiscip Rev Dev Biol. 1(4):533-557.
Manié E, Vincent-Salomon A, Lehmann-Che J, Pierron G, Turpin E, Warcoin M, Gruel N, Lebigot I, Sastre-Garau X, Lidereau R et al. 2009. High frequency of tp53 mutation in brca1 and sporadic basal-like carcinomas but not in brca1 luminal breast tumors. Cancer Res. 69(2):663-671.
Mannix DP, Cowley M. 1962. Black cargoes: A history of the atlantic slave trade. New York, New York, U.S.A: The Viking Press.
Massagué J. 1998. Tgf-beta signal transduction. Annu Rev Biochem. 67:753-791.
Massagué J. 2000. How cells read tgf-beta signals. Nat Rev Mol Cell Biol. 1(3):169-178.
Massagué J. 2012. Tgfβ signalling in context. Nat Rev Mol Cell Biol. 13(10):616-630.
Massagué J, Seoane J, Wotton D. 2005. Smad transcription factors. Genes Dev. 19(23):2783-2810.
McCall MN, Bolstad BM, Irizarry RA. 2010. Frozen robust multiarray analysis (frma). Biostatistics. 11(2):242-253.
McCrea PD, Gu D. 2010. The catenin family at a glance. J Cell Sci. 123(Pt 5):637-642.
Mebius RE, Kraal G. 2005. Structure and function of the spleen. Nat Rev Immunol. 5(8):606-616.
Medina D. 1996. The mammary gland: A unique organ for the study of development and tumorigenesis. J Mammary Gland Biol Neoplasia. 1(1):5-19.
Mehlen P, Puisieux A. 2006. Metastasis: A question of life or death. Nat Rev Cancer. 6(6):449-458.
Miyashita Y, Ozawa M. 2007. Increased internalization of p120-uncoupled e-cadherin and a requirement for a dileucine motif in the cytoplasmic domain for endocytosis of the protein. J Biol Chem. 282(15):11540-11548.
Miyazono K, Olofsson A, Colosetti P, Heldin CH. 1991. A role of the latent tgf-beta 1-binding protein in the assembly and secretion of tgf-beta 1. EMBO J. 10(5):1091-1101.

Mo YY, Reynolds AB. 1996. Identification of murine p120 isoforms and heterogeneous expression of p120cas isoforms in human tumor cell lines. Cancer research. 56(11):2633-2640.

Morris VL, Koop S, MacDonald IC, Schmidt EE, Grattan M, Percy D, Chambers AF, Groom AC. 1994. Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. Clin Exp Metastasis. 12(6):357-367.

Moses HL, Serra R. 1996. Regulation of differentiation by tgf-beta. Curr Opin Genet Dev. 6(5):581-586.

Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. 2011. Cyclin d as a therapeutic target in cancer. Nat Rev Cancer. 11(8):558-572.

Mócsai A. 2013. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med. 210(7):1283-1299.

Nagafuchi A, Ishihara S, Tsukita S. 1994. The roles of catenins in the cadherin-mediated cell adhesion: Functional analysis of e-cadherin-alpha catenin fusion molecules. J Cell Biol. 127(1):235-245.

Nagane M, Coufal F, Lin H, Bögler O, Cavenee WK, Huang HJ. 1996. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. Cancer Res. 56(21):5079-5086.

Nakano K, Vousden KH. 2001. Puma, a novel proapoptotic gene, is induced by p53. Mol Cell. 7(3):683-694.

Navarrete MA, Maier CM, Falzoni R, Quadros LG, Lima GR, Baracat EC, Nazário AC. 2005. Assessment of the proliferative, apoptotic and cellular renovation indices of the human mammary epithelium during the follicular and luteal phases of the menstrual cycle. Breast Cancer Res. 7(3):R306-313.

Nazario AC, De Lima GR, Simões MJ, Novo NF. 1995. Cell kinetics of the human mammary lobule during the proliferative and secretory phase of the menstrual cycle. Bull Assoc Anat (Nancy). 79(244):23-27.

Network CGA. 2012. Comprehensive molecular portraits of human breast tumours. Nature. 490(7418):61-70.

Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L et al. 2004. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 10(16):5367-5374.

Noren NK, Liu BP, Burridge K, Kreft B. 2000. P120 catenin regulates the actin cytoskeleton via rho family gtpases. J Cell Biol. 150(3):567-580.

O'Reilly EA, Gubbins L, Sharma S, Tully R, Guang MH, Weiner-Gorzel K, McCaffrey J, Harrison M, Furlong F, Kell M et al. 2015. The fate of chemoresistance in triple negative breast cancer (tnbc). BBA Clin. 3:257-275.

Oakman C, Viale G, Di Leo A. 2010. Management of triple negative breast cancer. Breast. 19(5):312-321.

Oft M, Heider KH, Beug H. 1998. Tgfbeta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol. 8(23):1243-1252.

Ogden SR, Wroblewski LE, Weydig C, Romero-Gallo J, O'Brien DP, Israel DA, Krishna US, Fingleton B, Reynolds AB, Wessler S et al. 2008. P120 and kaiso regulate helicobacter pylori-induced expression of matrix metalloproteinase-7. Molecular biology of the cell. 19(10):4110-4121.

Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. 2008. Loss of e-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer research. 68(10):3645-3654.

Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H. 1998. Brca1 regulates p53-dependent gene expression. Proc Natl Acad Sci U S A. 95(5):2302-2306.

Owens MB, Hill AD, Hopkins AM. 2013. Ductal barriers in mammary epithelium. Tissue Barriers. 1(4):e25933.

Padua D, Massague J. 2009. Roles of tgfbeta in metastasis. Cell research. 19(1):89-102.

Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, Massagué J. 2008. Tgfbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell. 133(1):66-77.

Palmer JR, Ruiz-Narvaez EA, Rotimi CN, Cupples LA, Cozier YC, Adams-Campbell LL, Rosenberg L. 2013. Genetic susceptibility loci for subtypes of breast cancer in an african american population. Cancer Epidemiol Biomarkers Prev. 22(1):127-134.

Pandya S, Moore RG. 2011. Breast development and anatomy. Clin Obstet Gynecol. 54(1):91-95.

Pang MF, Georgoudaki AM, Lambut L, Johansson J, Tabor V, Hagikura K, Jin Y, Jansson M, Alexander JS, Nelson CM et al. 2016. Tgf-β1-induced emt promotes targeted migration of breast cancer cells through the lymphatic system by the activation of ccr7/ccl21-mediated chemotaxis. Oncogene. 35(6):748-760.

Pannu J, Gore-Hyer E, Yamanaka M, Smith EA, Rubinchik S, Dong JY, Jablonska S, Blaszczyk M, Trojanowska M. 2004. An increased transforming growth factor beta receptor type i:Type ii ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type ii in scleroderma. Arthritis Rheum. 50(5):1566-1577.

Pantel K, Brakenhoff RH. 2004. Dissecting the metastatic cascade. Nat Rev Cancer. 4(6):448-456.

Papageorgis P. 2015. Tgfβ signaling in tumor initiation, epithelial-to-mesenchymal transition, and metastasis. J Oncol. 2015:587193.

Pareja Fresia GFC, Marchiò Caterina, Burke Kathleen A, Weigelt Britta, S. R-FJ. 2016. Triple-negative
breast cancer: The importance of molecular and histologic subtyping, and
recognition of low-grade variants. *npj Breast Cancer*

Park HS, Jang MH, Kim EJ, Kim HJ, Lee HJ, Kim YJ, Kim JH, Kang E, Kim SW, Kim IA et al. 2014. High egfr gene copy number predicts poor outcome in triple-negative breast cancer. Mod Pathol. 27(9):1212-1222.

Park JI, Kim SW, Lyons JP, Ji H, Nguyen TT, Cho K, Barton MC, Deroo T, Vleminckx K, Moon RT et al. 2005. Kaiso/p120-catenin and tcf/beta-catenin complexes coordinately regulate canonical wnt gene targets. Developmental cell. 8(6):843-854.

Perez-Moreno M, Jamora C, Fuchs E. 2003. Sticky business: Orchestrating cellular signals at adherens junctions. Cell. 112(4):535-548.

Perou CM. 2010. Molecular stratification of triple-negative breast cancers. Oncologist. 15 Suppl 5:39-48.

Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA et al. 2000. Molecular portraits of human breast tumours. Nature. 406(6797):747-752.

Pierce DF, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ, Moses HL. 1995. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci U S A. 92(10):4254-4258.

Pierre CC, Longo J, Bassey-Archibong BI, Hallett RM, Milosavljevic S, Beatty L, Hassell JA, Daniel JM. 2015a. Methylation-dependent regulation of hypoxia inducible factor-1 alpha gene expression by the transcription factor kaiso. Biochim Biophys Acta. 1849(12):1432-1441.

Pierre CC, Longo J, Mavor M, Milosavljevic SB, Chaudhary R, Gilbreath E, Yates C, Daniel JM. 2015b. Kaiso overexpression promotes intestinal inflammation and potentiates intestinal tumorigenesis in apc(min/+) mice. Biochim Biophys Acta. 1852(9):1846-1855.

Pike MC, Spicer DV, Dahmoush L, Press MF. 1993. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev. 15(1):17-35.

Polyak K, Kalluri R. 2010. The role of the microenvironment in mammary gland development and cancer. Cold Spring Harb Perspect Biol. 2(11):a003244.

Pozner A, Terooatea TW, Buck-Koehntop BA. 2016. Cell-specific kaiso (zbtb33) regulation of cell cycle through cyclin d1 and cyclin e1. J Biol Chem. 291(47):24538-24550.

Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X, Perou CM. 2010. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 12(5):R68.

Prokhortchouk A, Hendrich B, Jorgensen H, Ruzov A, Wilm M, Georgiev G, Bird A, Prokhortchouk E. 2001. The p120 catenin partner kaiso is a dna methylation-dependent transcriptional repressor. Genes & development. 15(13):1613-1618.

Prokhortchouk A, Sansom O, Selfridge J, Caballero IM, Salozhin S, Aithozhina D, Cerchietti L, Meng FG, Augenlicht LH, Mariadason JM et al. 2006. Kaiso-deficient mice show resistance to intestinal cancer. Molecular and cellular biology. 26(1):199-208.

Raghav SK, Waszak SM, Krier I, Gubelmann C, Isakova A, Mikkelsen TS, Deplancke B. 2012. Integrative genomics identifies the corepressor smrt as a gatekeeper of adipogenesis through the transcription factors c/ebpβ and kaiso. Mol Cell. 46(3):335-350.

Rakha EA, Elsheikh SE, Aleskandarany MA, Habashi HO, Green AR, Powe DG, El-Sayed ME, Benhasouna A, Brunet JS, Akslen LA et al. 2009. Triple-negative breast cancer: Distinguishing between basal and nonbasal subtypes. Clin Cancer Res. 15(7):2302-2310.

Reynolds AB, Daniel J, McCrea PD, Wheelock MJ, Wu J, Zhang Z. 1994. Identification of a new catenin: The tyrosine kinase substrate p120cas associates with e-cadherin complexes. Mol Cell Biol. 14(12):8333-8342.

Rhodes LV, Tate CR, Hoang VT, Burks HE, Gilliam D, Martin EC, Elliott S, Miller DB, Buechlein A, Rusch D et al. 2015. Regulation of triple-negative breast cancer cell metastasis by the tumor-suppressor liver kinase b1. Oncogenesis. 4:e168.

Rhodes LV, Tate CR, Segar HC, Burks HE, Phamduy TB, Hoang V, Elliott S, Gilliam D, Pounder FN, Anbalagan M et al. 2014. Suppression of triple-negative breast cancer metastasis by pan-dac inhibitor panobinostat via inhibition of zeb family of emt master regulators. Breast Cancer Res Treat. 145(3):593-604.

Robinson SC, Klobucar K, Pierre CC, Ansari A, Zhenilo S, Prokhortchouk E, Daniel JM. 2017. Kaiso differentially regulates components of the notch signaling pathway in intestinal cells. Cell Commun Signal. 15(1):24.

Roczniak-Ferguson A, Reynolds AB. 2003. Regulation of p120-catenin nucleocytoplasmic shuttling activity. Journal of cell science. 116(Pt 20):4201-4212.

Rodova M, Kelly KF, VanSaun M, Daniel JM, Werle MJ. 2004. Regulation of the rapsyn promoter by kaiso and delta-catenin. Molecular and cellular biology. 24(16):7188-7196.

Rojas A, Padidam M, Cress D, Grady WM. 2009. Tgf-beta receptor levels regulate the specificity of signaling pathway activation and biological effects of tgf-beta. Biochim Biophys Acta. 1793(7):1165-1173.

Rosales C, Demaurex N, Lowell CA, Uribe-Querol E. 2016. Neutrophils: Their role in innate and adaptive immunity. J Immunol Res. 2016:1469780.

Roy PG, Thompson AM. 2006. Cyclin d1 and breast cancer. Breast. 15(6):718-727.

Ruzov A, Dunican DS, Prokhortchouk A, Pennings S, Stancheva I, Prokhortchouk E, Meehan RR. 2004. Kaiso is a genome-wide repressor of transcription that is essential for amphibian development. Development (Cambridge, England). 131(24):6185-6194.

Ruzov A, Savitskaya E, Hackett JA, Reddington JP, Prokhortchouk A, Madej MJ, Chekanov N, Li M, Dunican DS, Prokhortchouk E et al. 2009. The non-methylated dna-binding function of kaiso is not required in early xenopus laevis development. Development. 136(5):729-738.

Sabatier R, Finetti P, Adelaide J, Guille A, Borg JP, Chaffanet M, Lane L, Birnbaum D, Bertucci F. 2011a. Down-regulation of ecrg4, a candidate tumor suppressor gene, in human breast cancer. PLoS One. 6(11):e27656.

Sabatier R, Finetti P, Cervera N, Lambaudie E, Esterni B, Mamessier E, Tallet A, Chabannon C, Extra JM, Jacquemier J et al. 2011b. A gene expression signature identifies two prognostic subgroups of basal breast cancer. Breast Cancer Res Treat. 126(2):407-420.

Saharinen J, Hyytiäinen M, Taipale J, Keski-Oja J. 1999. Latent transforming growth factor-beta binding proteins (ltbps)--structural extracellular matrix proteins for targeting tgf-beta action. Cytokine Growth Factor Rev. 10(2):99-117.

Sarrio D, Perez-Mies B, Hardisson D, Moreno-Bueno G, Suarez A, Cano A, Martin-Perez J, Gamallo C, Palacios J. 2004. Cytoplasmic localization of p120ctn and e-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. Oncogene. 23(19):3272-3283.

Sasaki Y, Tsuda H. 2009. Clinicopathological characteristics of triple-negative breast cancers. Breast Cancer. 16(4):254-259.

Savage C, Das P, Finelli AL, Townsend SR, Sun CY, Baird SE, Padgett RW. 1996. Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. Proc Natl Acad Sci U S A. 93(2):790-794.

Schmidt EV. 1999. The role of c-myc in cellular growth control. Oncogene. 18(19):2988-2996.

Schuster N, Krieglstein K. 2002. Mechanisms of tgf-beta-mediated apoptosis. Cell Tissue Res. 307(1):1-14.

Schwab LP, Peacock DL, Majumdar D, Ingels JF, Jensen LC, Smith KD, Cushing RC, Seagroves TN. 2012. Hypoxia-inducible factor 1α promotes primary tumor growth and tumor-initiating cell activity in breast cancer. Breast Cancer Res. 14(1):R6.

Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM. 1995. Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in drosophila melanogaster. Genetics. 139(3):1347-1358.

Seoane J. 2006. Escaping from the tgfbeta anti-proliferative control. Carcinogenesis. 27(11):2148-2156.

Seoane J, Pouponnot C, Staller P, Schader M, Eilers M, Massagué J. 2001. Tgfbeta influences myc, miz-1 and smad to control the cdk inhibitor p15ink4b. Nat Cell Biol. 3(4):400-408.

Serra R, Crowley MR. 2003. Tgf-beta in mammary gland development and breast cancer. Breast Dis. 18:61-73.

Shah SP, Roth A, Goya R, Oloumi A, Ha G, Zhao Y, Turashvili G, Ding J, Tse K, Haffari G et al. 2012. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature. 486(7403):395-399.

Shekhar MP, Pauley R, Heppner G. 2003. Host microenvironment in breast cancer development: Extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. Breast Cancer Res. 5(3):130-135.

Shi Y, Massagué J. 2003. Mechanisms of tgf-beta signaling from cell membrane to the nucleus. Cell. 113(6):685-700.

Shibata MA, Liu ML, Knudson MC, Shibata E, Yoshidome K, Bandey T, Korsmeyer SJ, Green JE. 1999. Haploid loss of bax leads to accelerated mammary tumor development in c3(1)/sv40-tag transgenic mice: Reduction in protective apoptotic response at the preneoplastic stage. EMBO J. 18(10):2692-2701.

Shumskaya VS, Zhigalova NA, Prokhorchouk AV, Prokhorchouk EB. 2015. Distribution of kaiso protein in mouse tissues. Histochem Cell Biol. 143(1):29-43.

Siegel PM, Massagué J. 2003. Cytostatic and apoptotic actions of tgf-beta in homeostasis and cancer. Nat Rev Cancer. 3(11):807-821.

Silberstein GB, Daniel CW. 1987. Reversible inhibition of mammary gland growth by transforming growth factor-beta. Science. 237(4812):291-293.

Silver DP, Richardson AL, Eklund AC, Wang ZC, Szallasi Z, Li Q, Juul N, Leong CO, Calogrias D, Buraimoh A et al. 2010. Efficacy of neoadjuvant cisplatin in triple-negative breast cancer. J Clin Oncol. 28(7):1145-1153.

Singh A, Settleman J. 2010. Emt, cancer stem cells and drug resistance: An emerging axis of evil in the war on cancer. Oncogene. 29(34):4741-4751.

Singh G, Singh SK, König A, Reutlinger K, Nye MD, Adhikary T, Eilers M, Gress TM, Fernandez-Zapico ME, Ellenrieder V. 2010. Sequential activation of nfat and c-myc transcription factors mediates the tgf-beta switch from a suppressor to a promoter of cancer cell proliferation. J Biol Chem. 285(35):27241-27250.

Snouwaert JN, Gowen LC, Latour AM, Mohn AR, Xiao A, DiBiase L, Koller BH. 1999. Brca1 deficient embryonic stem cells display a decreased homologous recombination frequency and an increased frequency of non-homologous recombination that is corrected by expression of a brca1 transgene. Oncogene. 18(55):7900-7907.

Soubry A, Staes K, Parthoens E, Noppen S, Stove C, Bogaert P, van Hengel J, van Roy F. 2010. The transcriptional repressor kaiso localizes at the mitotic spindle and is a constituent of the pericentriolar material. PloS one. 5(2):e9203.

Soubry A, van Hengel J, Parthoens E, Colpaert C, Van Marck E, Waltregny D, Reynolds AB, van Roy F. 2005. Expression and nuclear location of the transcriptional repressor kaiso is regulated by the tumor microenvironment. Cancer research. 65(6):2224-2233.

Spring CM, Kelly KF, O'Kelly I, Graham M, Crawford HC, Daniel JM. 2005. The catenin p120ctn inhibits kaiso-mediated transcriptional repression of the

beta-catenin/tcf target gene matrilysin. Experimental cell research. 305(2):253-265.

Stacey DW. 2003. Cyclin d1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr Opin Cell Biol. 15(2):158-163.

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Möröy T, Bartek J, Massagué J, Hänel F et al. 2001. Repression of p15ink4b expression by myc through association with miz-1. Nat Cell Biol. 3(4):392-399.

Stark A, Kleer CG, Martin I, Awuah B, Nsiah-Asare A, Takyi V, Braman M, Quayson SE, Zarbo R, Wicha M et al. 2010. African ancestry and higher prevalence of triple-negative breast cancer: Findings from an international study. Cancer. 116(21):4926-4932.

Strasser A, Harris AW, Bath ML, Cory S. 1990. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature. 348(6299):331-333.

Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS et al. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 98(19):10869-10874.

Talbot LJ, Bhattacharya SD, Kuo PC. 2012. Epithelial-mesenchymal transition, the tumor microenvironment, and metastatic behavior of epithelial malignancies. International journal of biochemistry and molecular biology. 3(2):117-136.

Talvinen K, Tuikkala J, Nykänen M, Nieminen A, Anttinen J, Nevalainen OS, Hurme S, Kuopio T, Kronqvist P. 2010. Altered expression of p120catenin predicts poor outcome in invasive breast cancer. J Cancer Res Clin Oncol. 136(9):1377-1387.

Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, Wakefield LM. 2003. Tgf-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest. 112(7):1116-1124.

Team RC. 2014. R: A language and environment for statistical computing. Vienna, Austria R Foundation for Statistical Computing.

Thiery JP, Acloque H, Huang RY, Nieto MA. 2009. Epithelial-mesenchymal transitions in development and disease. Cell. 139(5):871-890.

Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB. 2000. Selective uncoupling of p120(ctn) from e-cadherin disrupts strong adhesion. J Cell Biol. 148(1):189-202.

Tian F, DaCosta Byfield S, Parks WT, Yoo S, Felici A, Tang B, Piek E, Wakefield LM, Roberts AB. 2003. Reduction in smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res. 63(23):8284-8292.

Tomaskovic-Crook E, Thompson EW, Thiery JP. 2009. Epithelial to mesenchymal transition and breast cancer. Breast cancer research : BCR. 11(6):213.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. 1998. Sara, a fyve domain protein that recruits smad2 to the tgfbeta receptor. Cell. 95(6):779-791.

Valenta T, Hausmann G, Basler K. 2012. The many faces and functions of β-catenin. EMBO J. 31(12):2714-2736.

Van den Bossche J, Malissen B, Mantovani A, De Baetselier P, Van Ginderachter JA. 2012. Regulation and function of the e-cadherin/catenin complex in cells of the monocyte-macrophage lineage and dcs. Blood. 119(7):1623-1633.

van Hengel J, Vanhoenacker P, Staes K, van Roy F. 1999. Nuclear localization of the p120(ctn) armadillo-like catenin is counteracted by a nuclear export signal and by e-cadherin expression. Proceedings of the National Academy of Sciences of the United States of America. 96(14):7980-7985.

Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P, Christgen M, Lehmann U, Daniel J, van Diest PJ et al. 2012. Nuclear kaiso expression is associated with high grade and triple-negative invasive breast cancer. PloS one. 7(5):e37864.

Visvader JE. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. Genes & development. 23(22):2563-2577.

Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M et al. 2002. Efficacy and safety of trastuzumab as a single agent in first-line treatment of her2-overexpressing metastatic breast cancer. J Clin Oncol. 20(3):719-726.

Vousden KH, Lane DP. 2007. P53 in health and disease. Nat Rev Mol Cell Biol. 8(4):275-283.

Wahba HA, El-Hadaad HA. 2015. Current approaches in treatment of triple-negative breast cancer. Cancer Biol Med. 12(2):106-116.

Wang EH, Liu Y, Xu HT, Dai SD, Liu N, Xie CY, Yuan XM. 2006. Abnormal expression and clinicopathologic significance of p120-catenin in lung cancer. Histol Histopathol. 21(8):841-847.

Wang H, Liu W, Black S, Turner O, Daniel JM, Dean-Colomb W, He QP, Davis M, Yates C. 2016. Kaiso, a transcriptional repressor, promotes cell migration and invasion of prostate cancer cells through regulation of mir-31 expression. Oncotarget. 7(5):5677-5689.

Wang Y, Li L, Li Q, Xie C, Wang E. 2012. Expression of p120 catenin, kaiso, and metastasis tumor antigen-2 in thymomas. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 33(6):1871-1879.

Wang Y, Zhou BP. 2013. Epithelial-mesenchymal transition---a hallmark of breast cancer metastasis. Cancer Hallm. 1(1):38-49.

Warner BJ, Blain SW, Seoane J, Massagué J. 1999. Myc downregulation by transforming growth factor beta required for activation of the p15(ink4b) g(1) arrest pathway. Mol Cell Biol. 19(9):5913-5922.

Watson, J, Christine. 2006. Key stages in mammary gland development - involution: Apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ.8doi:doi:10.1186/bcr1401.

Watson CJ, Khaled WT. 2008. Mammary development in the embryo and adult: A journey of morphogenesis and commitment. Development. 135(6):995-1003.

Weiss A, Attisano L. 2013. The tgfbeta superfamily signaling pathway. Wiley Interdiscip Rev Dev Biol. 2(1):47-63.

Wong-Brown MW, Meldrum CJ, Carpenter JE, Clarke CL, Narod SA, Jakubowska A, Rudnicka H, Lubinski J, Scott RJ. 2015. Prevalence of brca1 and brca2 germline mutations in patients with triple-negative breast cancer. Breast Cancer Res Treat. 150(1):71-80.

Wrana JL, Attisano L, Cárcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massagué J. 1992. Tgf beta signals through a heteromeric protein kinase receptor complex. Cell. 71(6):1003-1014.

Wu Q, Liu X, Yan H, He YH, Ye S, Cheng XW, Zhu GL, Wu WY, Wang XN, Kong XJ et al. 2014. B-cell lymphoma 6 protein stimulates oncogenicity of human breast cancer cells. BMC Cancer. 14:418.

Xu J, Chen Y, Olopade OI. 2010. Myc and breast cancer. Genes Cancer. 1(6):629-640.

Yanagisawa M, Kaverina IN, Wang A, Fujita Y, Reynolds AB, Anastasiadis PZ. 2004. A novel interaction between kinesin and p120 modulates p120 localization and function. J Biol Chem. 279(10):9512-9521.

Yap AS, Niessen CM, Gumbiner BM. 1998. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol. 141(3):779-789.

Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massagué J, Mundy GR, Guise TA. 1999. Tgf-beta signaling blockade inhibits pthrp secretion by breast cancer cells and bone metastases development. J Clin Invest. 103(2):197-206.

Yoon HG, Chan DW, Reynolds AB, Qin J, Wong J. 2003. N-cor mediates dna methylation-dependent repression through a methyl cpg binding protein kaiso. Molecular cell. 12(3):723-734.

Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. 2001. Puma induces the rapid apoptosis of colorectal cancer cells. Mol Cell. 7(3):673-682.

Yu Q, Stamenkovic I. 2004. Transforming growth factor-beta facilitates breast carcinoma metastasis by promoting tumor cell survival. Clin Exp Metastasis. 21(3):235-242.

Zarzynska JM. 2014. Two faces of tgf-beta1 in breast cancer. Mediators Inflamm. 2014:141747.

Zavadil J, Böttinger EP. 2005. Tgf-beta and epithelial-to-mesenchymal transitions. Oncogene. 24(37):5764-5774.

Zhang J. 2013. The role of brca1 in homologous recombination repair in response to replication stress: Significance in tumorigenesis and cancer therapy. Cell Biosci. 3(1):11.

Zhang PX, Wang Y, Liu Y, Jiang GY, Li QC, Wang EH. 2011. P120-catenin isoform 3 regulates subcellular localization of kaiso and promotes invasion in lung cancer cells via a phosphorylation-dependent mechanism. International journal of oncology. 38(6):1625-1635.

Zhang YE. 2009. Non-smad pathways in tgf-beta signaling. Cell research. 19(1):128-139.

Zhou ZQ, Hurlin PJ. 2001. The interplay between mad and myc in proliferation and differentiation. Trends Cell Biol. 11(11):S10-14.