Characterization of the BTB/POZ protein ZBTB4 as a transcriptional regulator of *cyclin D1*

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ABSTRACT

The POZ-ZF transcription factor ZBTB4 was initially identified due to its sequence homology to the dual-specificity DNA-binding transcription factor Kaiso. Subsequent characterization of ZBTB4 revealed that it is also a dual-specificity DNA-binding protein; it recognizes a specific oligonucleotide sequence $C^{T}/_{C}GCCATC$, coined the **Z**BTB**4** **B**inding **S**equence (Z4BS) as well as methylated CpG-dinucleotides. Interestingly, ZBTB4 also binds to the highly similar consensus **K**aiso **B**inding **S**ite (KBS) *in vitro*.

ZBTB4 is misexpressed in cancer, and follows a stage-specific pattern of expression in breast carcinoma tissues; low ZBTB4 levels are found in late stages while high ZBTB4 expression is detected in early stages of disease progression. Ongoing studies have begun to elucidate the molecular interactions that mediate ZBTB4's apparent tumour suppressor role in tumourigenesis, however no study has investigated the nature of ZBTB4's ability to interact with both the Z4BS and the KBS *in vivo*, and how this may expand ZBTB4's repertoire of potential target genes.

Recently Kaiso has been characterized as a transcriptional repressor of the cell cycle regulatory gene *cyclin D1*, and thus we used *cyclin D1* as a model to investigate the nature of ZBTB4's interaction with the KBS *in vivo*. The *cyclin D1* minimal promoter contains two partial Z4BS at the same location as the KBS sites and we found that ZBTB4 binds to the +69 Z4BS/KBS site, but not to the -1067 site. Because the +69 Z4BS/KBS is immediately flanked by a CpG dinucleotide, this interaction may be a methylation-dependent interaction. To determine the consequence of this interaction, we conducted minimal promoter luciferase assays, and observed that ZBTB4 mediates an activation of the -1748-CD1 minimal promoter activity.

In conclusion, ZBTB4 does not bind to the -1067 Z4BS/KBS, however does bind to the +69 Z4BS/KBS of the *cyclin D1* promoter. ZBTB4 mediated a sequence-independent activation of the -1748-CD1 minimal promoter activity, although because the construct was not methylated it is not certain that this is the true consequence of the interaction. Future research should provide insight into whether ZBTB4's interaction with the +69 Z4BS/KBS is methylation dependent, and address the functional significance of this interaction on *cyclin D1* expression.

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CONTRIBUTIONS BY OTHERS

Stained **T**issue **M**icro**A**rrays (TMAs) were analysed using **A**utomated **QU**antitative **A**nalysis (AQUA) at the Yale Cancer Center Tissue Microarray Facility by Hallie Wimberly. Kaplan Meier Curves were produced from the resulting data.

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LIST OF ABBREVIATIONS

American **T**ype **C**ulture **C**ollection……ATCC

Automated **Qu**antitative **A**nalysis……..AQUA

Bric-a-brac, **T**ramtrack, and **B**road complex/**Po**xvirus and **Z**inc finger……BTB/POZ

Chromatin **I**mmuno**P**recipitation……...ChIP

cyclin D1 gene name……..CCND1

cyclin-**d**ependent **k**inase **4** ……CDK4

cyclin-**d**ependent **k**inase **6** ……CDK6

Dulbecco's **M**odified **E**agle **M**edium……DMEM

Enhanced **C**hemi**L**uminescence system……ECL

Electrophoretic **M**obility **S**hift **A**ssays……...EMSA

Estrogen **R**eceptor **A**lpha……ERα

Fetal **B**ovine **S**erum……FBS

Immuno**F**luorescence……..IF

Immuno**H**isto**C**hemical……..…….IHC

Kaiso **B**inding **S**ite……..KBS

Mouse **M**ammary **T**umor **V**irus……..MMTV

Nuclear **L**ocalization **S**ignal……NLS

ortho-**N**itro**p**henyl-β-**g**alactoside……ONPG

p120-**c**a**t**eni**n**……$p120^{ctn}$

Phosphate-**B**uffered-**S**aline……..PBS

Pixel-based **L**ocale **A**ssignment for **C**ompartmentalization of **E**xpression……PLACE

pRetro**S**uper……pRS

Rapid **E**xponential **S**ubtraction **A**lgorithm……..RESA

retino**b**lastoma protein……Rb

Specificity **P**rotein **1**…….Sp1

Specificity **P**rotein **3**…….....Sp3

Tissue **M**icro**A**rray……...TMA

Tris-**B**uffered-**S**aline……TBS

Vascular **E**ndothelial **G**rowth **F**actor……VEGF

Vascular **E**ndothelial **G**rowth **F**actor **R**eceptor **1**……..VEGFR1

Wild **T**ype……..WT

Zinc **F**inger……ZF

Zinc finger and **BTB** domain containing **4**……ZBTB4

ZBTB**4** **B**inding **S**equence……..Z4BS

INTRODUCTION

1.1 Breast Cancer Prevalence and Progression

Breast cancer is the most common type of cancer afflicting Canadian women, accounting for 26 % of all new cancer cases reported in Canada in 2011 [11,30]. In 2012, 22,000 women are predicted to be diagnosed with breast cancer, and 5,100 women are expected to die. Rates of breast cancer detection have steadily increased since the early 1980's, largely due to the development of highly sensitive screening technologies in conjunction with an increase in screening programs and public concern [11]. Similarly, breast cancer mortality rates have also declined during the same time period, partly as a consequence of early detection as well as due to the continual sophistication of treatment options [11,30].

Metastatic breast cancer is a disease with great heterogeneity since factors such as tissue histology and specific genetic aberrations can vary between individuals and ethnicities [5,7,28]. While different subcategories of breast cancer can influence clinical outcomes and treatment options, the disease as a whole follows a similar course of progression [4,7,15,47]. A series of molecular events mediate a loss of internal and external growth controls of breast epithelial cells, resulting in rapid proliferation and the formation of a primary tumour [5,6,7,15]. The second step, called intravasation, requires the entry of breast tumour cells into the circulatory system, be it via the vascular or lymphatic system [5,6,26]. Extravasation naturally follows intravasation as the tumour cells leave the circulatory system to inhabit and thrive in a secondary tissue such as the lung or liver [5,6,11]. Taken together, the process of breast cancer progression is one from the development of a primary site neoplasia to the spread of multiple secondary site metastases, utilizing the circulatory system as a mechanism for transportation [5,6,11,27]. The primary tumour within the breast tissue is rarely the cause for death due to breast cancer, but rather the

large number of secondary metastases that settle in vital organs such as the lungs and liver, and ultimately interferes with their normal function [5].

Although the number of proteins and other molecules that have been characterized to mediate the events of breast carcinogenesis is vast, one such group of proteins, the BTB/POZ domain family of proteins, will be discussed in depth throughout the thesis.

1.2 BTB/POZ Family of Transcription Factors

The **B**ric-a-brac, **T**ramtrack, and **B**road complex/**Po**xvirus and **Z**inc finger (BTB/POZ) domain is a highly conserved protein-protein interaction domain, found widely throughout organisms from viruses to humans [31]. The domain was concurrently identified by two separate groups in two separate organisms; in *Drosophila melanogaster* by Laski *et al.*, 1994 when they observed that three *Drosophila* transcription factors Bric-a-brac, Tramtrack, and Broad Complex all shared high sequence homology at their N-terminals, and by Bardwell & Treisman, 1994 when they noticed that various Poxvirus proteins are similar in sequence to particular Zinc finger proteins, namely GAGA and ZF5 [38,46]. Laski *et al.,* coined the domain BTB for **B**ric-a-brac, **T**ramtrack, and **B**road complex while Bardwell & Treisman referred to it as the POZ domain after **Po**xvirus and **Z**inc finger [38,46]. To date, both terms are used interchangeably to refer to the domain.

While high sequence variability exists amongst BTB/POZ domains in various organisms, their secondary and tertiary structure remain highly similar, conferring similar functional characteristics [38]. Although some proteins such as Skp1 are comprised solely of a BTB/POZ domain, it is much more common to find BTB/POZ domains existing in a protein along with other functional domains [38]. There are five domains that are frequently found in conjunction with the BTB/POZ domain in various BTB/POZ domain-containing proteins; they are MATH,

Kelch, NPH3, Ion transport, and **Z**inc **F**inger (ZF) domains [38]. The BTB/POZ domain acts as a protein-protein interaction interface, allowing for either homo/heterodimerization or oligomerization, while the alternative functional domains found within BTB/POZ domain containing proteins contribute to vastly different functions [31,38,46]. For example, the Kelch domain mediates interaction with actin filaments, allowing for BTB/POZ-kelch proteins to function in actin filament stability, while the MATH domain interacts with the TNF-α receptor of BTB/POZ-MATH proteins and plays important roles in cytoplasmic signal transduction pathways [46].

Another major class of BTB/POZ domain-containing proteins are the POZ-ZF proteins that largely act as transcription factors. The ZF domain, found almost exclusively at the C-terminal end of POZ-ZF molecules, confers DNA-binding ability to the protein [31,38]. Consequently, different ZF-containing proteins interact with different regions of DNA in a primarily sequence-specific manner. However, for three POZ-ZF proteins, namely Kaiso and the Kaiso-like proteins ZBTB4 and ZBTB38, binding to DNA occurs in a sequence-specific and methylation-dependent manner [31]. The BTB/POZ domain, found at the N-terminal end of POZ-ZF molecules, plays an important regulatory role in the transcriptional activity of the protein, allowing for homo- or heterodimerization with molecules that enhance or diminish DNA binding and thus determine transcriptional outcome [31]. With over 80 known POZ-ZF proteins encoded in the human genome and others being continually identified and characterized, it is not surprising that the POZ-ZF protein family has a widespread effect on cell function [38]. To date, only a subset of POZ-ZF proteins have been implicated in tumourigenesis; these include HIC-1 (effector of p53, lost in neuroblastomas), BCL6 (regulator of lymphocyte survival in B-Cell

Lymphomas), Kaiso and the **Z**inc finger and **BTB** domain containing **4** (ZBTB4) (implicated for their roles in breast, colon and lung carcinogenesis) [31,36,41,48].

1.3 The Transcription Factor Kaiso

Kaiso is a unique member of the POZ-ZF transcription factor protein family; it was the first to be characterized with dual-specificity DNA-binding since it recognized both the sequence-specific **K**aiso **B**inding **S**ite (KBS) TCCTGCnA and methylated CpG-dinucleotides [16,17,39]. Kaiso was originally identified through its interaction with the Armadillo repeat containing protein **p120**-**c**a**t**eni**n** ($p120^{ctn}$); the interaction occurred between the ZF domain of Kaiso, and Arm repeats 1-7 of $p120^{ctn}$ [16,17,18]. As is typical of POZ-ZF proteins, Kaiso contains a highly conserved protein-protein interaction BTB/POZ domain at its amino terminus, and three c-terminal Kruppel-like C_2H_2 ZFs involved in DNA-binding (Figure 1). Located between Kaiso's BTB/POZ and ZF domains is a **N**uclear **L**ocalization **S**ignal (NLS) required for the targeting of Kaiso to the nucleus [18,32].

It was originally postulated that Armadillo repeat containing catenins, such as $p120^{ctn}$, functioned exclusively as cell adhesion co-factors, given their ability to bind to E-cadherin and mediate adhesion to the actin cytoskeleton, as well as regulate E-cadherin stability and turnover [2,16,17]. Alternative functions for Armadillo catenins were considered when extensive characterization of the *Drosophila* Armadillo protein, and its vertebrate homolog β–catenin, revealed their ability to translocate to the nucleus and activate gene expression [17]. Thus upon the identification of Kaiso as a $p120^{ctn}$ interaction partner, a nuclear role for $p120^{ctn}$ was postulated [16,17]. It was determined that localization of $p120^{ctn}$ to the nucleus allows it to regulate gene expression through its interaction with Kaiso's ZF domain, thereby blocking the C-terminal C_2H_2 zinc finger DNA-binding domain of Kaiso [32]. This action is thought to

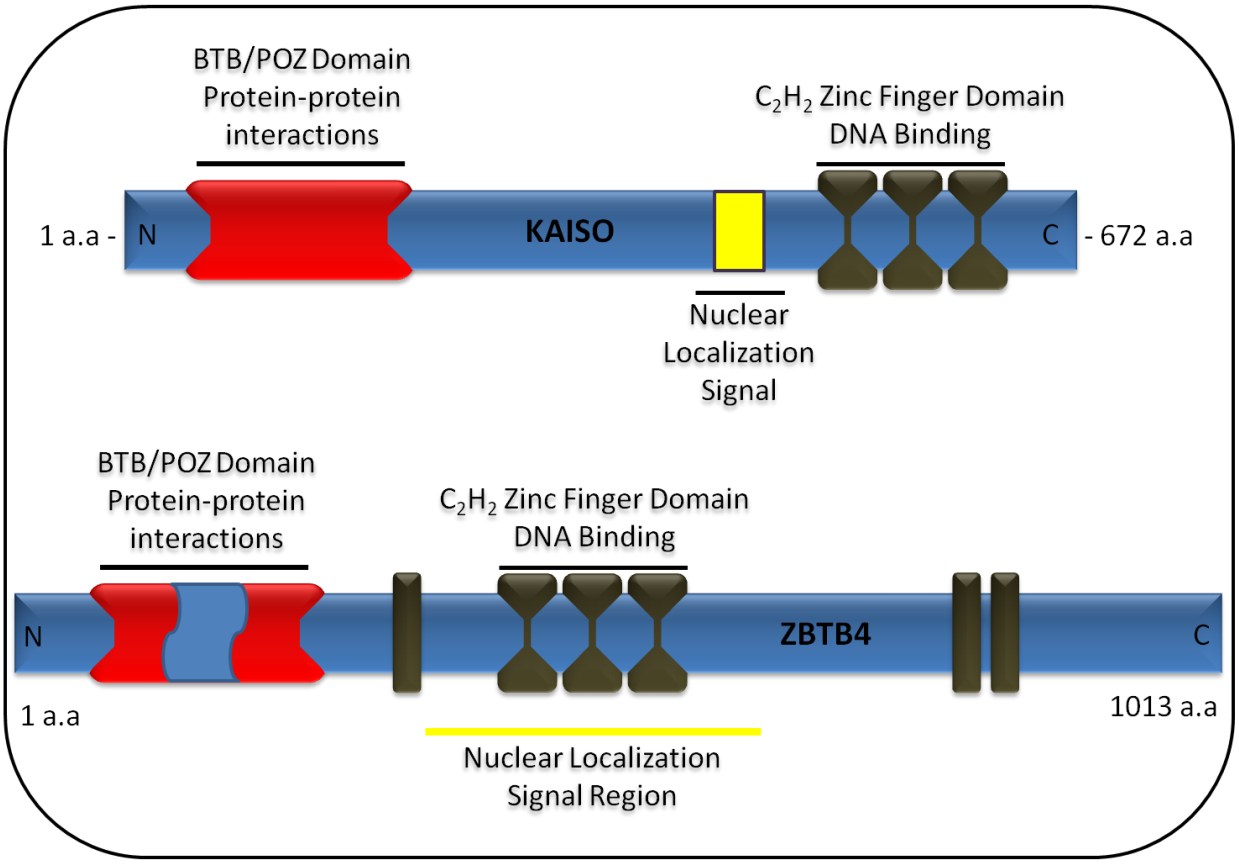


Figure 1: Schematic representation of Kaiso and ZBTB4. The BTB/POZ domain facilitates protein-protein interactions, including homo- and hetero-dimerization with other POZ and non-POZ domain proteins. The BTB/POZ domain of ZBTB4 contains a 60 residue insert of mainly serines and alanines, which could potentially alter its interaction abilities with other BTB/POZ containing proteins. The nature of this insertion is currently uncharacterized however. The Kaiso ZF domain facilitates sequence-specific DNA-binding to the KBS and methyl-CpG dinucleotides, whereas the ZBTB4 ZF DNA-binding domain mediates binding to both the Z4BS and KBS, as well as to methyl-CpG nucleotides. ZBTB4 also contains three other ZF motifs that are distinct from the ZF DNA-binding domains found in both Kaiso and ZBTB4, and likely mediate alternative binding activities. While the NLS of ZBTB4 has not been definitively mapped, the segment of ZBTB4 from amino acid 251 to 595, which spans the Kaiso-like zinc finger domain, is required for ZBTB4 nuclear localization.

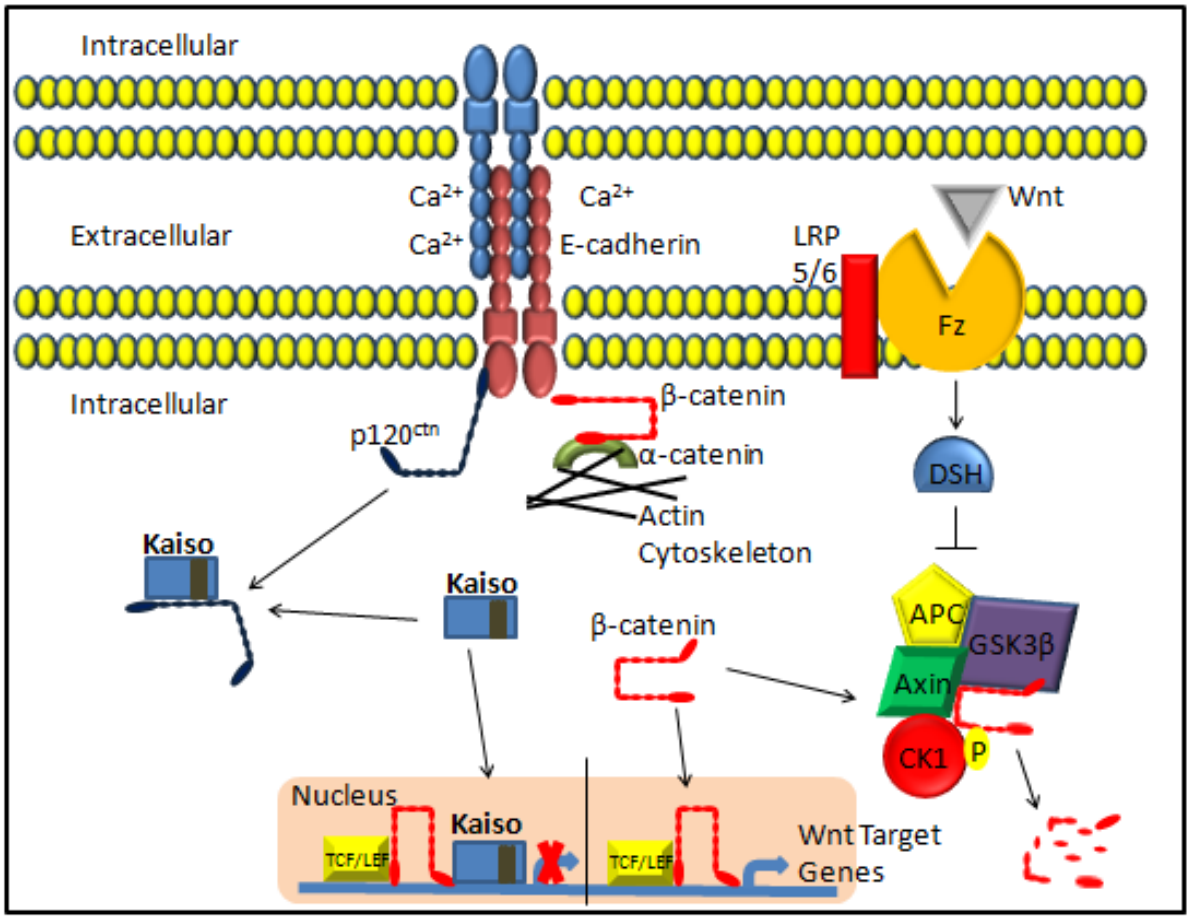
prevent Kaiso from associating with the promoters of its target genes, therefore providing a link between the cell-cell adhesion role of $p120^{ctn}$ with the transcriptional regulation role of Kaiso and suggesting that Kaiso may be a signalling molecule conveying information regarding cell-cell adhesion [2,3,32,44].

1.4 Kaiso as a Regulator of Wnt Signaling

The canonical Wnt signaling pathway is an extensively studied pathway as a result of its ties to cancer development and progression [8,10,14,23]. It is important in the regulation of cell growth, motility and differentiation, and as such, is implicated in tumourigenesis [8,10,14,23]. The Arm-repeat domain containing protein β-catenin is the key downstream signalling molecule in the canonical Wnt/β-catenin signalling pathway [8,10,24]. The function of β-catenin is dependent on its subcellular localization, as it plays a role in cell adhesion by interacting with cadherins at the membrane as well as in gene expression by interacting with TCF/LEF transcription factors to activate transcription in the nucleus [8,10,14]. In the absence of Wnt ligands, cytoplasmic β-catenin is bound by a destruction complex of proteins and is subsequently degraded [10]. In the presence of Wnt ligands however, β-catenin is not degraded by the destruction complex, but instead translocates to the nucleus where it activates gene expression of target genes [10].

Wnt target genes are involved in the regulation of cell proliferation and tumour progression, and hence many of the genes that regulate the Wnt signalling pathway act as proto-oncogenes or tumour suppressor genes [8,14,24,35]. Mis-regulation of the Wnt pathway can alter cell proliferation along with key developmental processes such as axis formation and gastrulation, resulting in tumourigenesis and developmental defects [8,14,24,35]. Kaiso was first

implicated in the regulation of multiple Wnt target genes through the identification of the KBS present in the promoters of such genes (Figure 2) [29,34,37,42]. Kaiso's role as a modulator of Wnt signaling was subsequently confirmed by studies in *Xenopus laevis* by demonstrating that the β-catenin double axis phenotype could be rescued by Kaiso overexpression [34,37]. This role for Kaiso was then expanded upon with further Kaiso depletion and overexpression experiments in *Xenopus laevis*; *cyclin D1*, *siamois*, and the non-canonical *Wnt 11* were all up-regulated after morpholino-induced depletion of Kaiso, resulting in gastrulation defects [34]. Subsequent injection of a dominant negative *Wnt 11* alleviated the defects observed in the Kaiso depletion experiments [34]. Ectopic expression of Kaiso inhibited β-catenin activation of *cyclin D1*, *siamois* and *Wnt 11*, resulting in gastrulation closure defects, and underscored Kaiso's role in the regulation of Wnt signalling [34].



<u>Figure 2:</u> Kaiso and the canonical Wnt signalling pathway. Activation of the canonical Wnt signalling pathway induces cytoplasmic accumulation and subsequent nuclear translocation of β-catenin. In the nucleus, β-catenin interacts with TCF/LEF, activating the expression of Wnt target genes like *cyclin D1* through relief of repression by TCF/LEF. Loss-of-function or gain-of-function mutations to various signalling molecules in the canonical Wnt signalling pathway can lead to constitutive cytoplasmic accumulation of β-catenin, and result in up-regulation of Wnt target genes that have been implicated in tumourigenesis. However, in contrast to β-catenin Kaiso represses the expression of Wnt target genes that harbour the KBS. Cytoplasmic $p120^{ctn}$ binds to the DNA-binding domain of Kaiso, thereby blocking Kaiso binding to the KBS and relieving Kaiso-mediated repression of Wnt target genes.

1.5 The characterization of the transcription factor ZBTB4

Given the functional significance of Kaiso as a transcriptional regulator of various Wnt target genes, an effort was made to identify additional proteins with similar sequence to Kaiso that may in turn confer similar functions. A BLAST search of the genome was conducted with the Kaiso ZF domain and two closely related Kaiso paralogs, ZBTB4 and ZBTB38 were identified [22]. ZBTB4 contains an N-terminal BTB/POZ domain that mediates homo/heterodimerization with interaction partners and co-repression machinery [22]. The domain differs notably from Kaiso's in that it is interrupted by a 60 amino acid stretch of mostly serines and alanines, a sequence that is absent in Kaiso's BTB/POZ domain (Figure 1) [22]. ZBTB4 shares 62% identity with Kaiso's three Kruppel-like C_2H_2 zinc fingers, suggesting that the two proteins share similar DNA-binding activities [22]. ZBTB4 contains three additional and distinct zinc fingers that are not found in Kaiso and therefore likely do not mediate Kaiso-like DNA binding activity (Figure 1) [22].

Like Kaiso, ZBTB4 also demonstrates dual-specificity DNA-binding activity; it binds methylated CpGs, as well as sequence-specific sites [41]. Because methylated DNA signals for gene repression through recruitment of repression machinery, it is likely that ZBTB4 and other methyl-DNA binding proteins act as transcriptional repressors [41,49]. Kaiso requires two CpG-dinucleotides (CGCG) for methylation-dependent binding, whereas ZBTB4 interacts with a single CpG (CG) dinucleotide sequence [41]. Kaiso and ZBTB4 also bind non-methylated sequence-specific DNA, interacting with the KBS, and the **Z**BTB**4** **B**inding **S**equence (Z4BS) $C^C/_T$GCCATC respectively [17,18,41]. Interestingly, in addition to binding to the Z4BS, ZBTB4 also binds to the KBS, which suggests that ZBTB4 may regulate Kaiso target genes, potentially in a functionally redundant manner to that of Kaiso [41].

As ZBTB4 continues to be characterized, its role as a tumour suppressor protein has become increasingly more clear. For example, ZBTB4's transcriptional repression of **S**pecificity **P**rotein (Sp) **1** & **3** exemplifies its role as a tumour suppressor protein [33]. Sp 1 & 3 have been characterized as transcriptional activators, and are well known for their roles in breast tumourigenesis by regulating pro-survival and pro-invasion genes such **V**ascular **E**ndothelial **G**rowth **F**actor (VEGF), **V**ascular **E**ndothelial **G**rowth **F**actor **R**eceptor **1** (VEGFR1), and survivin, among others [33]. The Sp 1 & 3 promoters contain CG-rich regions with which ZBTB4 interacts in a methylation-dependent manner to repress their expression [33]. Overexpression of ZBTB4 induces a down-regulation of Sp1 and 3 genes at the mRNA and protein level, *in vivo* [33]. In MCF7 cells, ZBTB4 overexpression reduced proliferation, while in MDA-231 cells, ZBTB4 overexpression reduced invasion potential [33,48]. Both of these phenotypes were attributed to ZBTB4's repression of Sp1 & 3, and their downstream target genes [33,48].

ZBTB4 has also been implicated in regulating p53-induced apoptosis by repressing p21 expression [48]. It was observed that ZBTB4 depletion provided a significant survival advantage to cancer cell lines in the presence of chemotherapeutic drugs [48]. The mechanism of action behind this observation was that ZBTB4 heterodimerizes with the BTB/POZ containing protein MIZ-1 to repress p21 expression which then induces a shift from p53-mediated cell cycle arrest to p53-mediated apoptosis [48]. Taken together, ZBTB4 functions as a tumour suppressor protein by repressing transcriptional activators and their target genes, and by functioning as a pro-apoptotic agent in the presence of p53 signaling [33,48].

1.6 Kaiso represses the cell cycle gene *cyclin D1*

Kaiso was originally identified as a modulator of *cyclin D1* expression levels following experiments in *Xenopus laevis* [37]. It was observed that a significant increase in *cyclin D1* expression occurred when xKaiso was depleted, suggesting that Kaiso modulates *cyclin D1* transcription levels *in vivo* [37]. Since the observed increase in *cyclin D1* expression may have been the result of an indirect or downstream effect, our lab performed additional studies to characterize the relationship between Kaiso and *cyclin D1*. We identified three KBS and eight CpG-dinucleotide sites within the *cyclin D1* promoter and demonstrated a direct interaction between Kaiso and the *cyclin D1* promoter *in vitro* using **E**lectrophoretic **M**obility **S**hift **A**ssays (EMSA) [19]. We determined that Kaiso interacts directly with the -1067 KBS in a sequence-specific but methylation-independent manner [19]. However, Kaiso interacted directly with the +69 KBS in a methylation-dependent but KBS-independent manner [19]. **Ch**romatin **I**mmuno**P**recipitation (ChIP) experiments confirmed that Kaiso interacts with the *cyclin D1* promoter in breast and colon tumour cell lines [19]. Luciferase assays in human breast tumour cells further confirmed that Kaiso represses transcription of a minimal *cyclin D1* promoter in a dose-dependent manner [19]. Together these data revealed that Kaiso directly interacts with and represses the *cyclin D1* promoter in a sequence-specific *and* methylation-dependent manner.

1.7 Cyclin D1 in Cell Cycle Regulation and Tumourigenesis

Cyclin D1 is a key player in cell cycle regulation, functioning to mediate passage from G1 (first growth) phase to S (DNA synthesis) phase of the cell cycle by directly activating G1 phase **c**yclin-**d**ependent **k**inase **4** or **6** (CDK4/6) [45]. Activated CDK4 or 6 then phosphorylate the **r**etino**b**lastoma protein (Rb), and trigger passage of the cell from G1 to S phase of the cell

cycle [4,21,45]. Cyclin D1 protein levels fluctuate dramatically throughout the cell cycle, with high levels being required to trigger passage into S phase, but low levels being required for DNA synthesis to occur (Figure 3) [1,21,45]. If conditions are optimum to support continued proliferation, Cyclin D1 protein levels rise in G2 phase in preparation for the following G1/S phase checkpoint [45]. As such, Cyclin D1 has been described to function as a molecular switch, requiring a pro-active induction of its expression and stabilization following S phase in order to permit passage through the cell cycle [45].

Experimental analysis has identified ***cyclin D1*** (CCND1) as an important oncogene in human cancer progression. CCND1 gene copy number is found to be amplified in ~20% of breast cancers, and is overexpressed in ~50% of invasive primary breast carcinomas [1,9,20]. Ectopic expression of *cyclin D1* in cell culture is sufficient to induce cell cycle progression, independent of external growth signals, whereas overexpression of *cyclin D1* in the mammary gland tissue of mice results in the development of adenocarcinomas [1,4]. Interestingly, *cyclin D1* knockout mice (cyclin D1-/-) display a striking resistance to tumourigenesis when c-neu or v-Ha-ras oncogenes are driven in the mammary gland by the **m**ouse **m**ammary **t**umor **v**irus (MMTV), as compared to wild type cyclin D1+/+ mice [50]. Taken together, these data implicate Cyclin D1 as a key regulatory molecule in breast tumourigenesis. Although *cyclin D1* has been identified as an important oncogene in breast tumourigenesis, a host of tumour-specific *cyclin D1* gene rearrangements in various other cancers including esophageal and B-cell lymphomas have also been described, underscoring the fact that *cyclin D1* activation provides a selective advantage to developing neoplasias across a wide range of tissue contexts [4].

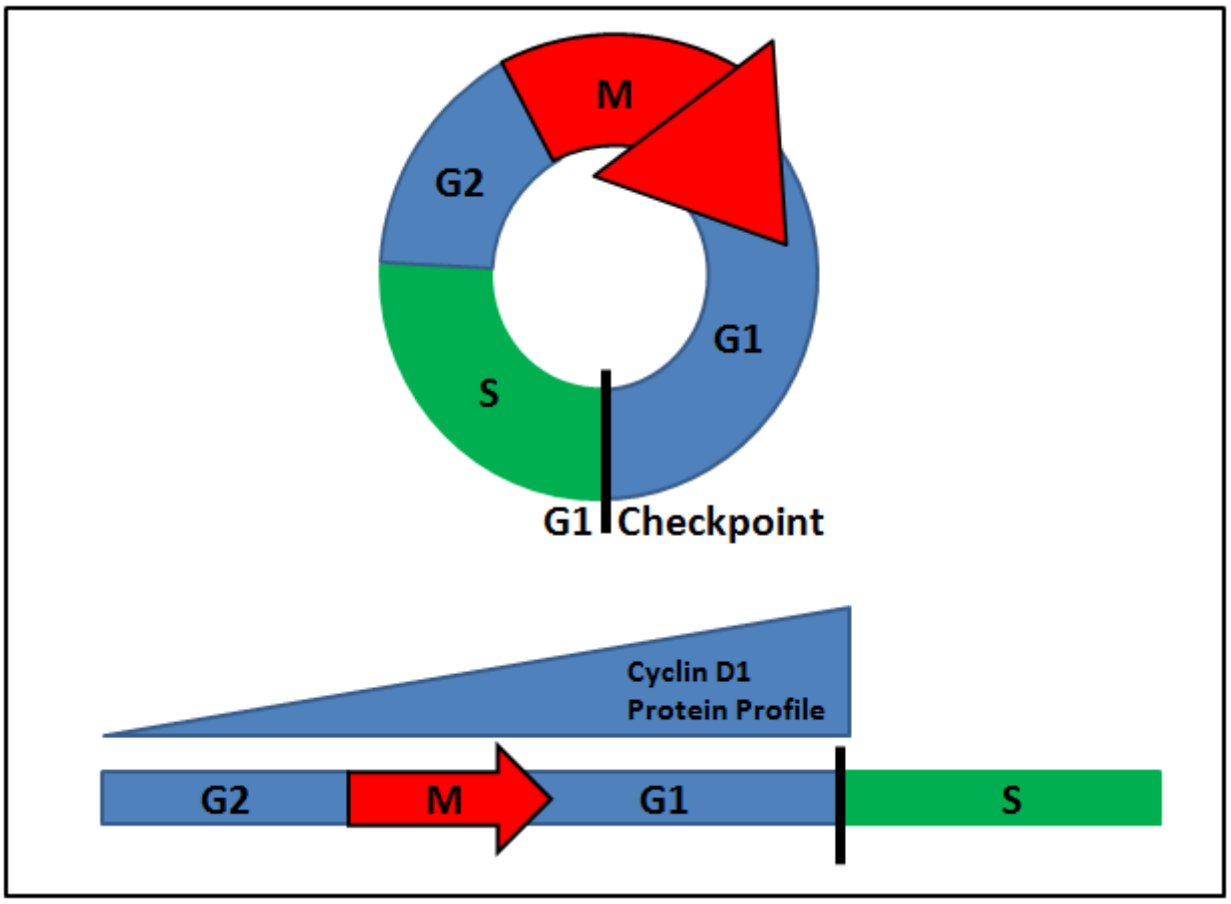


Figure 3: Cyclin D1 functions as a molecular switch in the cell cycle. Cyclin D1 plays a critical role in the regulation of cell cycle progression by functioning as a molecular switch. For the cell to cross the G1 checkpoint into S phase, Cyclin D1 protein levels must reach a critical threshold to activate the downstream signalling molecules CDK 4 and 6. Following passage into S phase, Cyclin D1 is rapidly degraded to allow DNA synthesis to occur. Finally, if conditions permit another round of cell division, Cyclin D1 protein levels will begin to increase throughout G2 and M phases, building toward another threshold level at the G1 checkpoint. Due to Cyclin D1's cyclic pattern of protein synthesis and degradation, it controls when cell division will and will not occur, resulting in its nickname, the molecular switch.

1.8 Kaiso and ZBTB4 are Misexpressed in Tumour Tissues

The tissue microenvironment has been shown to play a significant and unexpected role in Kaiso's function as a transcriptional repressor. In cultured cell lines, Kaiso localizes almost exclusively to the nucleus, where it interacts specifically with target genes to repress gene expression [18,32,43]. However across a wide spectrum of both normal and tumour tissues including breast carcinoma tissues, Kaiso sub-cellular localization was predominantly cytoplasmic [43]. Interestingly when cultured cell lines with nuclear Kaiso were xenografted into nude mice, Kaiso was detected in the cytoplasm rather than the nucleus, suggesting that the microenvironment influenced Kaiso's translocation from the nucleus to the cytoplasm [43]. A regulatory role for the tissue microenvironment was proposed, as relief of repression of tumour-promoting target genes occurs when Kaiso translocates from the nucleus to the cytoplasm [18,43]. These findings suggest that Kaiso's localization is highly dynamic, and may be tissue specific.

Interestingly, Kaiso's highly dynamic and tissue-specific subcellular localization has been shown to have clinical and prognostic significance as well. A study investigating correlations between clinical histology and Kaiso's subcellular localization in 477 human invasive breast carcinoma samples described significant correlations found between nuclear Kaiso and (i) high histological grade, (ii) ERα negativity and (iii) triple negative breast cancers [47]. All three described categories are linked with poor prognosis. Conversely, in non-small cell lung carcinomas a correlation was found between cytoplasmic Kaiso and poor overall prognosis [47]. It is clear that Kaiso demonstrates highly dynamic and tissue-specific subcellular localization, although this data is novel in demonstrating that this dynamic nature has a prognostic and clinically relevant impact that varies in different types of cancer.

In addition to Kaiso's varied subcellular localization in different tissues, Kaiso appears to be misexpressed in tumour tissues compared to matched normal tissues. A Cancer Profiling Array representing 12 different human tissues was analysed by our laboratory, and it was determined that Kaiso expression was decreased in ~30% of human breast cancer tissues, and ~50% in human ovarian cancer tissues [Daniel Lab unpublished data].

Mounting evidence suggests that ZBTB4 is also down-regulated in cancer, and that its expression levels bear significant predictive value for prognostic outcome. Patterns of stage-specific ZBTB4 expression were identified in breast tumour tissue, with ZBTB4 expression being the lowest in advanced tumour stages, and the highest in early stages [48]. Kaplan-Meier survival analysis of multiple independent breast and neuroblastoma data sets identifies a correlation between high ZBTB4 expression and longer relapse-free survival, and conversely, between low ZBTB4 expression and shorter relapse-free survival [48]. Furthermore, immunohistochemical analysis of ZBTB4 protein in breast tumour and non-tumour tissue revealed reduced levels of protein staining in breast tumour versus matched non-tumour tissue from the same patient [33]. Together with data on Kaiso's transient patterns of localization in tissue, it appears that Kaiso is mislocalized and down-regulated in a wide range of tumour tissues, and that ZBTB4 expression levels follow a stage-specific pattern, providing prognostic value for relapse free survival.

1.9 Rationale

ZBTB4 was initially identified as a Kaiso-like protein due to its high sequence similarity with Kaiso's zinc-finger DNA-binding domain [22]. This similarity in their DNA-binding domains suggested that the two proteins share similar gene regulatory activities. Indeed, although each protein binds a unique sequence-specific consensus element, and therefore

regulate different subsets of genes, ZBTB4 also interacts with the KBS *in vitro*, suggesting that ZBTB4 may co-regulate Kaiso target genes via the KBS [22]. Indirect relationships have been demonstrated between ZBTB4 and the Kaiso target gene *cyclin D1*, however it is unknown whether *cyclin D1* is a also a target gene of ZBTB4. Hence this thesis sought to elucidate whether ZBTB4 was a direct regulator of *cyclin D1* expression.

HYPOTHESIS:

ZBTB4 regulates activity of the cyclin D1 promoter via interaction with the KBS.

SPECIFIC AIMS

Specific Aim 1: Determine if *cyclin D1* is a putative ZBTB4 target gene.

1.1: Determine if ZBTB4 binds to the -1067 or +69 KBS in the cyclin D1 promoter.

Specific Aim 2: Determine if ZBTB4 regulates transcription of *cyclin D1*.

2.1: Determine ZBTB4's effect on the expression of the -1748 CD1 minimal reporter construct.

2.2: Determine the effect of ZBTB4's binding to the cyclinD1 promoter on Kaiso-mediated repression of cyclin D1.

MATERIALS AND METHODS

2.1 Cell Culture

The human breast carcinoma cell line MCF7 and the human colon carcinoma cell line HCT-116 were both purchased from **A**merican **T**ype **C**ulture **C**ollection (ATCC) (Manassas, VA) and used in various experiments. MCF7 cells were cultured in **D**ulbecco's **M**odified **E**agle **M**edium (DMEM) (Invitrogen, California) supplemented with 10% **F**etal **B**ovine **S**erum (FBS) (Hyclone, Utah), 0.1 mg/mL penicillin/streptomycin and 0.25 µg/mL Fungizone (Invitrogen, California). The ZBTB4-depleted cell line **pR**etro**S**uper-ZBTB4 (pRS-ZBTB4) and its control (pRS-ZBTB4 scrambled) were grown in Puromycin (Invitrogen) selection media once every 4 passages, or DMEM as described above. All cell lines were grown in a 5% CO_2 humidified incubator at 37°C.

2.2 Immunofluorescence

MCF7 cells were seeded onto 22x22 mm coverslips in 6-well cell culture dishes and incubated at 37°C and 5% CO_2 until cells reached 70-80% confluency. Media was aspirated and washed twice with cold 1X **P**hosphate **B**uffered **S**aline (PBS), and then fixed with 2mL of ice cold 100% methanol for 7 minutes at -20°C. Methanol was aspirated and washed away with cold 1X PBS twice. Non-specific binding was blocked with 3% milk-PBS at room temperature for 10 minutes, and then aspirated. Kaiso monoclonal (6F) and ZBTB4 polyclonal primary antibodies were diluted in 3% milk-PBS each at 1:500, and added directly to the coverslips for 30 minutes at room temperature. Primary antibody solutions were washed off slides twice with 1X PBS, after which slides were briefly washed once with 3% milk-PBS solution to aid the secondary antibody solution in coating the coverslips. Alexafluor 594 and 488-conjugated goat

anti-mouse and goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, Ore) were diluted in 3% milk-PBS at 1:1000 along with Hoescht stain at 1:300, and added directly to the coverslips at room temperature for 30 minutes in the dark. The secondary antibody solutions were washed twice with 1X PBS, once with double distilled water, and mounted on microscope slides with Prolong Gold Antifade Reagent (Invitrogen, Eugene, Ore). Slides were then imaged using a Zeiss Axiovert 200 inverted fluorescent microscope.

2.3 Chromatin Immunoprecipitation (ChIP)

MCF7 cells were grown to 70-80% confluency in 100 mm culture dishes, using DMEM supplemented with 10% FBS, 0.1 mg/mL penicillin/streptomycin and 0.25 µg/mL Fungizone, as described above. Media was aspirated from plates and the cells were fixed with 10 mL of a 1% formaldehyde in DMEM solution for 10 minutes at room temperature on a belly dancer. The formaldehyde solution was removed, and cells were washed with 5 mL of cold 1X PBS before stopping the fixation process by adding glycine to a final concentration of 125 mM and incubating for 5 minutes at room temperature.. The glycine solution was aspirated, and the plates were washed with 5 mL of cold 1X PBS. 1 mL of lysis buffer (1 Complete Mini protease inhibitor cocktail tablet (Roche, Germany), in 10 mL 1X PBS) was added to each plate, prior to scraping cells with a cell scraper, and collecting cell suspension with a pipette. The collected cell suspension was centrifuged at 4°C for 10 minutes at 2500 rpm, and the supernatant was removed. The cell pellet was re-suspended in 350 µL of cold lysis/IP buffer (150 mM NaCl, 5 mL of 0.5 M 8.0 pH EDTA, 25 mL of 1 M 7.5 pH Tris-HCl, 25 ml of 10% NP-40, and 50 mL of 10% Trition X-100 in 400 mL of double de-ionized water) and incubated on ice for 30 minutes to allow for membrane lysis. Cells were transferred to a chilled dounce homogenizer and gently dounced for 10 strokes to liberate nuclei. The cell solution was transferred to microcentrifuge

tubes and centrifuged at 4°C for 5 minutes at 5000 rpm. The supernatant was discarded and the nuclei were re-suspended in 250 µL of cold nuclear lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, SDS 0.8% vol/vol, and Complete Mini protease inhibitor cocktail) followed by an incubation on ice for 10 minutes. Samples were sonicated for 5 rounds of 15 pulses on ice, with sonication settings optimized as follows: 90% duty cycle, microtip set to 5% max power output. Following sonication, samples were centrifuged at 4°C for 15 minutes at 13,000 rpm and supernatant was transferred to new microcentrifuge tubes in 200 µL aliquots. To confirm efficacy of sonication, 20 µL of chromatin was removed from each sample and added to 150 µL of autoclaved water, 8 µL of 5 M NaCl, and 1 µL of 10 µg/µL RNase A, and incubated for 16 hours at 65°C. Samples were cooled to room temperature, and 10 µL of 0.5 µg/µL proteinase K was added to each sample before incubation for 1.5 hours at 42°C. 200 µL of phenol-chloroform was then added to each sample, and centrifuged at room temperature for 5 minutes at 14,000 rpm. The supernatant was transferred to fresh tubes, and 20 µL of 3M sodium acetate and 500 µL of 100% ethanol were added to precipitate the DNA. The samples were chilled at -20°C for 1 hour, and then centrifuged at 4°C for 10 minutes at 14,000 rpm. The supernatant was carefully discarded, and the chromatin pellet was dried at 30°C for 10 minutes using a speed vacuum. The pellet was re-suspended in 30 µL of autoclaved water, analysed with a spectrophotometer, and 10 µL was loaded into a 2% agarose gel to assess chromatin quality and fragmentation. From the sonicated chromatin aliquots, 6 µg of chromatin was removed per immunoprecipitation trial, and added to 400 µL of lysis/IP buffer. 10% of the sheared chromatin sample was transferred to a fresh microcentrifuge tube, and frozen at -80°C to serve as the 10% input. 5 µg of rabbit IgG was added to each trial, and samples were rotated end-over-end at 4°C for 1 hour to block non-specific antibody binding. 4 µg of Histone H3, ZBTB4, Kaiso, and Mouse IgG (negative

control) antibodies were added to each respective chromatin sample, and rotated end over end at 4°C overnight. Protein-A beads were prepared either with a rabbit anti-mouse IgG bridge (for mouse monoclonal primary antibodies) or without (for rabbit polyclonal primary antibodies) by adding 10 µg of rabbit anti-mouse IgG to washed beads, and rotating end-over-end at 4°C for 30 minutes. 50 µL of Protein-A beads (with or without antibody bridge) were added to the respective chromatin samples and rotated end-over-end at 4°C for 1 hour. Samples were then centrifuged at 5000 rpm for 3 minutes at 4°C to pellet beads. The chromatin was then re-suspended in 1 mL of RIPA solution (50 mM Tris pH 8, 150 mM NaCl, SDS 0.1% vol/vol., deoxycholate 0.5% vol/vol., NP40 buffer 1% vol/vol., 1mM EDTA pH 8, and 8 mL double distilled water) and rotated end-over-end at 4°C for 10 minutes. Each sample was centrifuged at 5000 rpm at 4°C for 3 minutes, and the supernatant was discarded. Samples were re-suspended in high salt buffer (50 mM Tris pH 8, 500 mM NaCl, SDS 0.1% vol/vol., deoxycholate 0.5% vol/vol., NP40 buffer 1% vol/vol., 1 mM EDTA pH 8, and 8 mL double distilled water) and rotated end-over-end at 4°C for 10 minutes. Samples were centrifuged at 5000 rpm for 3 minutes at 4°C, and the supernatant was discarded. The chromatin was re-suspended in LiCl buffer (50 mM Tris pH 8, 250 mM LiCl, NP40 buffer 1% vol/vol., deoxycholate 0.5% vol/vol., 1 mM EDTA pH 8, and 8 mL double distilled water) and rotated end-over-end for 5 minutes at 4°C. Samples were then centrifuged at 5000 rpm for 3 minutes at 4°C, and the supernatant was discarded. The chromatin was re-suspended in TE buffer (10 mM Tris-Cl and 1 mM EDTA) and rotated end-over-end at 4°C for 10 minutes. The samples were then centrifuged at 5000 rpm for 3 minutes and the supernatant was discarded. 300 µL of TE buffer and 15 µg of RNase was added to Immunoprecipitated chromatin samples, as well as the thawed 10% input chromatin, and incubated at 37°C for 30 minutes. 75 µg of proteinase K and SDS 1% vol/vol. were then

added to each Immunoprecipitated sample and 10% input tube, and incubated at 37°C for 4 hours. Formaldehyde-induced crosslinks were then reversed by incubating samples at 65°C for 6 hours. Using a phenol chloroform extraction protocol, DNA was isolated as follows. 300 µL of phenol chloroform was added to each sample and vortexed shortly to mix. Samples were centrifuged at 13,000 rpm for 5 minutes and the aqueous layer was then transferred to a new tube. The previous step was repeated twice to ensure maximum DNA extraction. 600 µL of ice cold 100% ETOH and 30 µL of 3M NaOAc (pH 5.2) were transferred to the retrieved aqueous layer in the new tube, and incubated at -80°C for 2 hours. The samples were then centrifuged at 13,000 rpm for 15 minutes at 4°C to pellet the DNA, and the supernatant was discarded. 1000 µL of ice cold 70% ETOH were added to each tube, and mixed by inverting. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C to pellet DNA, and the supernatant was discarded. The previous rinse step was repeated twice. The pelleted chromatin was left on the bench to air dry. The DNA pellet was then resuspended in 50 µL of Gibco water, and stored at -20°C prior to PCR amplification with primers specific to the CCND1 -1067 or +69 KBS, followed by resolution on a 2% agarose gel. Primers used in the PCR are listed in table 1 below.

Table 1: PCR Primers for ChIP

Mobix ID	Primer Name	Sequence
ML 4850	CD1-1067-for	5’ TTT ACA TCT GCT TAA GTT TGC G 3’
ML 4851	CD1-1067-rev	5’ TTA GAA TTT GCC CTG GGA CT 3’
ML 6191	CD1+69-for	5’ CAC ACG GAC TAC AGG GGA GTT 3’
ML 6192	CD1+69-rev	5’ CTC GGC TCT CGC TTC TGC TG 3’

2.4 Western Blot

293T, HCT-116, HeLa, MCF7, MDA-231, and T47D cells were grown to 70 % confluency in 100 mm tissue culture dishes as described above. Media was aspirated off each plate and cells were washed once with 5 mL of ice-cold PBS. The cells were then lysed with 600 µL of Nonidet P-40 lysis buffer (0.5% NP-40, 50 mM Tris & 150 mM NaCl) with protease and phosphatase inhibitors (1 mM PMSF, 10 µg/mL Leupeptin, 10 µg/mL Aprotinin, 1 mM sodium orthovanadate, and 25 mM EDTA) for 7 minutes on ice. Plates were scraped with cell scraper and lysates were transferred to fresh 1.5 mL Eppendorf tubes. Cell lysates were cleared of debris by centrifugation at 4ºC for 5 minutes at 13,200 rpm. Cleared cell lysates were then transferred to fresh microcentrifuge tubes. A Bradford Assay (Biorad, California) was performed to determine protein concentrations within each lysate sample. 10 µg of protein from each cell lysate was combined with 2 x Laemmli Sample Buffer (0.1 M Tris [pH 6.8], 2 % SDS, 10 % sucrose, 0.008 % Bromophenol blue & 0.24 M β-mercaptoethanol) vortexed, and boiled for 10 minutes. Samples were allowed to cool to room temperature on bench top for 20 minutes, and then resolved on a 7% SDS-polyacrylamide gel for 4 hours at 35 mA. The gel was then transferred to a nitrocellulose membrane (Perkin Elmer) using a Hoeffer semi-dry transfer apparatus (Amersham/Pharmacia, California) for 2 hours at 125 mA. The nitrocellulose membrane was then cut into pieces, specific to protein size, and blocked for non-specific antibody binding with 3% milk in **T**ris-**B**uffered-**S**aline (TBS) [pH 7.4] for 30 minutes at room temperature. The blocked membranes were then incubated with primary antibody in 3% milk-TBS solution overnight at 4ºC. Primary antibodies were used at the following concentrations: Kaiso rabbit polyclonal (1:30,000) (Daniel Lab), ZBTB4-120 rabbit polyclonal (1:15,000) (Pierre-Antoine Defossez Lab) and β-actin mouse monoclonal (1:5,000) (Sigma-Aldrich, MO).

The membranes were washed 5 times for 5 minutes each with 1X TBS at room temperature to remove residual primary antibody solution. The membranes were incubated with horseradish peroxidase-(HRP) conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch, PA) in 3% milk-TBS solution (1:40,000) for 2 hours at room temperature. The membranes were washed 5 times for 5 minutes each with 1X TBS to remove residual secondary antibody solution. The membranes were then incubated with the **E**nhanced **C**hemi**L**uminescence system (ECL) (Amersham/Pharmacia) for 1 minute at room temperature, as per manufacturer's protocol. Finally, the membranes were developed on XAR film (KODAK BioMax, NY).

2.5 Luciferase Assay Transfections

MCF7 cells were seeded at 250,000 cells per well in 6-well dishes and incubated at 37ºC overnight, or until cells were approximately 70% confluent. The cells in each well were transfected with pGluc-Basic or pGluc-Basic-1748CD1 reporter constructs, as well as varying amounts of effector plasmids (pcDNA3-Kaiso, pEGFP-ZBTB-4, pRSV-β-Gal or empty vectors) as follows: the DNA vectors were diluted in DMEM without FBS or antibiotics and vortexed briefly before adding Turbofect transfection reagent (MBI Fermentas) as per manufacturer's recommendations. The solution was vortexed for 15 seconds before incubating at room temperature for 15 minutes. Media was aspirated from cells and 900 µL of serum supplemented DMEM media was added to each well, followed by 100 µL of DNA vector solution, added drop-wise. The cells were incubated at 37ºC for 24 hours. 25 µL of supernatant media from each well was then assayed for luciferase activity as per the New England Biolab protocol. Media was aspirated from each well, and cells were lysed using 1X Reporter Lysis Buffer (Promega, WI) for standardization as part of the β-Galactosidase assay. Briefly, cell lysates were transferred

into fresh microcentrifuge tubes, and centrifuged at 13,000 rpm for 5 minutes at 4°C. 50 µL of supernatant from each sample was then transferred to a corresponding well of a 96-well plate. An **o**rtho-**N**itro**p**henyl-β-**g**alactoside (ONPG) colourimetric solution was made as follows: 33 µL ONPG (4 mg/ml) (Sigma-Aldrich, MO), 1.5 µL $MgCl_2$ Buffer, and 65.5 µL of 0.1M Na_2HPO_4. 100 µL of ONPG solution was added to each well, and the plate incubated at 37°C for the colourimetric reaction to occur. The reaction was stopped by adding 150 µL of 1M Tris (pH 11) and the reaction was read using a Spectramax plate reader. The values were used as a measure of transfection efficiency to relate the luciferase assay data.

RESULTS

3.1 Kaiso and ZBTB4 co-localize in the nucleus in MCF7 Cells.

To determine if spatial overlap occurs between ZBTB4 and Kaiso, we first examined the subcellular localization patterns of both proteins in MCF7 cells. Given that both Kaiso and ZBTB4 harbour DNA-binding ZF domains and have been widely characterized for their roles in transcriptional regulation of various target genes, it was expected that they would each localize predominantly to the nucleus in cell culture, and share similar patterns of localization. We performed IF experiments using Kaiso-specific and ZBTB4-specific antibodies. As expected, both Kaiso and ZBTB4 localize predominantly to the nucleus in MCF7 cells (Figure 4A). A weaker signal was observed in the cytoplasm for both Kaiso and ZBTB4, suggesting that the subcellular localization of these transcription factors is dynamic and not static (Figure 4A). Interestingly and unexpectedly, when MCF7 cells were seeded at a lower density (100,000 cells/well as compared to 300,000 cells/well) we observed that both Kaiso and ZBTB4 were excluded from the nucleus, and instead localized predominantly in the cytoplasm and perinuclear space (Figure 4B). While this pattern of localization is opposite to that observed when cells were seeded at higher concentrations, both ZBTB4 and Kaiso displayed similar patterns of localization (Figure 4B).

3.2 High ZBTB4 protein levels are found across a panel of cell lines.

We next preformed western blot analysis to assess the patterns and levels of protein expression for ZBTB4 and Kaiso across a panel of cell lines including breast carcinoma (MCF7, MDA-231 and T47D), a cervical carcinoma (HeLa), a colon carcinoma (HCT-116), and an embryonic kidney (HEK 293T) line. ZBTB4 was highly expressed across all the cell lines

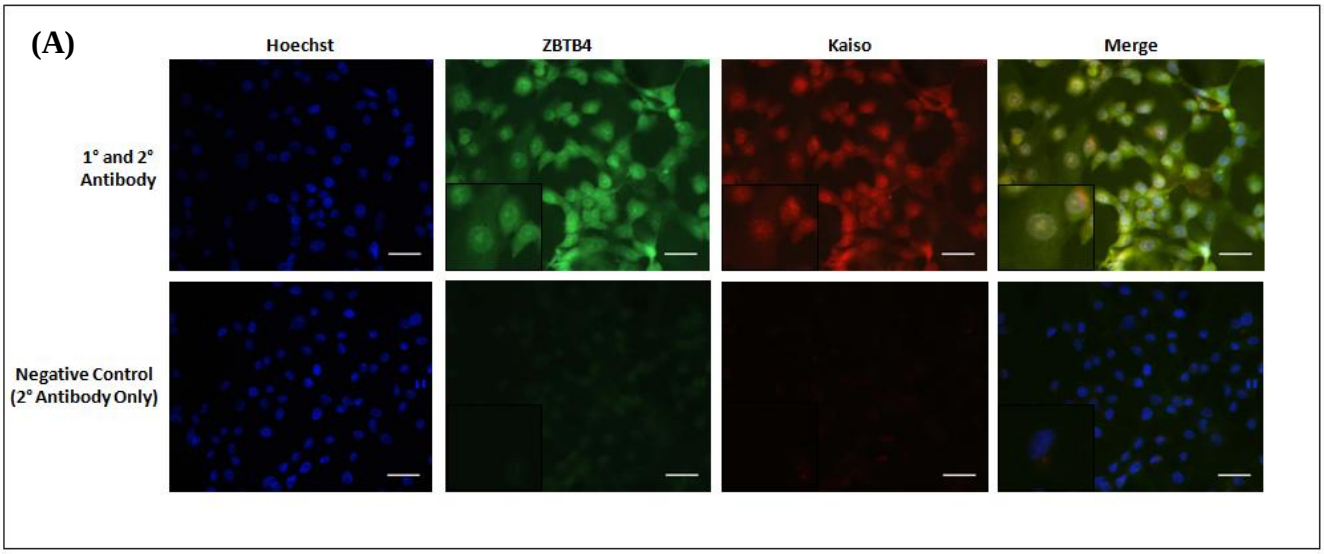
assayed, whereas Kaiso displayed a more varied pattern of expression across cell lines (Figure 5). 293T and HCT-116 cell lines both express high levels of Kaiso as compared to HeLa, MCF7, MDA-231 and T47D lines which all display similar, but lower levels Kaiso (Figure 5).

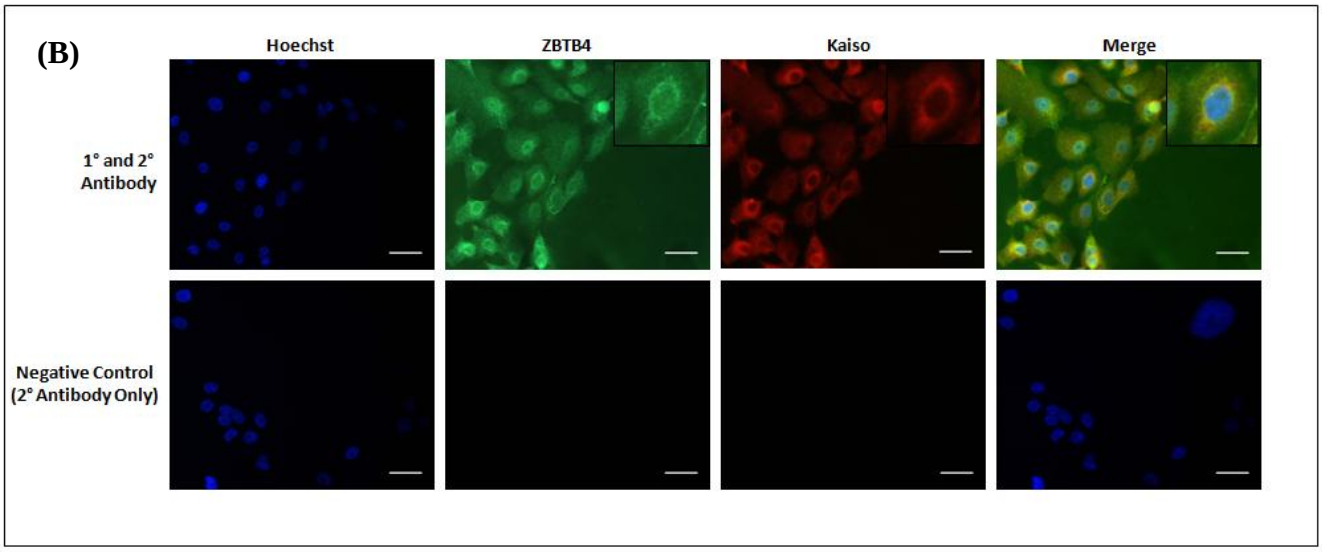
3.3 The CCND1 minimal promoter sequence contains a -1065 and +69 Z4BS.

The CCND1 minimal promoter sequence was analyzed to determine whether putative binding sequences for ZBTB4 exist. Two previously identified KBS are shown at -1067 and +69 positions of the CCND1 minimal promoter (Figure 6, red text). Due to high sequence homology shared between the KBS and the Z4BS, it is not surprising that most of the Z4BS (7 of 8 nucleotides) was found located at the same site as the -1067 KBS (to be referred to as the -1065 Z4BS) (Figure 6, underlined), while 5 of 8 nucleotides (encompassing all 4 nucleotides previously described as being essential for ZBTB4 binding) were found at the +69 KBS of the CCND1promoter (to be referred to as the +69 Z4BS/KBS) (Figure 1, underlined). The presence of these two partial Z4BS suggests that ZBTB4 binds to and regulates CCND1 expression.

3.4 ZBTB4 interacts with the CCND1 promoter *in vivo*.

Kaiso has previously been shown to interact with the CCND1 promoter both in a methylation-dependent and sequence-specific manner, and it has been characterized as a transcriptional regulator of CCDN1 [19]. ZBTB4 has been demonstrated to bind specifically to an artificial KBS fragment *in vitro*, however this binding activity has not yet been demonstrated *in vivo*. Thus to determine if ZBTB4 interacts with the KBS sequence *in vivo*, we performed chromatin immunoprecipitation (ChIP) experiments using Kaiso and ZBTB4 specific antibodies in MCF7 cells, and PCR primers specific to the -1067 and +69 KBS located within the CCND1 promoter region. As previously demonstrated by Donaldson et al., 2012, PCR fragments





<u>Figure 4:</u> Kaiso and ZBTB4 Co-localize in MCF7 Cells. (A) ZBTB4 and Kaiso display similar patterns of subcellular localization; they both predominantly localize to the nucleus, but also localize to the cytoplasm. **(B)** MCF7 cells seeded at a lower seeding density (100,000 cells/well) show Kaiso and ZBTB4 co-localizing with predominantly perinuclear localization.

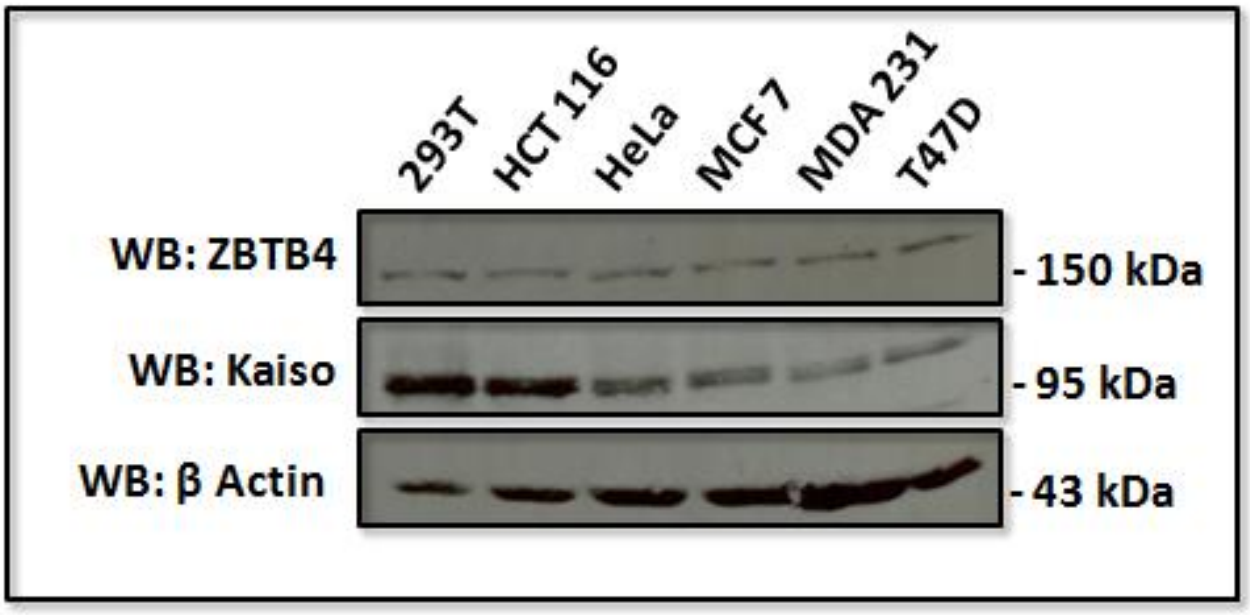


Figure 5: Comparison of Kaiso and ZBTB4 protein levels in a panel of human cell lines. 293T, HCT-116, HeLa, MCF7, MDA-231 and T47D cell lines were assayed for Kaiso and ZBTB4 expression levels by western blot analysis. ZBTB4 expression levels were similar across all cell lines assayed, while Kaiso was highly expressed in 293T and HCT-116 cell lines but showed moderate expression in HeLa, MCF7, MDA-231 and T47D lines.

(A)

Element	Sequence
-1067 KBS	-1070- GGCTCCTGCCAGCCCCCTCA -1050
-1065 Z4BS	-1070- GGCTCCTGCCAGCCCCCTCA -1050
+69 KBS	+61- CGGCGCAGTAGCAGCGAGCA +80
+69 Z4BS	+61- CGGCGCAGTAGCAGCGAGCA +80

(B)

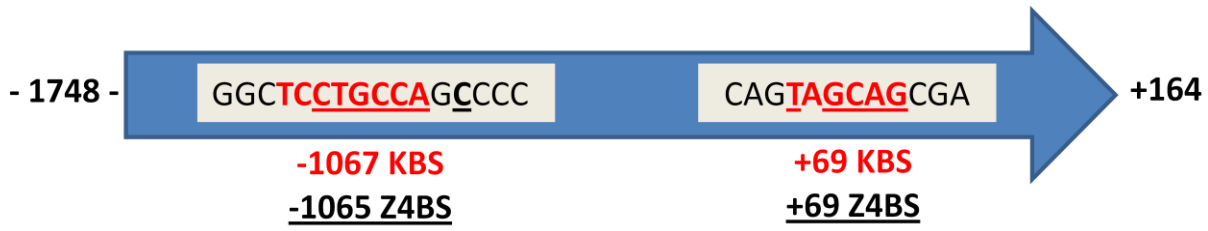


Figure 6: ***cyclin D1*** **minimal promoter sequence highlighting the KBS and Z4BS.** **(A)** The *cyclin D1* minimal promoter sequence was analyzed for the presence of the Z4BS. Two previously characterized KBS (-1067 and +69 KBS) are shown in red text. Due to the high degree of sequence homology shared between the KBS and Z4BS, 2 partial Z4BS are found at -1065 and +69 (shown above as underlined). **(B)** A schematic diagram illustrates the spatial organization of both the KBS (illustrated in red text) and Z4BS (illustrated as underlined text) on the CCND1 minimal promoter sequence.

corresponding to the -1067 or +69 KBS were amplified from Kaiso-precipitated chromatin (Figure 7, lane 5, upper & lower panel). Interestingly, we also amplified +69 KBS-specific DNA fragments from ZBTB4 precipitated chromatin (Fig. 7, lane 4, lower panel), suggesting that ZBTB4 interacts specifically with the CCND1 promoter via interaction with the +69 Z4BS/KBS. However we failed to amplify specific fragments corresponding to the -1067 KBS (Figure 7, lane 4, upper panel). Amplicons present in the negative control lane are likely due to non-specific binding of chromatin to the negative control antibody during the immunoprecipitation. Collectively this data indicates that ZBTB4 interacts specifically with the CCND1 promoter via interaction with the +69 Z4BS/KBS, but not with the -1067 Z4BS/KBS and supports the idea that ZBTB4 may be a physiologically relevant regulator of *cyclin D1 in vivo*.

3.5 ZBTB4 and Kaiso demonstrate opposite patterns of CCND1 gene regulation in a dose-dependent manner.

To elucidate the functional consequence of ZBTB4 binding to the CCND1 promoter, we conducted minimal promoter luciferase reporter assays to quantify the effects of Kaiso and ZBTB4 overexpression on CCND1 minimal promoter activity. The -1748-CD1 minimal promoter construct was cloned into the pGLuc-Basic expression vector and this plasmid was then transfected into MCF7 cells along with the effector overexpression vectors pcDNA3-Kaiso or pBICEP-CMV-2-ZBTB4. As was previously described, Kaiso represses CCND1 minimal promoter activity in a dose-dependent manner (Figure 8A). Given that the -1748-CD1 minimal promoter construct is unmethylated, we can conclude that the observed repression is due to a sequence-specific and not methylation-dependent transcriptional repression. Interestingly, when ZBTB4 was co-expressed with the CCND1 minimal promoter-reporter, we observed a modest activation of minimal promoter activity in a dose-dependent manner (Figure 8B). Thus, ZBTB4

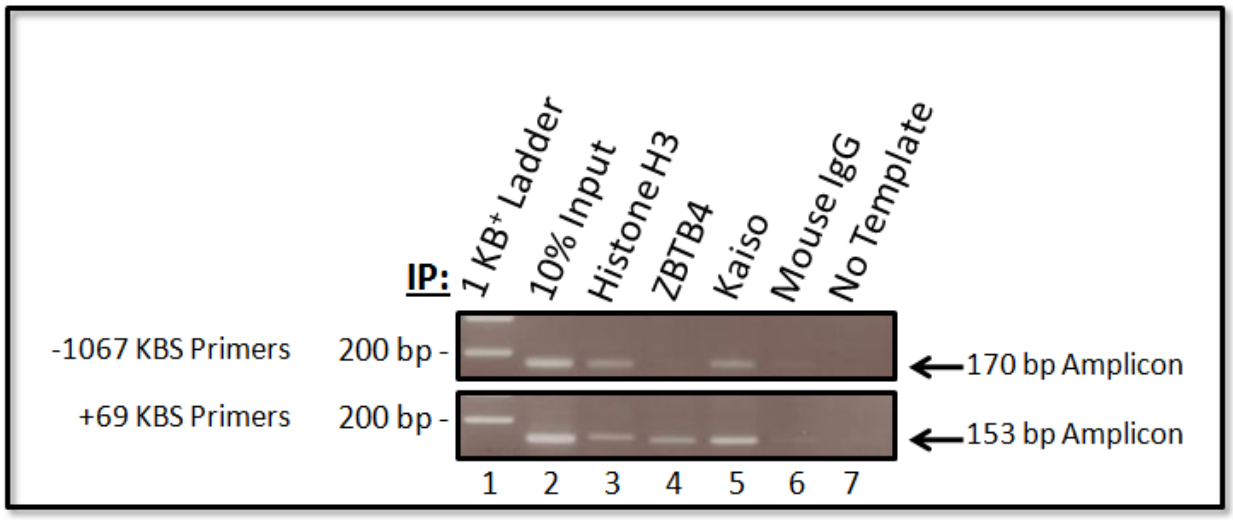
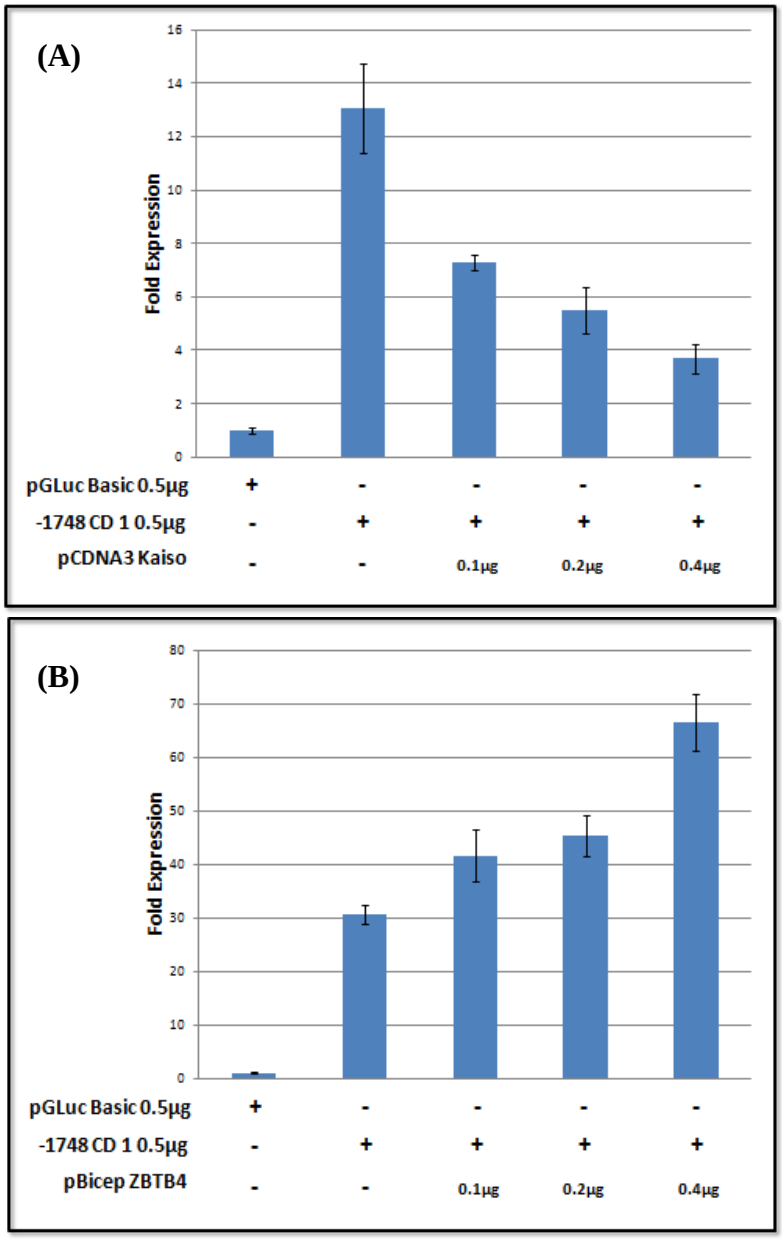


Figure 7: ZBTB4 interacts with the +69 but not the -1067 CCND1 KBS *in vivo*. To determine if ZBTB4 interacts with the CCND1 promoter sequence via the -1067 or +69 KBS *in vivo*, a ChIP experiment was performed using chromatin from MCF7 cells and antibodies directed against ZBTB4 and Kaiso. Primers specific to the +69 KBS amplified size-specific amplicons from ZBTB4 immunoprecipitated chromatin, suggesting that like Kaiso, ZBTB4 binds to the +69 KBS of the CCND1 promoter *in vivo*. However, primers specific to the -1067 KBS failed to amplify size-specific amplicons from ZBTB4 immunoprecipitated chromatin, suggesting that unlike Kaiso, ZBTB4 does not bind to the -1067 KBS of the CCND1 promoter *in vivo*.

may be activating the minimal CCND1 promoter, or ZBTB4 may be antagonizing endogenous Kaiso-mediated repression of CCND1.

3.6 ZBTB4 modulates CCND1 expression in a KBS-independent manner.

Kaiso and ZBTB4 display dual-specificity DNA-binding properties, as each have been widely characterized to bind DNA in a sequence-specific as well as a methylation-dependent manner [41]. To determine if ZBTB4 is modulating the minimal CCND1 promoter by interacting with the KBS/Z4BS in a sequence-specific manner, we conducted minimal promoter luciferase reporter assays using the pGluc-1748 CD1 MUTANT reporter vector, in which both KBS sites have been mutated. As was observed in Figure 8, both Kaiso and ZBTB4 modulate the minimal CCND1 promoter in a dose-dependent manner when the KBS/Z4BS sites are intact. As previously reported, Kaiso's dose-dependent repression was abolished when both KBS binding sites are mutated in the -1748 CD1 MUTANT reporter construct, however the ZBTB4 dose-dependent activation persisted even when both KBS/Z4BS sites are mutated (Figure 9A,B). This suggests that Kaiso mediates repression of the -1748 minimal CCND1 promoter construct via interaction with the KBS/Z4BS sequences, whereas ZBTB4 may mediate activation of the minimal CCND1 promoter by binding to alternative elements, or via alternative mechanisms.



<u>Figure 8:</u> Kaiso and ZBTB4 modulate CCND1 minimal promoter activity in MCF7 Cells. Kaiso and ZBTB4 modulate CCND1 minimal promoter activity, although in opposing manners. Kaiso represses the minimal CCND1 promoter **(A)**, while ZBTB4 activates the minimal CCND1 promoter in a dose-dependent manner **(B)**.

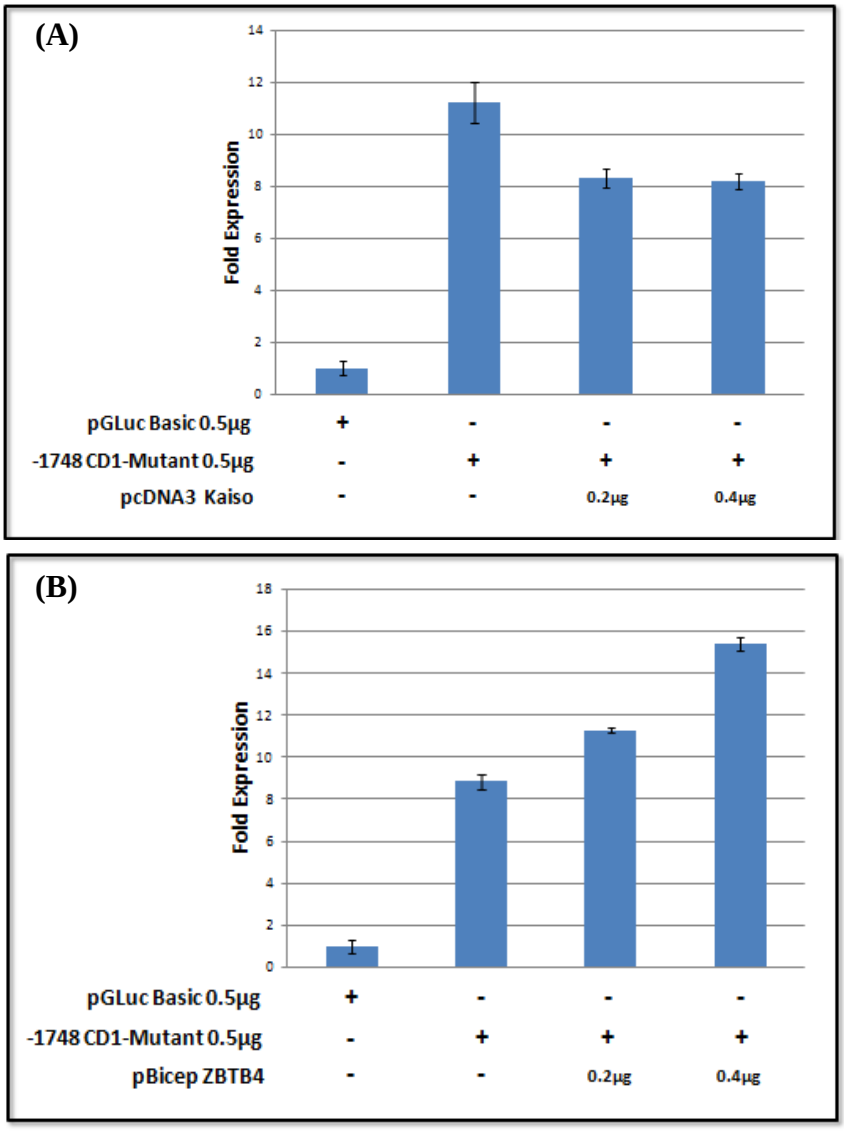


Figure 9: ZBTB4 interacts with the CCND1 promoter in a KBS independent manner. (A) Kaiso represses the minimal CCND1 promoter in a KBS-specific manner. When the KBS are mutated in the -1748-CD1 minimal promoter construct, the dose-dependent repression of the minimal CCND1 promoter is lost, suggesting that Kaiso mediates repression in a KBS-specific manner. **(B)** ZBTB4 activates the minimal CCND1 promoter in a KBS-independent manner. Mutation of the KBS does not interfere with activation of the -1748-CD1 construct by ZBTB4, suggesting that ZBTB4 interacts with the CCND1 promoter in a KBS-independent manner.

DISCUSSION

Interest in Kaiso as a tumour suppressor protein stems from several pieces of evidence. First, the identification of the KBS in the promoter region of various Wnt target genes including *cyclin D1* and *matrilysin*, both of which have been characterized for their roles in tumourigenesis (proliferation and invasion, respectively) implied that Kaiso is involved in their regulation [18,29,34,44]. Characterization of the functional relevance of these KBS using EMSA, ChIP, and minimal promoter luciferase assays led to the confirmation that Kaiso functions as a transcriptional repressor of these genes, and supported the role of Kaiso as a tumour suppressor protein in tumourigenesis [19,44]. Second, Kaiso's down-regulation in 30% of breast carcinoma tissue and 50% of ovarian carcinoma tissue further underscored its possible role as a tumour suppressor protein in breast carcinogenesis [Daniel Lab, unpublished data].

Given the data implicating Kaiso as a tumour suppressor protein, a BLAST search of the genome was conducted using the ZF domain of Kaiso in the interest of identifying proteins with similar characteristics to Kaiso. ZBTB4 was identified as a Kaiso-like protein after the BLAST search determined that it shared 62% identity with Kaiso over the ZF domain [22]. Characterization of its binding properties determined that ZBTB4 interacts with a specific DNA sequence, coined the Z4BS, but also with the highly similar KBS *in vitro* [41]. Subsequent research has focused mainly on the consequences of ZBTB4 misexpression in tumourigenesis, for example identifying a tumour-stage-specific down-regulation of ZBTB4 mRNA levels in a number of tumour tissues including breast carcinomas and neuroblastomas [48]. While investigation into Z4BS-containing and CpG rich genes has begun to describe the molecular function of ZBTB4 as a tumour suppressor protein, it remains unclear the physiological relevance, if any, of ZBTB4 recognizing and binding the sequence-specific KBS. The focus of

my research thesis was therefore to determine if the *in vitro* interaction of ZBTB4 with the KBS extends to an *in vivo* effect on the KBS-containing target gene, *cyclin D1*.

4.1 Kaiso and ZBTB4 share patterns of subcellular localization

To determine what similarities exist between Kaiso and ZBTB4, we first investigated their expression levels and patterns of subcellular localization in a panel of different cell lines. As expected, both Kaiso and ZBTB4 localize primarily to the nucleus in MCF7 cells, and share an overall pattern of subcellular co-localization. Unexpectedly, when MCF7 cells were seeded at a lower density (100,000 cells/well) we observed in shift in the subcellular localization patterns of both Kaiso and ZBTB4 from mainly nuclear to mainly cytoplasmic with predominant perinuclear staining. Although Kaiso is typically found to localize to the nucleus in cell culture, this perinuclear subcellular localization of Kaiso and ZBTB4 as observed in Figure 4B is not a unique occurrence. Kaiso displays this same localization pattern in the human endothelial cell line HBMEC and the bovine endothelial cell line BPAEC [51]. Co-immunoprecipitation experiments using HBMEC and BPAEC cells demonstrated that Kaiso was interacting strongly with $p120^{ctn}$, suggesting that $p120^{ctn}$ was mediating its translocation from the nucleus to the cytoplasm [51]. While this perinuclear pattern of subcellular localization has not been previously demonstrated for ZBTB4, it is possible that the cell microenvironment may play a role in ZBTB4's subcellular localization, as has been demonstrated for Kaiso. For example, Soubry et al., 2010 demonstrated that Kaiso localized to the nucleus in tumour cells grown in culture, but translocated to the cytoplasm when those cultured tumour cells were injected into nude mice. Given that Kaiso has been characterized as a transcriptional repressor of Wnt target genes such as *cyclin D1* that play roles in cell proliferation, it is possible that Kaiso and ZBTB4 are exported out of the nucleus when cells are at low density and proliferation is required, but

then translocate back to the nucleus when cell-cell contacts are formed, and contact inhibition has been achieved. This nucleocytoplasmic trafficking event may be mediated by binding partners like $p120^{ctn}$. Ultimately it seems that the transcriptional activity of Kaiso is regulated by its binding partners and its subcellular localization, and this pattern of transcriptional regulation may also occur for the Kaiso-like protein ZBTB4.

4.2 The minimal CCND1 promoter contains a -1065 and +69 Z4BS

Cyclin D1 is a widely studied protein due to its immense importance as a regulator of the mammalian cell cycle and cell proliferation, and its subsequent links to various neoplasias and tumourigenesis [1,4,9]. The protein functions as a molecular switch, signalling the passage of the cell from G1 to S phase by activation of CDK4 & 6, followed by the subsequent phosphorylation of the Rb protein [45].

Sequence analysis of the CCND1 promoter sequence revealed that it possessed two Z4BS sites; a nearly complete Z4BS located at -1065 relative to the transcriptional start site, and a core Z4BS site located at +69 relative to the transcriptional start site. This finding was not surprising considering that the KBS and the Z4BS are very similar to one another. The -1065 Z4BS contains 7 out of 8 nucleotides that were previously described to constitute the Z4BS by Sasai et al., 2010. While nucleotide 7 of the -1065 Z4BS is a Guanine where Thymine is expected, it was also determined by Sasai et al., 2010 that only nucleotides 2, 3, 4, and 6 are critical for ZBTB4 recognition of the Z4BS. Moreover, when an unlabeled Z4BS oligonucleotide with nucleotide 7 mutated from T to G (as is the case in the CCND1 -1065 Z4BS) was used to outcompete the labelled wild type Z4BS, they observed nearly complete competition, suggesting that ZBTB4 can bind the -1065 Z4BS sequence *in vitro*.

The +69 Z4BS is a partial site, containing nucleotides 1-4 and 6 of the 8 base pairs for an optimal Z4BS. Interestingly, the +69 Z4BS encompasses all 4 nucleotides deemed to be essential in mediating ZBTB4 recognition to the Z4BS [41]. The 3 mismatched nucleotides in the CCND1 +69 Z4BS that do not match the published optimal Z4BS were proven to be not critical for ZBTB4 binding to the site; unlabelled oligonucleotides bearing point mutations at nucleotide 5, 7, or 8 did not significantly compete with the labelled wild type probe for ZBTB4 binding [41]. While all 3 mismatched nucleotides likely reduce the efficiency in which ZBTB4 binds to the +69 Z4BS, it is important to note that ZBTB4 has been shown to bind to the KBS *in vitro*, and that the +69 site has a complete core KBS [22]. This suggests that the four essential nucleotides present in the +69 Z4BS are sufficient to mediate binding, at least in *in vitro* studies.

Studies on Kaiso as a transcriptional regulator of CCND1 revealed that its binding to the +69 KBS is methylation-dependent [19]. Kaiso bound to a mutated +69 KBS that was methylated at CpG sites flanking the KBS, although surprisingly it did not bind to a wild type +69 KBS that was not methylated at the flanking CpG sites [19]. Although optimal binding occurred with a methylated, wild type probe, this result indicated that Kaiso binds the +69 KBS in the CCND1 promoter in a methylation-dependent but not sequence-specific manner. Due to the fact that ZBTB4 has a very similar ZF domain to Kaiso, and ZBTB4 was also characterized as a methyl-binding-protein, it is possible that ZBTB4 interacts with the +69 Z4BS in a manner analogous to that of Kaiso; i.e. in a methylation-dependent but not sequence-specific manner. Thus, the role of the 4 core nucleotides of the +69 Z4BS may be to stabilize the interaction between ZBTB4 and the flanking methylated CpG dinucleotides.

Finally, it should be noted that a second type of Z4BS was published by Sasai et al., 2010, called the methyl-Z4BS. This Z4BS differs from the non-methyl Z4BS by only a single

nucleotide (C**C**GCCATC as opposed to C**T**GCCATC) which allows for methylation of the site as part of a CpG dinucleotide, whereas no methylation occurs with the thymine present [41]. Sasai et al., 2010, determined that ZBTB4 binds with greater affinity to the methyl-Z4BS than to the non-methylated Z4BS, although they observed equal binding affinity when the methyl-Z4BS was enzymatically demethylated [41].

4.3 ZBTB4 binds to the CCND1 promoter *in vivo* via interaction with the +69 Z4BS, but not the -1065 Z4BS

While ZBTB4 has been demonstrated to interact with the KBS *in vitro*, there has been no examination of this data in the *in vivo* environment. To this end, we sought to determine if ZBTB4 can interact with the Kaiso target gene *cyclin D1* and regulate its expression. Because the Z4BS and the KBS are highly similar to each other, it was possible that ZBTB4 could bind to the CCND1 promoter region at these sites. To expand on this data, we conducted Chromatin Immunoprecipitation experiments using cleared chromatin from MCF7 cells and PCR primers specific to the CCND1 -1067 and +69 KBS. Indeed, we found that ZBTB4 associated with the +69 but not the -1067 Z4BS/KBS regions of the CCND1 promoter. Although it has been demonstrated that ZBTB4 can bind to an oligonucleotide possessing the sequence-specific -1065 Z4BS *in vitro*, it is possible that binding was not detected at this site because it is not a methyl-Z4BS site [41]. Considering the complex events and circumstances that govern *in vivo* binding of a transcription factor to promoters (such as steric hindrance) it is possible that the partial -1065 Z4BS is insufficient to attract ZBTB4 to the CCND1 promoter, and that a methyl-Z4BS is required.

The +69 specific primers did amplify DNA from ZBTB4 precipitated chromatin, suggesting that ZBTB4 does interact with the CCND1 promoter at the +69 KBS/Z4BS. It is possible that ZBTB4 could interact with the +69 site in a methylation-dependent manner, as is the case for Kaiso [19]. Given that ZBTB4 is a bimodal binding protein, it is expected that it may bind to any of the 8 CpG dinucleotides along the minimal CCND1 promoter sequence. Although methylated CpG sites are very common within the human genome (around 50,000,000 individual sites) it is likely that dual-specificity binding proteins such as ZBTB4 and Kaiso exist to specify which CpG sites should be bound; the sequence surrounding the CpG site may not be necessary for binding, although it may play a role in stabilizing or increasing the likelihood of the interaction [22,41]. Hence, ZBTB4 could interact with the +69 Z4BS as a result of the three CpG sites immediately flanking this location.

4.4 ZBTB4 overexpression mediates a dose-dependent loss of repression of the minimal CCND1 promoter

To characterize the functional significance of ZBTB4 binding to the CCND1 promoter (either via the methylated +69 KBS/Z4BS or otherwise) we sought to perform minimal promoter assays using either the -1748-CD1 minimal promoter construct, or the mutant -1748-CD1 minimal promoter construct in which both KBS sites are mutated. When ZBTB4 and the wt -1748-CD1 minimal promoter construct were transfected in MCF7 cells, we observed a dose-dependent increase in the minimal CCND1 promoter activity. Furthermore, when ZBTB4 was transfected into MCF7 cells with the mutant -1748-CD1 minimal promoter construct, a similar dose-dependent increase in minimal promoter activity was observed. This is in contrast to the effect exogenous Kaiso had on both the wt and mutant -1748-CD1 minimal promoter construct,

where a dose-dependent repression was observed with the wt construct, although not with the mutant construct.

The apparent ZBTB4-mediated dose-dependent increase in the minimal CCND1 promoter activity can be interpreted either as a mild activation, or as a loss of repression of the minimal promoter. A mild activation scenario is certainly not unprecedented when considering POZ-ZF proteins. Although most POZ-ZF proteins function as transcriptional repressors, some are activators, while others (such as Kaiso) have been characterized to perform both functions [18]. For example, while Kaiso typically functions as a transcriptional repressor protein, it was found to activate expression of the postsynaptic gene *rapsyn* via a muscle cell specific interaction with δ-catenin [40]. ZBTB4 is yet to be characterized for a transcriptional activation role like its close relative Kaiso, although its requirement for interaction partners is well documented. As a transcriptional repressor of the cell cycle regulatory gene *p21CIP1*, ZBTB4 first must complex with the BTB/POZ protein Miz1 and Sin3 to mediate repression [48]. When Miz1 or Sin3 was experimentally depleted, repression of *p21CIP1* was lost [48]. Moreover, when ZBTB4 was depleted from the system, Miz1 bound directly to the *p21CIP1* promoter and functioned as a powerful activator of expression [48]. Thus the functional role of BTB/POZ transcription factor proteins is highly dependent on their interaction partners and tissue specificity and it is possible that while ZBTB4 typically functions to repress expression of target genes, the environment in MCF7 cells may induce ZBTB4 to mediate a mild activation of CCND1 minimal promoter activity.

Alternatively, the increase in CCND1 minimal promoter activity can be interpreted as a loss of repression by endogenous repressors such as Kaiso. This view point is most consistent with the data obtained. When you consider that ZBTB4 mediates a dose-dependent increase in

CCND1 minimal promoter activity when transfected with either the wt or the mutated -1748-CD1 minimal promoter construct, this suggests that the minimal promoter activity is not due to sequence-specific binding. If ZBTB4 were interacting with the -1748-CD1 minimal promoter construct in a sequence-specific manner (via the -1067 or +69 Z4BS/KBS) we would expect this interaction to be lost when transfected with the mutated minimal promoter construct. Instead, we find a similar dose-dependent pattern of minimal promoter activity with the mutated-1748-CD1 construct as with the wt -1748-CD1 minimal promoter activity, suggesting that ZBTB4 may be interacting with the -1748-minimal promoter sequence in a sequence-independent manner. This observation is consistent with the ChIP data; ZBTB4 was found to bind to the +69 Z4BS/KBS, although not to the -1067 Z4BS/KBS site. Contrary to our beliefs at the time, the -1748-CD1 minimal promoter was not methylated. This is because the pGluc-1748-CD1 plasmid was stored and replicated in a bacterial strain that was unable to methylate CpG sites. Because of this fact, and with the data obtained from the ChIP, we would expect Kaiso to repress the -1748-CD1 minimal promoter construct in a sequence-specific manner (via binding to the -1067 KBS) but we would not expect ZBTB4 to regulate activity of the -1748-CD1 minimal promoter construct because it does not bind to the -1067 Z4BS/KBS, and likely requires methylation of the flanking CpG sites surrounding the +69 Z4BS/KBS to mediate binding. A possible explanation to the ZBTB4-mediated sequence-independent increase in CCND1 minimal promoter activity is that a loss of repression of endogenous repressors on CCND1 is occurring. As stated earlier, ZBTB4 does not act in isolation, but instead as part of complexes [40,48]. It is possible that ZBTB4 is sequestering binding partners such as co-repressor machinery, or potentially *bona fide* endogenous CCND1 transcriptional repressor proteins, and preventing them from repressing the CCND1 minimal promoter activity

Conclusions and Overall Significance

This study was the first to investigate the biological consequences of ZBTB4 binding to the KBS element *in vivo*. Both KBS within the minimal CCND1 promoter were determined to also be partial Z4BS sites as determined by sequence analysis. While *in vitro* studies have demonstrated that ZBTB4 binds to oligonucleotides containing both the -1065 Z4BS and the +69 Z4BS, we wanted to determine if this relationship extends to the *in vivo* environment. Interestingly, we found that ZBTB4 binds to the +69 KBS/Z4BS but not the -1067 KBS/Z4BS. Using minimal promoter luciferase assays, we found that ZBTB4 likely mediates a loss of repression of CCND1 minimal promoter activity, possibly due to sequestration of co-repressor machinery. Our minimal promoter construct was not methylated, and therefore, this finding was in accordance with our ChIP data which demonstrated that ZBTB4 bound to the +69 Z4BS/KBS likely in a methylation dependent manner. Finally we sought to determine the biological consequence of both ZBTB4 and Kaiso bind to the KBS. Due to time limitations, this experiment was not completed.

We also found that Kaiso and ZBTB4 demonstrate similar patterns of subcellular localization, co-localizing to the nucleus in MCF7 cells. Interestingly, under a low cell density situation, the localization patterns of both proteins shifted significantly to the cytoplasm and perinuclear space. Kaiso's sequestration in the cytoplasm has been theorized as a mechanism to regulate its activity, although this is the first indication that the same process may be regulating the activity of the Kaiso-like protein ZBTB4. Furthermore, this finding supports previous research indicating that the activity of both Kaiso and ZBTB4 are highly susceptible to both internal and external environmental fluctuations such as presence of binding partners and co-factors, tissue microenvironment, and possibly cell density.

Overall, like Kaiso, ZBTB4's activity may be susceptible to the cellular context and environment. It binds to the CCND1 +69 KBS, likely in a methylation dependent manner, as has been described for Kaiso. Further study is required to elucidate the nature of ZBTB4's interaction with the +69 Z4BS/KBS, as well as the biological consequences of both proteins interacting with the same element.

FUTURE DIRECTIONS

To elucidate the natural consequences of both Kaiso and ZBTB4 recognizing the same binding element, we wanted to determine if ZBTB4 interferes with Kaiso's repression of the minimal CCND1 promoter (by steric hindrance or sequestration of binding partners) or if Kaiso and ZBTB4 cooperate to regulate CCND1 minimal promoter activity (potentially by stabilization or recruitment of binding factors). To address this question, we transfected the -1748-CD1 minimal promoter construct into HCT-116 cells stably depleted for ZBTB4, and measured the effect of the absence of ZBTB4 on Kaiso's repression of the minimal promoter activity, as compared to wt and PRS-ZBTB4-scrambled HCT-116 lines. Due to time limitations, this experiment was not completely optimized. The purpose in conducting this experiment was to shed light on the nature of the interaction between Kaiso and ZBTB4 at the KBS. What is the biological function of ZBTB4 interacting with the KBS *in vivo*? Do ZBTB4 and Kaiso work in cooperation with one another, or instead does ZBTB4 function as a competitive inhibitor to Kaiso by binding to the KBS but not mediating differential gene expression. The data produced from this experiment was intended to satisfy some of these questions.

Furthermore, it should be determined if the interaction between ZBTB4 and the CCND1 +69 KBS is methylation-dependent or sequence specific. Treatment of the cells with 5-

azacytadine prior to a ChIP experiment will demethylate the flanking CpG sites surrounding the +69 KBS/Z4BS, and shed light on the importance of these methylated sites in mediating ZBTB4 binding to the +69 KBS/Z4BS. Determining the nature of ZBTB4's interaction with the +69 Z4BS/KBS can be elucidated by artificially methylating the -1748-CD1 minimal promoter construct using Sss1 methylase and conducting minimal promoter luciferase assays. This data would help to clarify the nature of the interaction between ZBTB4 and the +69 Z4BS/KBS, and determine if its binding is methylation dependent.

Another area of interest is to investigate the relative importance of the methyl-Z4BS and the non-methyl Z4BS in *in vivo* target gene regulation. Minimal artificial promoter-reporter constructs containing either the methyl-Z4BS or the non-methyl Z4BS could be used to characterize the functional consequence of these elements in target gene regulation, and aid in streamlining the identification of putative target genes.

Finally, investigating the binding properties of ZBTB4 to alternative Kaiso target genes will broaden the depth of this research, and help to further understand the biological relevance of the Z4BS and the KBS with respect to ZBTB4.

APPENDIX

Examining correlations between Kaiso and its target genes in breast tumour tissue

The data presented here are a summary of the results obtained during the course of my thesis project that are beyond the scope of my current project. They may be of interest for future studies.

APPENDIX

Examining correlations between Kaiso and its target genes in breast tumour tissue

INTRODUCTION

A.1 Kaiso's Regulation of Wnt Target Genes and Tumourigenesis

The identification of the KBS in the promoter regions of various Wnt target genes such as *cyclin D1* and *matrilysin* suggested their transcriptional regulation by Kaiso [29,37]. Further proof of the physiological relevance of these sites using minimal promoter constructs possessing the KBS identified a potential cross-regulatory role for Kaiso in the canonical Wnt signalling pathway [29,37]. Kaiso gain-of-function and loss-of-function experiments in *Xenopus laevis* exhibited a clear effect on the expression of endogenous Wnt target genes such as *siamois* and *Xnr3*, underscoring Kaiso's apparent physiological role in the cross regulation of the Wnt signalling pathway [37].

Matrilysin is a proteolytic enzyme belonging to a group of zinc-dependent transmembrane enzymes, which function in tissue remodelling [44]. Matrilysin targets and cleaves adhesive molecules of the extracellular matrix and basement membrane, allowing for tumour invasion and metastasis [44]. The protein has been found to be mis-regulated in carcinomas of the breast and gastrointestinal tract, as well as in tumours of the esophagus [44]. Interestingly, Kaiso has been shown to interact with *matrilysin*, as two copies of the KBS are found in the promoter region of the gene, and electrophoretic mobility shift assays show *matrilysin*-derived oligonucleotide probes complex with GST-Kaiso fusion proteins [44]. Given the misexpression of *matrilysin* in breast carcinomas, and that Kaiso interacts with the promoter

region of the gene, it is possible that Kaiso may be important in the regulation of *matrilysin* expression in breast carcinoma tissue.

A second target gene of interest, Cyclin D1, is a key player in cell cycle regulation and has been found to be overexpressed in a wide number of human cancers, from carcinomas to lymphomas [1,4,9]. Cyclin D1 directly activates G1 phase cyclin-dependent kinases (CDK 4 and 6), resulting in the induction of the cell cycle and subsequent proliferation [45]. Cyclin D1 has oncogenic potential independent of cdks as well, by interacting and regulating the activity of transcription factors such as androgen receptor, and STAT3 [21]. Regulation of the expression of these receptor proteins implicates Cyclin D1 in the cross regulation of many different signalling pathways, and represents an expanded potential for oncogenicity [1,4,21]. Cyclin D1 expression in breast carcinomas has been found to be robust, and associated with malignancy and poor overall prognosis [21]. Three KBS are found in the promoter region of *cyclin D1* in conjunction with multiple methylated CpG dinucleotide islands, implicating Kaiso in its regulation [19].

A.2 Tissue Microarray Technology

Immuno**h**isto**c**hemical (IHC) analysis of extracted tumour tissue is an essential step in the identification and assessment of biomarkers which may provide insight into disease progression and prognosis [25]. Potential biomarkers may first be identified by means of DNA microarray technology, whereby large throughput applications allow for thousands of genes to be assessed for expression in various contexts, such as tumourigenic versus non-tumourigenic cell lines. However a critical step in the bench to bedside process is the validation of these identified targets of interest at the protein level for use as legitimate biomarkers in disease progression [13,25].

Traditional analysis of protein biomarkers in extracted tumour tissue was to perform IHC analysis on fresh, formalin-fixed and paraffin-embedded blocks of tissue, which would then be sliced and mounted on microscope slides [25]. This low throughput technique is susceptible to high error amongst trials, and exhaustion of finite tissue resources [25]. For example, if 20 identified biomarkers of interest were to be analysed for protein expression level in 400 tumour tissue samples, it would require massive amounts of whole mount tissue samples and reagents, and be subject to high variability in reagents used and lack of standardization of factors such as temperatures and incubation times between tissue samples [25]. Furthermore, the experiment would likely take weeks to execute, and months to process and organize the resulting data. Due to these limitations in quality and feasibility, an alternative technique has been created to eliminate error between samples, increase preservation of scarce tissue reserves, and reduce waste of reagents used [13,25]. **T**issue **M**icro**a**rray (TMA) technology allows for the arrangement of 600 or more tissue sample cores to be assembled onto a standard microscope slide, providing the researcher to assess protein expression in hundreds to thousands of tissue samples, using a single batch of reagents and over a single day [13,25]. This technology drastically reduces error between tissue samples, and significantly increases efficiency while reducing cost [25].

TMA construction begins with standard formalin-fixed and paraffin-embedded tissue blocks, called donor blocks [25]. A pathologist analyses the blocks and selects areas of interest. The identified area of interest is then extracted using a manual arraying device, which punches 0.6 mm diameter cores in specific locations straight through the tissue block [13,25]. This core is then assembled into a recipient master block which contains cores collected from hundreds to thousands of other donor tissue blocks [13,25]. The recipient master block is then sectioned and

mounted onto traditional microscope slides, ready for high throughput IHC analysis of protein expression.

A.3 Automated Quantitative Analysis

Although TMAs have vastly reduced the error that occurs between tissue samples when conducting IHC for protein expression levels, the process is still vulnerable to a great deal of bias. Following the IHC procedure to stain for potential biomarkers, a pathologist must then review the images taken and score the expression of target proteins [12,25]. Given that this is a human based analysis of expression level, great subjectivity can be unintentionally introduced to the data. For example, there are many factors which can influence the pathologist's score, such as the context of the tissue, the background staining intensity of the tissue, and the order in which the slides are assessed [12]. Furthermore, it takes significant amounts of time and effort for the pathologist to assess hundreds to thousands of spots, essentially undermining the advancements in efficiency that the TMA technology provides [12,13,25]. These issues in traditional pathologist based screening of IHC stained tissues identified the need for rapid and accurate automated analysis and quantification of target protein expression levels.

Many different computer based programs have attempted to satisfy this need, among which is **A**utomated **QU**antitative **A**nalysis (AQUA). AQUA is a series of algorithms which accurately quantify protein expression levels as well as subcellular localization in tissue, and dramatically increases the efficiency and accuracy of quantification as compared to traditional pathologist-based scoring methods [12]. Briefly, the **P**ixel-based **L**ocale **A**ssignment for **C**ompartmentalization of **E**xpression (PLACE) algorithm defines subcellular compartments such as membrane, cytoplasm and nucleus, and eliminates background stroma from quantification by

using fluorescent markers [12]. It then quantifies biomarker expression in a given identified compartment by dividing the sum of the target signal intensity within a compartment by the total area of that compartment [12]. A second algorithm that plays an important role in AQUA is the **R**apid **E**xponential **S**ubtraction **A**lgorithm (RESA) which accounts for tissue section depth [12]. Because an image will typically capture cellular compartments overlapping one another, it is important to define a specific depth of interest, and then subtract away compartments and target signals that are not in line with this given depth. RESA accomplishes this task by subtracting out-of-focus images from in-focus images, using factors such as pixel intensity, signal-to-noise ratio, and expected compartment size to make objective judgments with [12].

Together, TMAs and the use of automated quantification software such as AQUA present a significant improvement in the efficiency and quality of data obtained in IHC based experiments. Their combined application increases the feasibility of conducting large scale, high throughput analysis of expression of multiple target proteins in tissue, streamlining the process of bench top to bedside research.

A.4 Rationale

Although many genes have been identified as putative Kaiso target genes, there is yet to be a proven correlation between Kaiso's subcellular localization and the expression of its putative target genes in breast carcinoma tissue. Statistically significant data proving a physiologically relevant role for Kaiso in the regulation of genes known to be involved in various pathways of tumourigenesis will provide valuable information to better understand Kaiso's role in tumour development. Identified Kaiso target genes such as *cyclin D1* and *matrilysin* are implicated in tumourigenic processes such as proliferation and metastasis, however understanding Kaiso's overall biological function requires identification of novel target

genes, which may be involved in further aspects of tumourigenesis such as apoptosis and angiogenesis. Hence, the main objective of this component of my thesis was to examine the correlation, if any, between Kaiso expression and subcellular localization with that of existing target genes using a human breast tumor tissue microarray (TMA).

MATERIALS AND METHODS

A.5 Immunofluoresence

Paraffin-coated breast tissue microarray slides were placed on a 60°C slide warmer for 10 minutes to remove excess wax. Slides were then washed with Xylene twice for 20 minutes each to remove any residual wax from tissue. The tissue was hydrated by washing twice in 100% ethanol for 1 minute each, once in 70% ethanol for 1 minute, and then rinsed under running tap water for 5 minutes. Antigen retrieval was performed on the tissues in a rice cooker with boiling water, with slides submerged in 10mM sodium citrate buffer for 10 minutes. Endogenous peroxidase activity was blocked by incubating slides in H_2O_2 3% vol/vol. solution in methanol for 30 minutes at room temperature. Non-specific binding sites were blocked by incubating the tissue in a 0.3% BSA in TBS-Tween solution in a humidified chamber for 30 minutes at room temperature. Primary antibodies were diluted in the 0.3% BSA in TBS-Tween solution and added to the tissue in the humidified chamber for 16 hours at 4°C. The Kaiso mouse monoclonal antibody 6F was diluted at 1:5000, while the Wide Spectrum Cytokeratin rabbit polyclonal antibody (Dako, Denmark) was diluted at 1:300. Primary antibodies were removed from the tissue by washing slides twice in Tween-1X TBS 0.05% vol/vol. for 10 minutes each on the belly dancer, followed by 1 wash in 1X TBS for 10 minutes on the belly dancer. The Alexafluor 546 conjugated goat anti-rabbit secondary antibody (Invitrogen, California) was diluted 1:100 in the

ready-to-use EnVision+ HRP conjugated goat anti-mouse secondary antibody solution (Dako, Denmark), which was then added to the tissue in the humidified chamber for 1 hour at room temperature. Secondary antibodies were removed from the tissue by washing slides twice in Tween-1X TBS 0.05% vol/vol. for 10 minutes each on the belly dancer, followed by 1 wash in 1X TBS for 10 minutes on the belly dancer. Cyanine 5-Tyramide (Perkin Elmer, Massachusetts) was diluted 1:50 in amplification buffer and added to the tissue in the humidified chamber for 10 minutes at room temperature. Excess Cyanine 5-Tyramide solution was removed from the tissue by washing slides twice in Tween-1X TBS 0.05% vol/vol. for 10 minutes each on the belly dancer, followed by 1 wash in 1X TBS for 10 minutes on the belly dancer. Hoechst was diluted 1:300 in 0.3% BSA in TBS-Tween, and added to the tissue in the humidified chamber for 30 minutes at room temperature. Hoechst was removed from the tissue by washing slides twice in Tween-1X TBS 0.05% vol/vol. for 10 minutes each on the belly dancer, followed by 1 wash in 1X TBS for 10 minutes on the belly dancer. Slides were dried with disposable tissue, preserved by adding 1 drop of Prolong Gold Anti-Fade reagent (Invitrogen, California) and covered with 22 x 22 mm cover slips. Slides were then sent to Dr. David Rimm's Laboratory at Yale University, where Hallie Wimberly processed the data using Automated Quantitative Analysis.

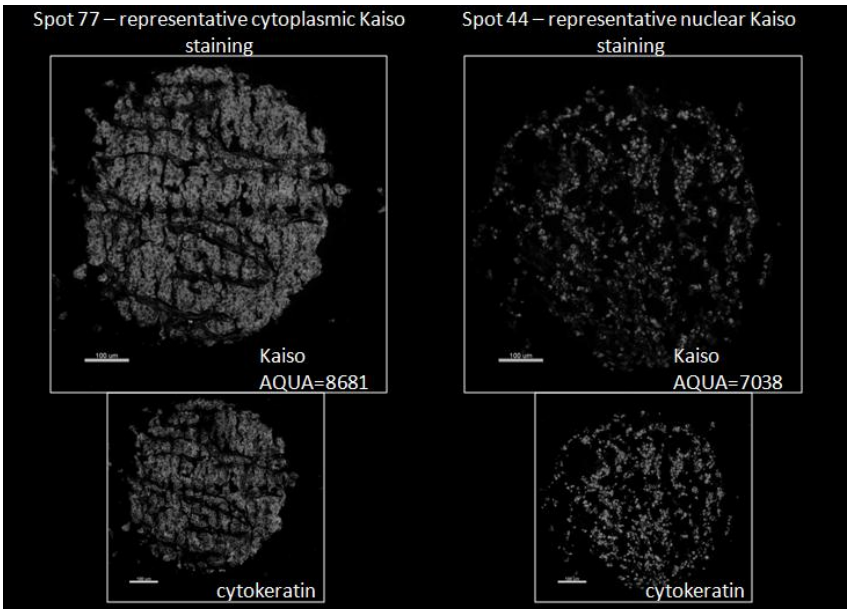
RESULTS AND DISCUSSION

A.6 Kaiso localization is predominantly cytoplasmic in breast carcinoma tissue

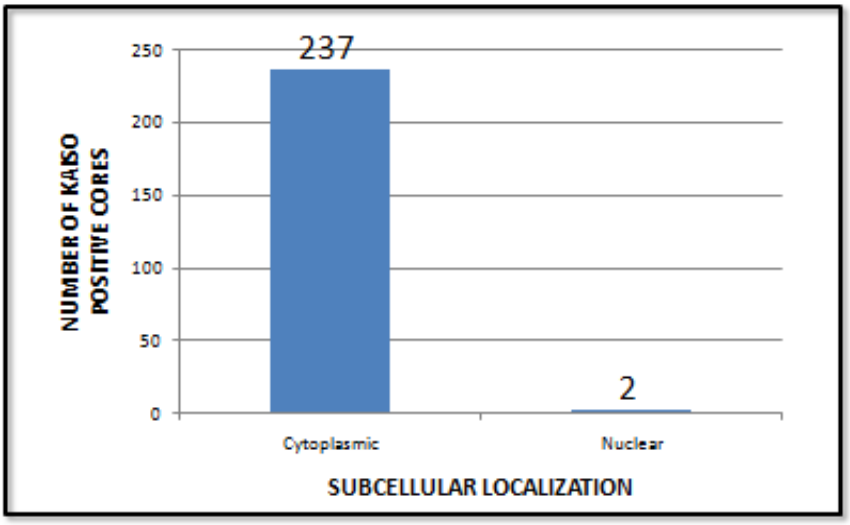
Kaiso protein localization was assayed in breast tumour tissue microarrays (TMA) using IF, after which the slides were then processed by AQUA to determine (i) patterns of Kaiso's subcellular localization in breast carcinoma tissue, and (ii) the relationship if any between Kaiso's subcellular localization and clinical outcome of the disease. Kaiso is predominantly

localized in the cytoplasm of the breast carcinoma tissue, with 237 out of 239 positively staining cores displaying vastly cytoplasmic localization (Figure 10). This finding is supported by findings from Soubry et al., 2005, whereby they observed diffuse cytoplasmic localization of Kaiso in tumour tissue, as compared to highly nuclear localization of Kaiso in cancer cell cultures. In fact, when cancer cells with nuclear Kaiso localization were taken from culture and xenografted onto nude mice, Soubry et al., 2005 observed a translocation of Kaiso from the nuclear to cytoplasmic compartment. The tissue microenvironment was proposed to play a significant but unexpected regulatory role in Kaiso's subcellular localization, and suggests that Kaiso is being inhibited from acting as a transcriptional regulator in breast carcinoma tissue by means of external signals [43]. A link between Kaiso and cadherin adhesion complexes has been well described since its discovery as a $p120^{ctn}$ binding partner, which interacts with both Kaiso and E-cadherin. It is possible that interactions such as this one mediate the regulation of Kaiso's subcellular localization by the microenvironment. This experiment was not performed in duplicate because staining between two cohort TMAs was not found to be reproducible. Therefore, the data were collected from one cohort array, which decreases its validity.

(A)



(B)



<u>Figure 10:</u> Kaiso's subcellular localization in breast tumour tissue microarray cores. (A) **Representative AQUA images of Kaiso subcellular localization in breast carcinoma cores.** Kaiso was found to be predominantly localized throughout the cytoplasm in breast carcinoma tissue cores, occurring in 237 out of 239 breast tumour tissue microarray cores. Only 2 cores displayed nuclear localization of Kaiso. One representative cytoplasmic and nuclear core are displayed below. (B) **Graphical representation of Kaiso's subcellular localization in breast carcinoma tissue.** Kaiso is predominantly localized to the cytoplasm in breast carcinoma tissue, with 315 out of 317 cores displaying cytoplasmic localization.

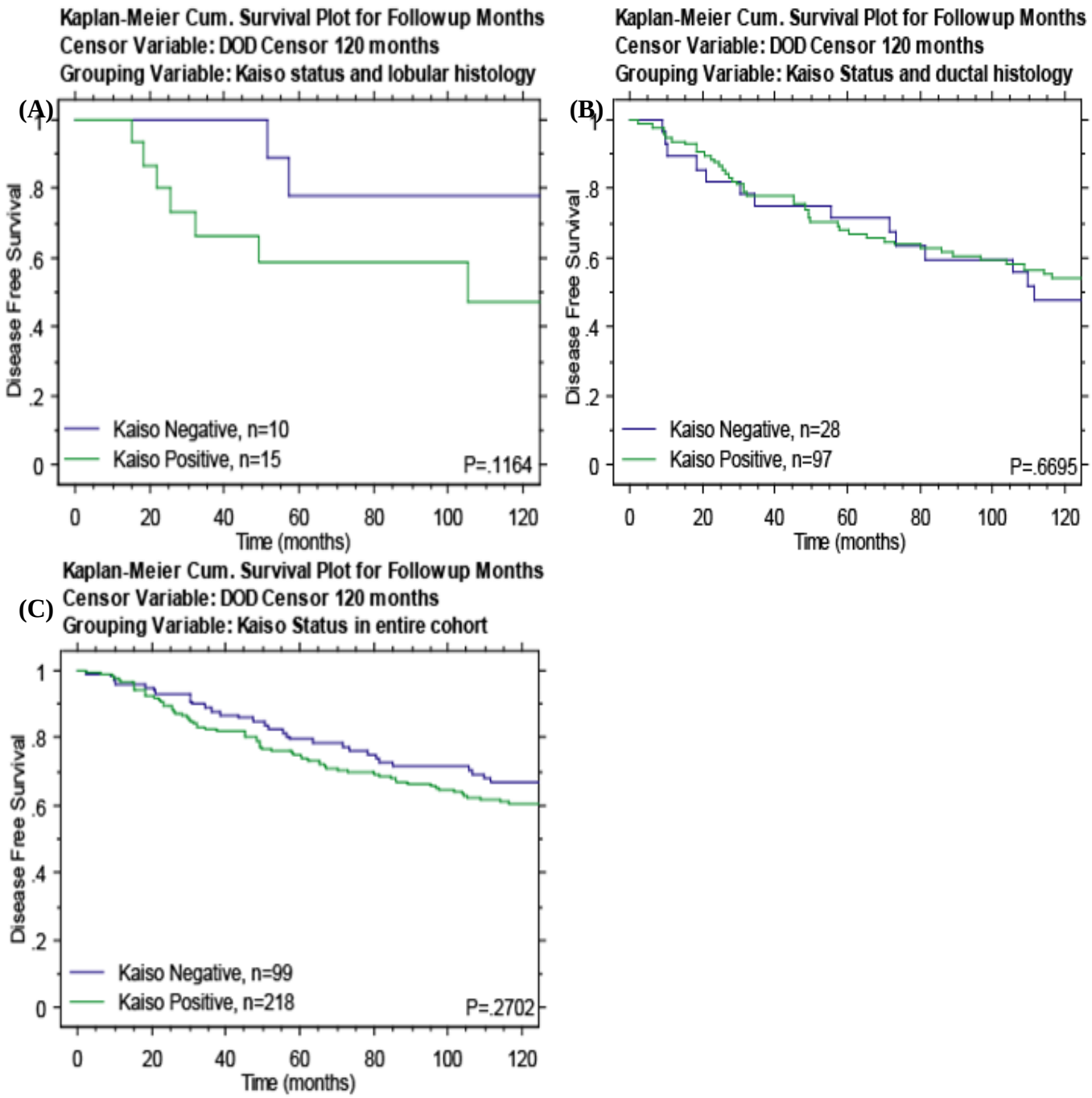
A.7 Kaiso expression in breast carcinoma tissue bears no significant correlation to disease free survival or clinical prognosis.

Kaiso's expression levels in breast carcinoma tissue bear no influence on clinical outcome of the disease. When clinical outcome was compared to Kaiso expression across the entire cohort of patients, there was no relationship found between Kaiso expression and disease free survival (Figure 11, C). Moreover, when the cohort was stratified into various clinical and histological sub-categories such as carcinoma histology type, or estrogen receptor status (data not shown), there was still no relationship between clinical outcome and Kaiso expression in breast carcinoma tissue (Figure 11, A,B). This data is supported by recent findings whereby publicly available breast cancer gene expression data sets were used to analyze correlations between gene expression and clinical prognosis of the disease. No correlation was found between the expression of various ZBTB genes such as Kaiso & ZBTB38 with patient survival [33]. Interestingly, a correlation was found between ZBTB4 expression and survival; higher ZBTB4 expression correlated with longer relapse free survival, whereas lower ZBTB4 expression correlated with shorter relapse free survival [33]. Together, these data demonstrate that Kaiso has no prognostic value in breast tumourigenesis, although ZBTB4 does. Given that disease progression is immensely complex and dependent upon various cellular processes, it may be that Kaiso's presence and functionality (or lack thereof) is simply lost amongst the vast number of potential players that influence overall disease progression. Alternatively, Kaiso may have no influence on breast tumourigenesis and its outcome.

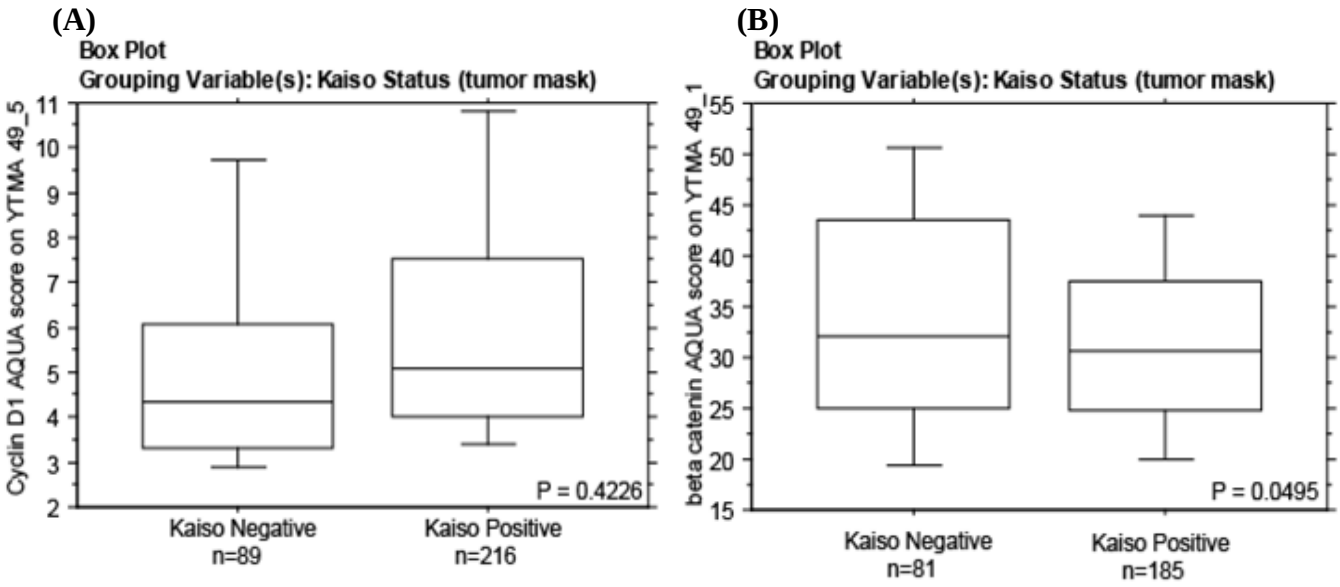
A.8 Kaiso status in breast carcinoma tissue is inversely proportional to β-catenin levels, but not to cyclin D1 protein levels

Next we sought to determine the relationship between Kaiso and Cyclin D1 protein levels in a cohort of breast carcinoma tissues. We found no relationship between Kaiso and Cyclin D1 protein levels across the cohort, however we did find an inverse relationship between Kaiso and β–Catenin protein levels in the tissue (Figure 12, A,B). While *cyclin D1* has been characterized as a legitimate Kaiso target gene, the lack of a correlation at the protein level may be due to post-transcriptional regulation, or alternative transcriptional regulators of *cyclin D1*. Considering Cyclin D1's cyclic pattern of expression and Kaiso's more stable and steady expression, it is not surprising that a correlation was not observed. Alternatively, because reproducibility was not obtained across experimental duplicates, it is possible that the data is inaccurate.

Kaiso and β-catenin likely oppose each other's function given that they share target genes and mediate opposite patterns of transcription. Because of this, the inverse correlation found between the two proteins may be due to separate regulatory mechanisms mediating their levels, and therefore the inverse correlation may be indirect. Considering the β-catenin promoter does not contain the KBS, it is unlikely the inverse relationship is via a direct repression of transcription.



<u>Figure 11:</u> Kaplan-Meier plots illustrating patient disease free survival as a function of Kaiso status in various groupings. (A) Disease free survival of patients with carcinomas of lobular histology as a function of Kaiso status in tissue. There is no relation between Kaiso status and overall disease free survival in patients with carcinomas of lobular histology. (B) Disease free survival of patients with carcinomas of ductal histology as a function of Kaiso status in tissue. There is no relation between Kaiso status and overall disease free survival in patients with carcinomas of ductal histology. (C) Disease free survival of the entire cohort as a function of Kaiso status in tissue. There is no relation between Kaiso status and overall disease free survival within the entire cohort of patients.



<u>Figure 12:</u> Protein levels of Cyclin D1 and β-catenin as a function of Kaiso status in breast carcinoma tissue. Kaiso status was correlated with Cyclin D1 **(A)** and β-catenin **(B)** protein levels across a cohort of breast carcinoma tissues. While no correlated existed between Kaiso and Cyclin D1 protein levels (p=0.4226), a statistical inverse correlation was determined between Kaiso and β-catenin protein levels (p=0.0495).

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