CYCLIN D1: A PUTATIVE KAISO TARGET GENE

CHARACTERIZATION OF *CYCLIN D1* AS A PUTATIVE KAISO TARGET GENE

By

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TITLE: Characterization of *cyclin D1* as a putative Kaiso target gene

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Abstract

Kaiso is a unique member of the BTB/POZ (**B**road complex, **T**ramtrak, **B**ric à brac,/**Po**x virus and **z**inc finger) zinc finger family of transcription factors with established roles in development and tumourigenesis. Kaiso was originally identified as a novel binding partner of the Armadillo catenin p120^{ctn}, a cytosolic co-factor and regulator of the cell-cell adhesion molecule and tumor suppressor E-cadherin. In addition to their roles in cell adhesion, the multifunctional Armadillo catenins also regulate gene expression, thus providing at least two mechanisms for their contribution to tumourigenesis. The discovery of a novel interaction between p120^{ctn} and the transcription factor Kaiso was therefore consistent with gene regulatory roles for Armadillo catenins. Interestingly, Kaiso represses transcription via a sequence-specific DNA binding site (TCCTGCnA) as well as through methylated CpG di-nucleotides, and one role of nuclear p120^{ctn} is to inhibit Kaiso DNA-binding and transcriptional repression. We recently identified sequence-specific Kaiso binding sites in a subset of Wnt/β-catenin/TCF tumour-associated target genes, and here we present data characterizing *cyclin D1* as a putative Kaiso target gene.

Kaiso binds the *cyclin D1* promoter *in vitro* and *in vivo,* and artificial promoter assays revealed that Kaiso overexpression results in the repression of a *cyclin D1* promoter luciferase reporter. Since *cyclin D1* is highly amplified in ~50% of human breast tumours, and a cancer profiling array demonstrated that Kaiso is misexpressed in ~40% of human breast tumours, we hypothesized that Kaiso represses and regulates *cyclin D1*

expression to inhibit breast tumourigenesis. In fact, examination of Kaiso expression in human breast cell lines demonstrated that *cyclin D1* mRNA levels were upregulated in Kaiso-depleted cells. My studies further revealed that methylation-dependent Kaiso-DNA binding may contribute to Kaiso's transcriptional repression of the *cyclin D1* promoter. We also determined that Kaiso inhibits, while $p120^{ctn}$ activates, β-catenin-mediated activation of the *cyclin D1* promoter. These findings further support a role for Kaiso and $p120^{ctn}$ in breast tumourigenesis via their modulation of the canonical Wnt signaling pathway which is highly implicated in human tumourigenesis. Together these findings support our hypothesis that Kaiso regulates *cyclin D1* expression. However, further studies are required to elucidate the mechanism employed by Kaiso to elicit *cyclin D1* repression and to examine how this activity may contribute to breast tumourigenesis.

Acknowledgments

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Contributions by Others

The pGL3 Basic 1748CD1 and pGL3 Basic 962CD1 luciferase reporter constructs were kind gifts of Dr. Frank McCormick (USCF). The pcDNA3-hKaiso and pcDNA3-hKaiso-NLSmutant constructs were subcloned and characterized by Kevin F. Kelly (Daniel lab). The semi-quantitative RT-PCR experiments were performed by Monica Graham (Technician, Daniel lab), and designed and optimized by Abena A. Otchere. All other experimental procedures were performed by Abena A. Otchere.

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Abbreviations

5-aza-dc	**5**-**aza**-**d**eoxy**c**ytidine
AR	**a**cidic **r**egion
Arm	**Arm**adillo
BTB/POZ	**B**ric a brac/**T**ramtrack/**B**road Complex/**Po**x viruses and **z**inc finger
CBD	**c**atenin **b**inding **d**omain
ChIP	**ch**romatin **i**mmuno**p**recipitation
CMS	**c**ongenital **m**yasthenic **s**yndromes
EC	**e**xtra **c**ellular
EGF	**e**pidermal **g**rowth **f**actor
EMSA	**e**lectrophoretic **m**obility **s**hift **a**ssay
GSK-3	**g**lycogen **s**ynthase **k**inase
GST	**g**lutathione-**S**-**t**ransferase
HDAC	**h**istone **dea**c**e**tylase
IP	**I**mmuno**p**recipitation
JMD	**j**uxta**m**embrane **d**omain
KBS	**K**aiso **b**inding **s**ite
LRP5/6	**l**ow-density-lipoprotein receptor **r**elated **p**roteins 5 or 6
MAPK	**m**itogen-**a**ctivated **p**rotein **k**inase
me	**Me**thylated
N-CoR	**n**uclear **cor**epressor
NES	**n**uclear **e**xport **s**ignal
NLS	**n**uclear **l**ocalization **s**ignal
pAb	**p**olyclonal anti**b**ody
PLB	**P**assive **l**ysis **b**uffer
RNAi	**RNA** **i**nterference

RTK	**r**eceptor **t**yrosine **k**inase
RT-PCR	**r**everse **t**ranscriptase **p**olymerase **c**hain **r**eaction
siRNA	**s**hort **i**nterfering **RNA**
TCF/LEF	**T**-**c**ell **f**actor/**l**ymphoid **e**nhancer **f**actor
WB	**W**estern **b**lot
ZF	**z**inc **f**inger

Introduction

1.1. Cell adhesion defects in tumourigenesis

Tumour progression from the benign to malignant stage is characterized by a multitude of molecular, cellular, and tissue level events. These events culminate in phenotypic changes that eventually lead to tumour metastasis from the primary tumour site to other parts of the body. It is usually when primary tumours metastasize to vital organs that cancers become fatal, due to organ malfunction or failure caused by tumour cell proliferation.

In tumour cells, the aberrant regulation of genes involved in cell proliferation, adhesion, and migration alters normal cellular processes. This allows individual tumour cells to break away from the primary tumour site, migrate to distant tissues, and establish secondary tumour sites. While a multitude of genes are dysregulated during the tumourigenic process, evidence suggests that the disruption of genes associated with normal cell-cell adhesion is a crucial turning point in the cascade of events that promote tumour progression and metastasis, reviewed in (12). The specific disruption of the cadherin family of cell adhesion molecules has been strongly implicated in promoting metastasis (12).

1.2. E-cadherin: The major epithelial cell adhesion molecule

Although there are many different classes of cell adhesion molecules, those of the cadherin family play a key role in the development and maintenance of strong cell-cell

adhesion junctions, reviewed in (12, 39, 98). Cadherins are tissue-specific cell adhesion molecules that are vital for the establishment and maintenance of normal cell-cell contacts and tissue structure, reviewed in (33). The most extensively studied cadherins are epithelial (E-), neuronal (N-), placental (P-), and vascular endothelial (VE-) cadherins. In epithelial cells, E-cadherin-based Adherens Junctions are responsible for maintaining strong cell-to-cell contacts and upholding proper epithelial tissue structure, reviewed in (140). E-cadherin is a 114 kDa protein consisting of a large extracellular (EC) domain that mediates E-cadherin homodimerization and homophilic interactions (58, 93), a single-pass transmembrane domain, and a cytoplasmic domain that complexes with cytoplasmic cell-cell adhesion co-factors (51, 85). During development, interaction between the same type of cadherin molecules on different cells dictates the sorting of cells into tissues. This is the initial point of cell-to-cell contact and provides the basis of proper tissue structure.

The E-cadherin extracellular domain is required for its adhesive activity (91, 113) and consists of 5 EC repeats that are successively linked together by Ca^{2+} binding sites (75, 124). Multiple EC repeats contribute to a low affinity interaction between cadherin monomers to form cadherin homodimers on the cell surface (17). Upon E-cadherin homodimer formation, the EC repeats mediate homophilic interactions with E-cadherin molecules on adjacent cells in a Ca^{2+}-dependent manner to form strong cell-cell contacts (17, 75). Homophilic binding of cadherin molecules involves extensive overlap between EC domains and may arise from multiple interactions or different combinations of interactions between EC domains, which are as yet not clearly defined (17). However,

EC repeats 1 and 2 are necessary for proper cadherin dimerization and homophilic interaction activity (75, 112, 113).

E-cadherin function is vital to the establishment and maintenance of tissue structure and as such its downregulation or malfunction is believed to be a major contributing factor to the motility and invasive characteristics of metastatic tumours, including tumours of the breast, colon, and prostate, reviewed in (11, 12, 32). Most metastatic tumours are of epithelial origin and in approximately 50% of these tumours E-cadherin expression and/or function is absent or reduced, reviewed in (12). In most of these cases E-cadherin expression is lost altogether, mostly through promoter inactivation by methylation, reviewed in (11). In the remaining 50% of metastatic tumours that exhibit normal E-cadherin expression and function, the loss of cell adhesion and increase in migration has been attributed to the catenins, the cadherin cytoplasmic cofactors, reviewed in (12).

1.3. The E-cadherin-catenin complex

The cytoplasmic tail of E-cadherin interacts with the catenins, which coordinately regulate E-cadherin stability, adhesion, and turnover, reviewed in (9). The catenins β-, γ-, and α-catenin were first identified based on their ability to co-precipitate with E-cadherin and were named based on their mobility by SDS-PAGE (68, 132). The catenin p120 ($p120^{ctn}$) was identified years later as a component of the E-cadherin-catenin complex due to its structural similarity to β- and γ-catenin (100, 102). β-catenin and γ-catenin are known as Armadillo catenins due to their high sequence homology to the

Drosophila segment polarity gene Armadillo (68) and they are characterized by repeated 42-amino acid **Arm**adillo (Arm) motifs (90). γ-catenin is also known as plakoglobin, which is a component of desmosomes (16, 57). In contrast, α-catenin is not an Armadillo catenin but is homologous to the actin binding protein vinculin (36, 74). E-cadherin interacts in a mutually exclusive manner with β-catenin or γ-catenin at the extreme C-terminal **c**atenin **b**inding **d**omain (CBD), whereas it interacts with p120-catenin (p120 ^{ctn}) at the **j**uxta**m**embrane **d**omain (JMD) (Figure 1) (28, 76, 141). The classical catenins β- and γ-catenin in turn interact with α-catenin to anchor the E-cadherin-catenin complex to the actin cytoskeleton and stabilize cell-cell adhesion (72, 73, 86, 121) (Figure 1).

Together the catenins play a crucial role in regulating E-cadherin-based cell-cell adhesion. Notably, cells expressing cadherin molecules that lack the cytoplasmic tail do not aggregate or form cell-cell contacts (72, 86, 121), underscoring the essential roles of catenins in cell-cell adhesion. Phosphorylation events further regulate the interplay between cadherin and catenin mediation of cell-cell adhesion. Several studies have suggested an association between cadherins and receptor tyrosine kinases, which reciprocally regulate each other's activities, reviewed in (132). Receptor tyrosine kinase phosphorylation of the E-cadherin catenin binding domain inhibits cadherin-catenin complex formation (121). Furthermore, the Armadillo catenins β-, γ-, and p120^{ctn} are phosphorylated by the non-receptor tyrosine kinase Src, and by receptor tyrosine kinases in responses to a variety of growth factors, reviewed in (22). Phosphorylation of β- and γ-catenin can lead to the dissociation of α-catenin from the complex, reducing the

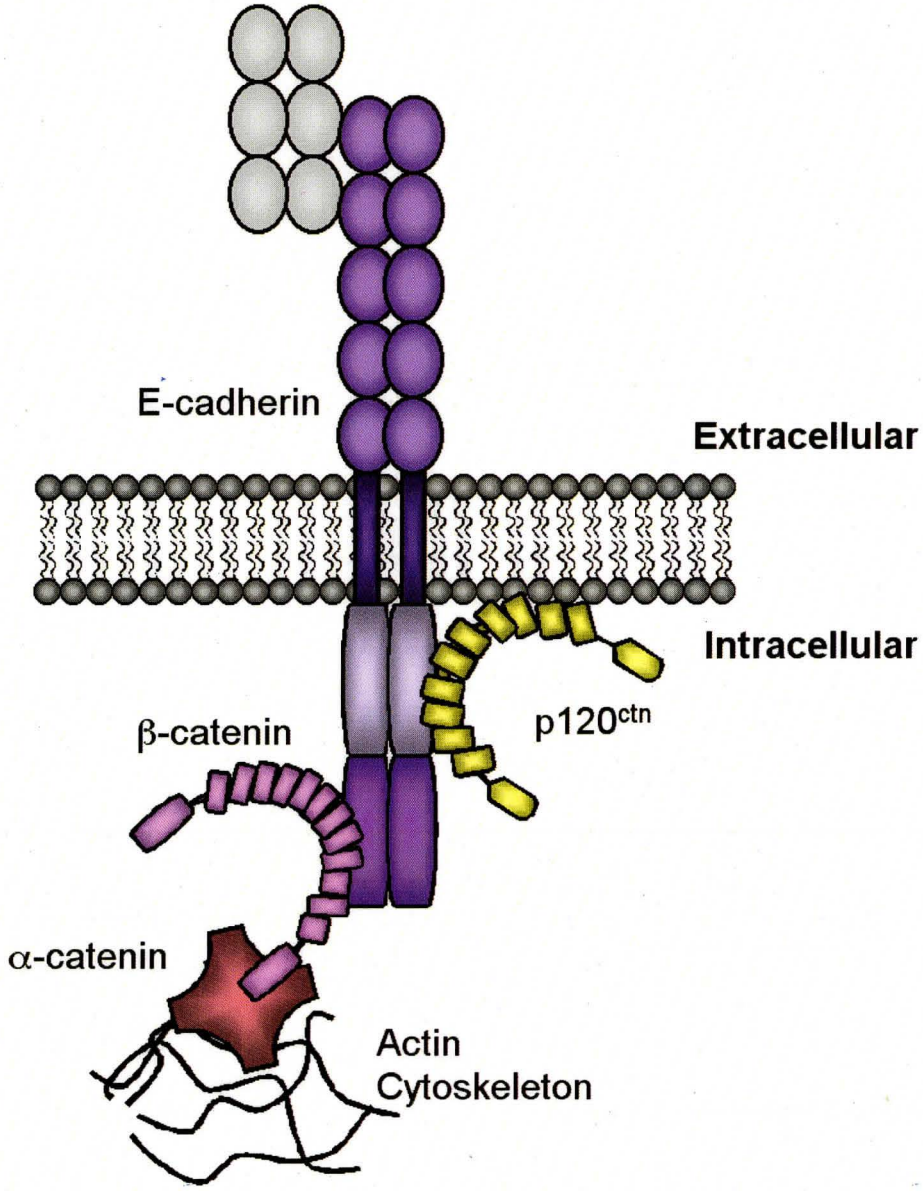


Figure 1: The E-cadherin-catenin complex. E-cadherin is a transmembrane cell adhesion molecule with five EC repeats that mediate E-cadherin dimer formation and homophilic interactions with E-cadherin molecules of adjacent cells. E-cadherin is anchored to the actin cytoskeleton through its cytoplasmic interactions with the catenins. β-catenin interacts with E-cadherin at the C-terminal catenin binding domain, and is interchangable with γ-catenin (not depicted). β- or γ-catenin in turn interact with α-catenin to link E-cadherin to the actin cytoskeleton. The catenin $p120^{ctn}$ interacts with E-cadherin at the juxtamembrane domain.

strength of cell adhesion, reviewed in (9); however, the role of $p120^{ctn}$ phosphorylation in modulating cadherin adhesive strength remains controversial. Initial studies hinted that p120 phosphorylation enhances cadherin junction assembly (55, 87, 117), but current studies suggest that p120 phosphorylation is not essential to stabilize cadherin function (24, 42, 64). Moreover it is still difficult to distinguish between the direct effects of p120 phosphorylation and phosphorylation of other components of the cadherin-catenin complex (4, 22), however, the development of new technologies may aid in elucidating how p120 phosphorlyation contributes to the function of the cadherin-catenin complex. Phosphorylation of the non-Armadillo catenin α-catenin is relatively low when compared to the Armadillo catenins (22) and the signficance of this phosphorylation is poorly understood.

Mounting evidence suggests that in addition to modulating cadherin function, the catenins are also involved in intracellular signaling events, leading to changes in gene expression, reviewed in (9). In support of this notion, aberrant catenin expression, subcellular localization, and phosphorylation have also been implicated in contributing to the invasive phenotype of tumours. β-, γ- and $p120^{ctn}$, are tyrosine phosphorylated upon **e**pidermal **g**rowth **f**actor (EGF) signaling in several tumour cell lines, reviewed in (22), a phenomenon which is associated with E-cadherin redistribution to the cytoplasm and suppression of its function (114, 115). Furthermore phosphorylation of β-catenin in v-Src- or Ras- transformed cells is associated with decreased cell adhesion, increased migration, and increased cell invasion, without affecting overall expression levels of the cadherins or catenins (10, 65).

1.4. β-catenin roles in intracellular signaling

The most extensively characterized catenin is β-catenin, which in addition to its role in cadherin-mediated cell adhesion, also participates as a key signaling molecule in the Wnt signaling pathway. The Wnt pathway regulates various cellular processes including cell growth, cell differentiation, organogenesis and tumourigenesis, reviewed in (63) (Figure 2). The Wnt signaling cascade initiates with the binding of the secreted Wnt ligand to the Frizzled transmembrane receptor, which is facilitated by the **l**ow-density-lipoprotein receptor **r**elated **p**roteins 5 or 6 (LRP5/6) (35). This binding in turn suppresses the activity of the serine threonine **g**lycogen **s**ynthase **k**inase 3 (GSK-3). In the absence of the Wnt signal, GSK-3 complexes with β-catenin and the tumour suppressor **a**denomatous **p**olyposis **c**oli (APC), targeting β-catenin for degradation via the ubiquitin-dependent proteosomal pathway (63).

However, upon Wnt signaling, GSK-3 suppression by Frizzled promotes the stabilization of cytosolic β-catenin. This increase in cytosolic β-catenin levels promotes its nuclear localization where it binds the **T**-**c**ell **f**actor/**l**ymphoid **e**nhancer **f**actor (TCF/LEF) transcriptional repressor (Figure 2). β-catenin interaction with TCF/LEF (hereafter TCF) derepresses genes involved in cell growth, differentiation, and migration such as *cyclin D1*, *matrilysin*, *ID2*, and *c-myc* (19, 34, 106, 126), leading to their upregulation in developmental as well as tumourigenic processes. Aberrant Wnt signaling is involved in promoting the progression of various tumours including those of the colon, breast, and intestine, reviewed in (81). Indeed, β-catenin and the β-

catenin/TCF complex are upregulated in many human tumours and tumour-derived cell lines, and have been implicated in the deregulation of cell growth and migration (81).

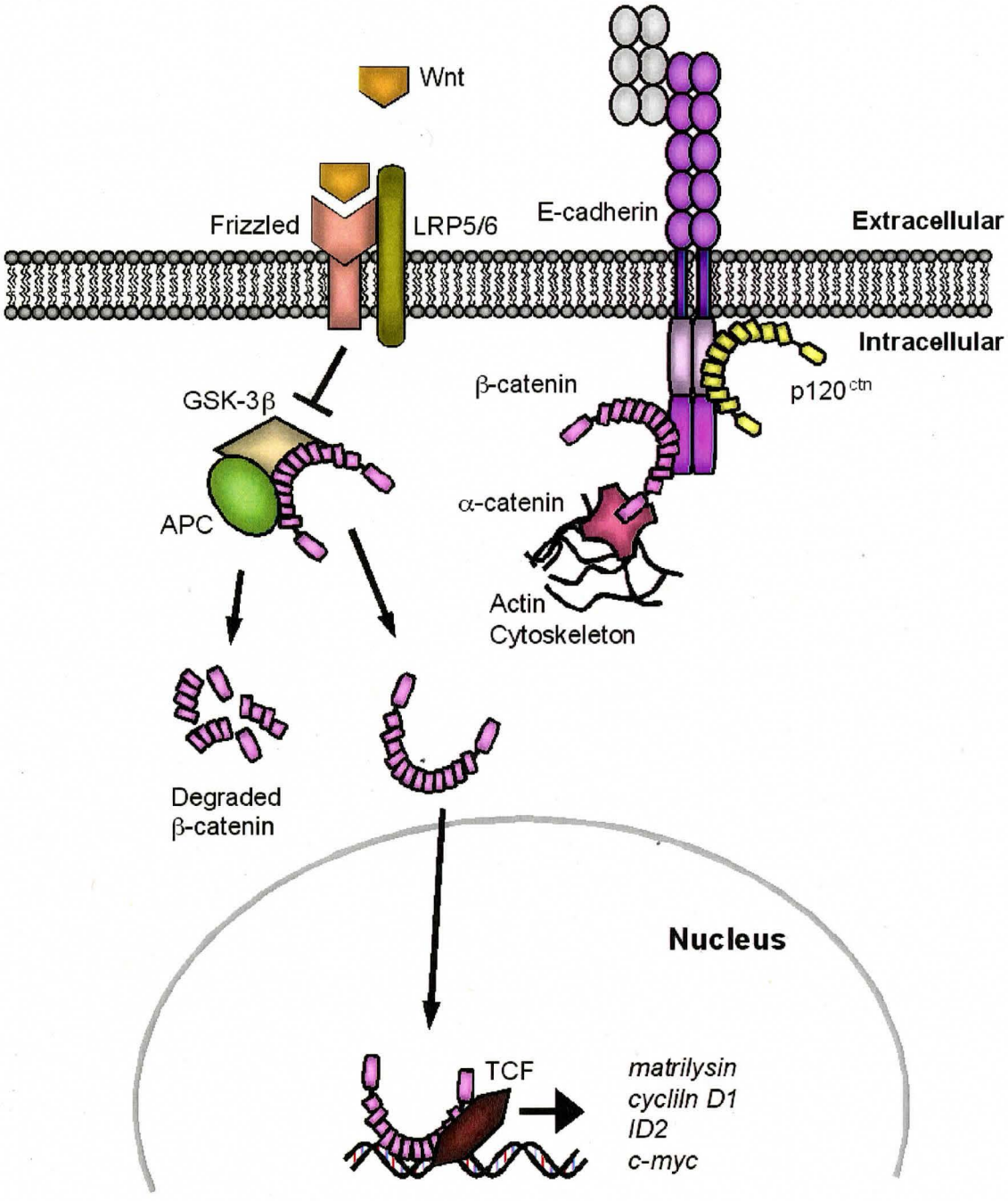


Figure 2: β-catenin and the Wnt signaling pathway. β-catenin, a well-known regulator of E-cadherin function and cell adhesion (right), is also a signaling molecule in the Wnt signaling pathway (left). Binding of the secreted Wnt ligand to the Frizzled receptor initiates a cascade of events that ends in β-catenin/TCF transcriptional de-repression of genes including *matrilysin, cyclin D1, ID2, and c-myc.*

1.5. p120-catenin: a versatile regulator of cadherin function

The catenin $p120^{ctn}$ (hereafter p120) was originally discovered as a substrate for the Src tyrosine kinase and was later found to be phosphorylated by various receptor tyrosine kinases (26, 102, 104). Further studies on p120 then revealed that it was also a component of the E-cadherin-catenin cell adhesion system (100), facilitating new research into the function of this intriguing protein. p120 is an Armadillo catenin and the prototypical member of the p120 subfamily of Armadillo proteins, that include ARVCF, plakophilin, and δ-catenin, and which are more related to each other than to other Armadillo proteins (4). The p120 Arm domain contains 10 Armadillo repeats (Figure 3) and shares 22% sequence homology with the β-catenin Arm domain (102). The peptide length of p120 is 911 amino acids and has a molecular weight of 120 kDa. Most cell types express multiple isoforms of p120, which are derived from alternative splicing and use of four different start codons of a single *p120* gene (48) (Figure 3). Epithelial cells preferentially express the smaller p120 isoform 3, while fibroblasts mainly express the larger p120 isoform 1 (1). The existence of cell-type specific p120 expression patterns hint at functional differences between the p120 isoforms.

Unlike the classical catenins β- and γ-catenin, p120 binds to E-cadherin at the **j**uxta**m**embrane **d**omain (JMD) (127, 141), a highly conserved region within the cadherin superfamily. Furthermore, p120 does not interact with α-catenin like the classical catenins do, and its expression does not seem to be influenced by, or related to, β- or α-catenin expression (21, 25). These unique characteristics of p120 led researchers to

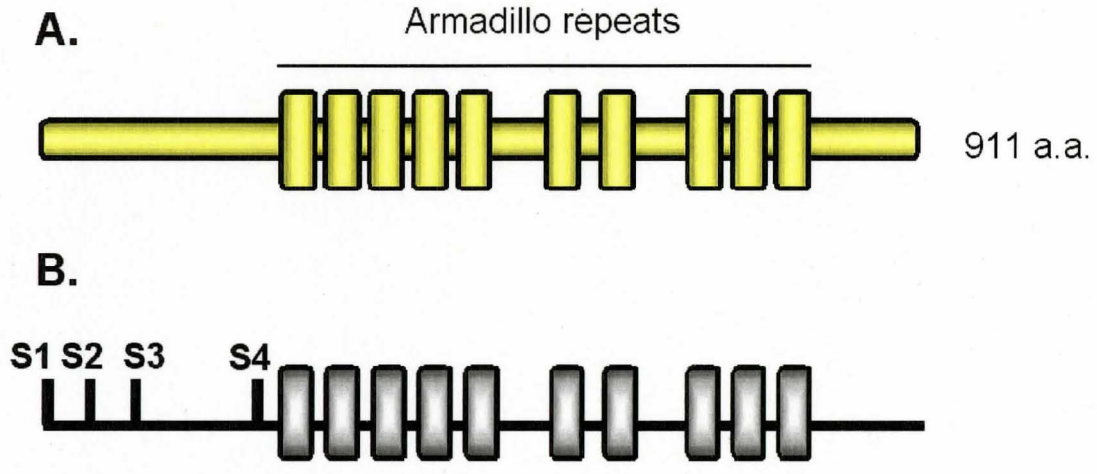


Figure 3: Schematic diagram of p120 structure. (A) p120 is 911 amino acids long, contains 10 Armadillo repeats, and has a molecular weight of 120 kDa. (B) The four major p120 isoforms are derived from alternative splicing of the same gene at different start codons (S1 – S4). S1 is the start site for translation of the largest isoform 1, and so forth.

postulate that p120 had a unique role in regulating cadherin function. While β- and γ-catenin are known for positively regulating E-cadherin adhesive strength (9, 145), several studies have implicated p120 as both a positive and negative regulator of E-cadherin adhesive strength, and as a key modulator of E-cadherin stability and turnover (7, 24, 42, 127).

A seminal study on p120 function demonstrated that p120 directly regulates E-cadherin adhesive strength, since uncoupling of p120 from the cadherin-catenin complex severely reduced the transition from loose to tight cell junctions (127). Moreover in a p120-deficient colon carcinoma cell line (SW48) the cadherin-catenin complex is severely impaired; however, expression of wild type p120 rescued the characteristic cobblestone epithelial morphology (42). These were the first indications that p120 has a critical function in regulating cell adhesive strength and establishing epithelial morphology. However, in the colon carcinoma cell line, Colo205, which lack normal E-cadherin function despite having normal levels of E-cadherin and the catenins, ectopic expression of N-terminally truncated p120 restored adhesion, whereas full length p120 did not (7). This implicated the N-terminus of p120 in negatively regulating E-cadherin adhesion, and points to the possibility that signaling events in different cell types may dictate whether p120 has a positive or negative effect on E-cadherin adhesive strength.

Research in recent years has revealed novel functions for p120 as a cadherin co-factor and point to an indispensable role for p120 in regulating cadherin stability and turnover. One key study concluded that E-cadherin expression levels are dependent upon p120 expression (24). Using **RNA** interference (RNAi) and p120 reconstitution, Davis *et*

al. (24) demonstrated that p120 depletion resulted in the dose-dependent reduction of not only E-cadherin, but also N-, P-, and VE-cadherins. Independent studies further revealed that p120 is involved in directing cadherin endocytosis and degradation (38); specifically, p120 controls the entry of cadherins into the endosomal-lysosomal pathway (139).

In addition to regulating cadherin internalization and degradation, p120 is also involved in directing cadherins to the cell membrane. p120 associates with the microtubule network and specifically interacts with kinesin, a molecular motor involved in trafficking proteins along the microtubule network (18, 30). In the cytosol, p120 interacts directly with vesicle-bound cadherins and kinesin simultaneously and supports the rapid delivery of cadherins to the cell surface (18). Furthermore observations that p120 associates with the earliest forms of N-cadherin (130) and co-localizes with N-cadherin at the Golgi apparatus (18) suggest that p120 is involved at various stages of transporting newly synthesized cadherins from the Golgi Apparatus to the cell surface. Together these studies demonstrate that p120 is involved in cadherin membrane trafficking, endocytosis, and degradation.

1.6. p120 regulation of actin cytoskeleton dynamics

Like its family member β-catenin, p120 is also involved in intracellular signaling. The E-cadherin JMD, which interacts with p120, has been implicated in modulating cell motility (141), and now p120 has also been implicated in this complex process. Previous work with p120 revealed that its overexpression in fibroblasts and epithelial cells induced a dendritic branching phenotype that is reminiscent of the formation of dendritic neuronal

extensions (101). Co-transfection of E-cadherin along with p120 suppressed the branching phenotype, whereas E-cadherin mutants lacking p120-binding ability were unable to suppress the phenotype (101). Additionally, cytoplasmic p120 was observed at significantly higher levels in the E-cadherin mutant cells when compared to wild type cells (101). This suggested that cytoplasmic p120 is responsible for this dendritic phenotype and that sequestration of p120 from the cytosol to the membrane is sufficient to suppress the phenotype. The formation of dendritic cellular extensions is a process that occurs through the remodelling of the actin cytoskeleton, which is mediated by small GTPases of the Rho family, reviewed in (105). Ectopic expression of p120 also results in increased cell migration and motility (79), another cellular process mediated by Rho GTPases. Together, the p120 effects on cellular branching and motility led researchers to question whether p120 is involved in modulating Rho GTPase function.

Initial studies demonstrated that p120 overexpression increased the activity of Rac1 and Cdc42, two members of the Rho GTPase family that inhibit the activity of RhoA (80). p120 was also shown to bind the Rho GTPase guanine nucleotide exchange factor Vav2 (80). Moreover p120 directly inhibits the activity of RhoA (5), which is involved in regulating myosin chain elongation, actin filament rearrangement, cell-shape determination, cell proliferation, and gene expression (79). The link between p120 and Rho GTPase activity, and direct evidence demonstrating that p20 interacts with various Rho GTPases provides a possible molecular mechanism by which p120 regulates cytoskeletal dynamics and cell motility. Overall these observations led to the hypothesis

that cytoplasmic p120 promotes cell migration and metastasis in E-cadherin-deficient cells through its interactions with Rho GTPases.

1.7. p120 nuclear functions

In addition to its role in regulating cytoskeletal dynamics, p120 has nuclear roles that are not yet fully understood. Several groups have detected nuclear p120 in various cell lines, suggesting that p120 may have a functional role in the nucleus (50, 107, 127, 128). Recent work from our laboratory revealed that p120 contains a **n**uclear **l**ocalization **s**ignal (NLS) between Arm repeats 6 and 7 that mediates p120 translocation to the nucleus (50). Interestingly, a minimal point mutation of this sequence not only abrogates p120 nuclear localization, but also abolishes the p120-induced branching phenotype (50). These observations suggest that the p120 NLS is not only important for p120 nuclear localization but also functions in p120's cytoskeletal remodelling functions. The discovery of the p120 NLS coupled with the identification of a putative **n**uclear **e**xport **s**ignal (NES) sequence in the carboxy terminus of p120 (128) demonstrates that p120 can shuttle in and out of the nucleus.

Currently, there is no known signaling event that specifically induces p120 nuclear import, although certain instrinsic and extrinsic factors affect p120 subcellular localization. For example, the different p120 isoforms display unique nuclear localization patterns; p120 isoform 1 is seldom observed in the nucleus, whereas isoform 3 is observed more often in the nucleus (107). Closer inspection of these isoforms reveals an N-terminal coiled-coil domain in isoform 1, which is not present in isoform 3.

Coiled-coil domains are known to be involved in protein-protein interactions (107), and it is possible that the isoform 1 coiled-coil domain anchors p120 to unknown cytoplasmic factors or binds nuclear export factors. Additional studies have indicated that phosphorylated forms of p120 preferentially localize to the nucleus (138), however other studies have shown that mutation of certain phosphorylation domains within p120 does not affect its nuclear shuttling activity (107). Furthermore, E-cadherin-deficient cell lines show a notable increase in cytoplasmic as well as nuclear p120, and p120 phosphorylation status is frequently altered in these cell lines (117, 127, 128). Together these observations provide a spatial and contextual link between p120 and the different extrinsic factors that may affect its intracellular signaling, nuclear localization and function during tumour progression (Figure 4).

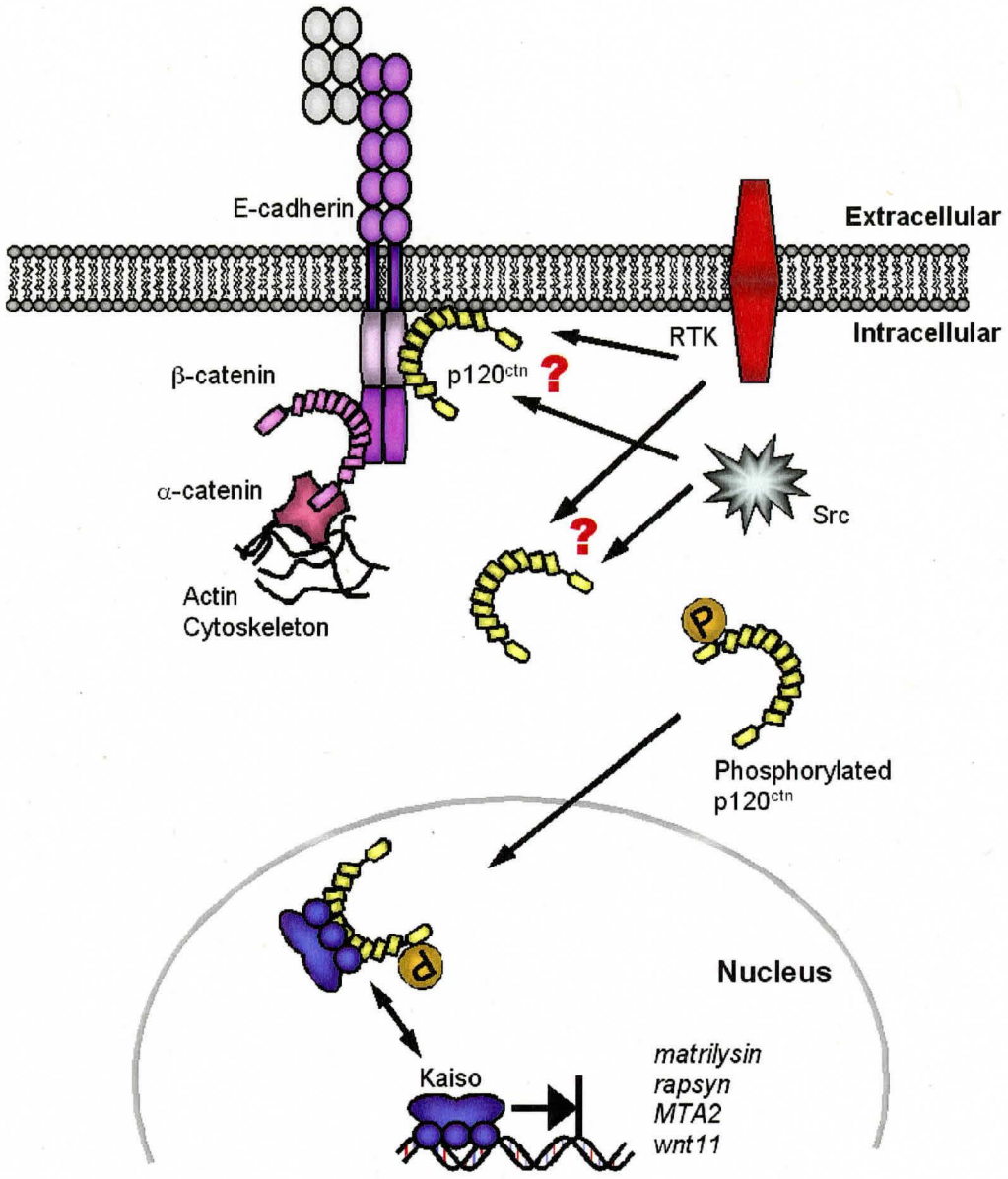


Figure 4: p120ctn intracellular signaling. The catenin p120ctn, a regulatory component of the E-cadherin-catenin complex, is emerging as an intracellular signaling molecule. p120ctn is phosphorylated by Src and receptor tyrosine kinases although it is currently unknown whether this occurs with E-cadherin-bound or free cytoplasmic p120ctn. Phosphorylated forms of p120ctn are known to localize to the nucleus and preferentially interact with Kaiso, a POZ-ZF transcriptional regulator, to abrogate Kaiso transcriptional activity. p120ctn nuclear localization and gene regulation may be the end-point of the long-suspected E-cadherin adhesion-based signaling pathway.

1.8. Kaiso: the unique dual-specificity POZ-ZF transcription factor

In light of the increasing body of data that hinted at a nuclear role for p120, an exciting discovery was made when Kaiso, a novel transcription factor, was identified as a p120-specific binding partner using a yeast-2-hybrid assay with the p120 Arm domain as bait (20). Kaiso belongs to the growing family of **B**ric à brac/**T**ramtrack/**B**road Complex and **Po**x viruses and **Z**inc finger (BTB/POZ) (hereafter POZ-ZF) transcription factors (20). This family of proteins is characterized by a POZ domain at the amino terminus and carboxy-terminal zinc finger motifs (2, 8). The POZ domain is a conserved hydrophobic domain of 120 amino acids that mediates protein-protein interactions and is associated with nucleosome/chromatin disruption, metamorphosis, and oogenesis in *Drosophila* (2, 8). The zinc finger domain is generally characterized as a DNA-binding domain, reviewed in (43). Kaiso is a 671 amino acid residue protein with a molecular weight of approximately 100 kDa that possesses the characteristic amino terminal POZ domain and three carboxy terminal C_2H_2 zinc fingers (20) (Figure 5).

After the initial discovery of Kaiso, further experiments revealed that it does not interact with p120 via its POZ domain, but rather via a region encompassing the Kaiso zinc finger domain (20). The catenin p120 binds to Kaiso via its Arm repeats 1-7 which are also involved in its binding to E-cadherin (42, 127), and as such, p120 binding to Kaiso and E-cadherin are believed to be mutually exclusive. In addition, Kaiso does not bind any other known component of the E-cadherin-catenin complex (20), suggesting that its interaction with p120 may mediate specific downstream effects of p120 signaling in epithelial cells. Interestingly, Kaiso interacts preferentially with the smaller p120

isoform 3; this isoform is mainly expressed in epithelial cells that express E-cadherin and is also observed more frequently in the nucleus (1, 107). The possibility exists that the p120/Kaiso interaction is dictated in part by p120 isoform expression, E-cadherin expression, and/or epithelial morphology. More excitingly this unique interaction may be a component or endpoint of the long-suspected epithelial cell-specific cell adhesion-signaling pathway (Figure 4).

Kaiso is a predominantly nuclear protein, although in certain cell lines some cytoplasmic Kaiso staining is also observed (20, 84). Kaiso possesses a basic monopartite NLS located just upstream of the zinc finger domain. This Kaiso NLS is highly conserved across species, and suggests an indispensable role for the NLS in Kaiso function (49) (Figure 5). Minimal point mutations within the NLS abolish Kaiso's nuclear localization and lead to a loss of its transcriptional regulatory abilities (49). Kaiso is a bimodal DNA-binding protein that recognizes a specific consensus sequence or **K**aiso **b**inding **s**ite (KBS) TCCTGCnA where "n" denotes any nucleotide, as well as CpG-methylated dinucleotides, establishing Kaiso as the first POZ-ZF protein to be recognized with dual-specificity DNA-binding capabilities (23). Artificial promoter assays revealed that Kaiso represses transcription via either sequence (94, 119) but to date only Kaiso-mediated repression via the KBS has been shown to be inhibited by p120 in a dose-dependent manner (23).

Our finding that Kaiso immunocomplexes possess **h**istone **d**e**ac**etylase (HDAC) transcriptional co-repressor activity in both epithelial and fibroblast cell lines (27),

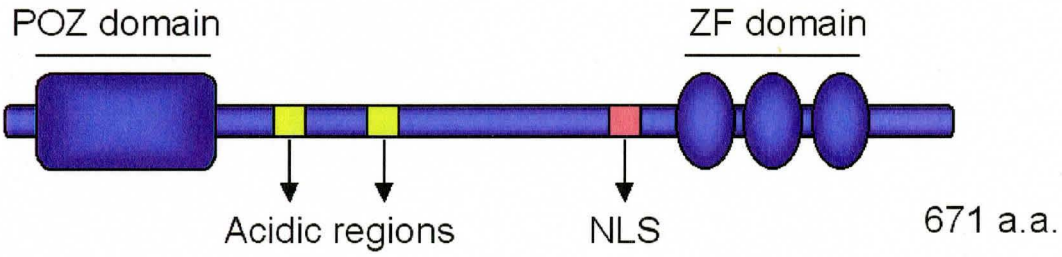


Figure 5: Schematic diagram of Kaiso structure. Kaiso has a peptide length of 671 amino acids. It has an amino terminal POZ domain and a carboxy terminal ZF domain with 3 C_2H_2 zinc fingers. There are two highly acidic sequences near the N-terminus of Kaiso which have an unknown function but which may play a role in transcription activation. Kaiso also possesses a basic monopartite **n**uclear **l**ocalization **s**ignal (NLS) upstream of the zinc finger domain.

suggests that Kaiso, like most POZ-ZF proteins, recruits the HDAC complex to repress transcription. Furthermore Kaiso complexes with the mSin3A co-repressor in epithelial but not fibroblast cell lines (27), suggesting that Kaiso co-repressors may be cell-type specific. Kaiso also exists as part of the methylation-specific **n**uclear **cor**epressor (N-CoR) transcriptional repression complex, which also involves HDAC, but lacks mSin3A (142). Together these findings suggest that different transcriptional repression complexes may mediate Kaiso sequence-specific and methylated DNA repression activities.

1.9. Sequence-specific Kaiso target genes

To date Kaiso has been shown to regulate a variety of genes involved in different cellular processes and most of these gene promoters have at least two copies of the sequence-specific KBS (Summarized in Figure 6). During our initial characterization of Kaiso, our lab identified two KBS elements within the promoter region of the matrix metalloproteinase gene *matrilysin*. The *matrilysin* gene product is involved in extracellular matrix degradation and is often dysregulated during tumourigenesis, reviewed in (67). Moreover, *matrilysin* is a well-known target of the Wnt/β-catenin/TCF signaling cascade (19) and the presence of Kaiso recognition sequences within this promoter provided a context in which to study the possible convergence of p120/Kaiso and β-catenin/TCF on the same target gene. Kaiso associates with the *matrilysin* promoter in various cell lines, and **e**lectrophoretic **m**obility **s**hift **a**ssays (EMSA) using Kaiso fusion proteins and *matrilysin* pomoter-derived probes show that this association

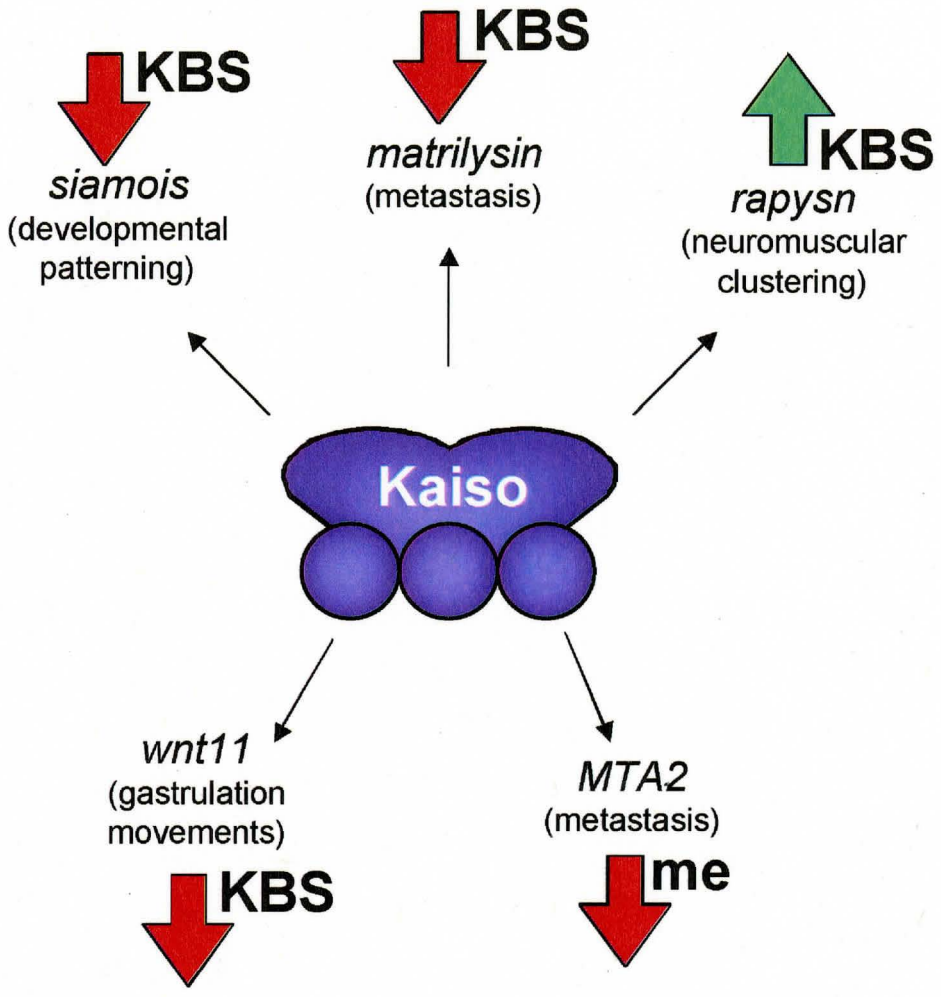


Figure 6: Kaiso regulates the expression of diverse genes. Kaiso is a transcription factor that has already been characterized to regulate a variety of target genes including *matrilysin*, *rapsyn*, *wnt11*, *siamois*, and *MTA2*. These genes are involved in different cellular functions, placing Kaiso as a key regulatory component of various cell processes. Red arrows indicate genes that are repressed by Kaiso while the green arrows indicate genes that Kaiso activates. "KBS" denotes sequence-specific DNA-binding while "me" denotes methyl-CpG binding.

most likely occurs through the KBS elements (119). Artificial promoter assays demonstrated that Kaiso is a dose-dependent and sequence-specific transcriptional repressor of the *matrilysin* promoter, since a minimal point mutation of the KBS elements within the promoter abolished this Kaiso-mediated repression (119). More importantly we found that Kaiso inhibits β-catenin-mediated activation of *matrilysin*, and Kaiso's repression of *matrilysin* and its inhibitory effect on β-catenin can be abrogated by nuclear p120 (119). Our findings were the first to report that Kaiso and p120 converged with β-catenin/TCF on a canonical Wnt target gene and raised the exciting possibility that Wnt and cadherin signaling events may result in the transcriptional regulation of similar genes.

Although several independent studies have established Kaiso as a transcriptional repressor, one study to date has also reported it as a transcriptional activator. Kaiso activates the synapse-specific gene *rapysn* via two KBS elements within its promoter region in murine and chicken myotubes (108). This establishes Kaiso as one of the few POZ-ZF transcription factors known to activate as well as repress gene expression; ZF-5 and Miz-1 also display transcriptional activation and repression activities (13, 47, 54, 136). The *rapsyn* gene product is involved in acetylcholine receptor clustering at neuromuscular junctions upon neural signaling, reviewed in (133). Several *rapysn* promoter mutations have been identified in patients with **c**ongenital **m**yasthenic **s**yndromes (CMS), and interestingly, one of these mutations occurs within one of the two *rapsyn* promoter KBS (108), implicating Kaiso as a key regulator of *rapysn* transcription and perhaps linking Kaiso to CMS. Minimal promoter assays using a *rapysn* promoter

harbouring the CMS promoter KBS mutation resulted in a reduced Kaiso ability to activate the promoter (108), which may provide an explaination as to why *rapysn* expression levels are reduced in CMS patients (108). Interestingly the p120 subfamily member δ-catenin, which is highly expressed in neuronal cells, was found to interact with *Kaiso* in murine myotube cells where it enhances Kaiso-mediated activation of *rapsyn* (59, 108). Since δ-catenin is a p120 subfamily member, it is possible that Kaiso is a specific effector of p120 subfamily signaling functions in specific cell types. The interaction may also hint at a more general ability for Kaiso, a ubiquitously expressed protein, to interact with cell-type specific p120 family members to propagate signals initiated by cell-type specific cadherins.

1.10. Methylation-dependent Kaiso gene regulation

Although several genes have been identified as Kaiso sequence-specific targets, thus far only one gene has been characterized as a DNA methylation-dependent Kaiso target; *MTA2* belongs to the metastasis-associated gene family and may be involved in the transcriptional activation or repression of genes involved in promoting metastasis (142). The MTA2 promoter contains a 22 nucleotide CpG island approximately 3 Kb upstream of the transcriptional start site and Kaiso and N-CoR associate with this CpG island in a methylation-dependent manner *in vivo* (142). Treatment of cells with **5-aza**-**d**eoxy**c**ytidine (5-aza-dC), a DNA methylation inhibitor, not only reduced Kaiso and N-CoR association with the MTA2 CpG island, but significantly increased MTA2 transcript levels (142), suggesting that Kaiso is a methylation-specific transcriptional repressor of

this gene (Figure 6).

Other genes have been postulated to be methylation-dependent Kaiso target genes including the *S100A4* gene and *E-cadherin* (94), both of which are known to contain heavily methylated promoter regions. Since *E-cadherin* is repressed by methylation in various human tumours that have acquired metastatic potential (32), the possibility that it is a Kaiso target gene is an intriguing notion. One can postulate that Kaiso-mediated transcriptional repression of *E-cadherin* through one or more of its CpG islands may be the result of a signal transduction feedback loop transmitted from the cell surface through p120 to result in E-cadherin downregulation.

Additional evidence supporting a role for methylation-dependent Kaiso transcriptional regulation was obtained using the *Xenopus* model system. Kaiso depletion in early *Xenopus* embryos led to the premature activation of normally methylated zygotic genes (109). Furthermore a large portion of these genes (~60%) function in cell growth control, chromatin structure, and signal transduction (109). These findings point to a crucial role for Kaiso in methylation-dependent transcriptional repression and suggest that further studies on Kaiso- methylation-dependent transcriptional repression are warranted.

1.11. Kaiso roles in development and cancer

Most mammalian POZ-ZF proteins are strongly implicated in development and cancer, and ongoing research on Kaiso function indicates that it too plays a role in these complex processes. Kaiso's role in vertebrate development has been studied thus far

using the *Xenopus* model system. The *Xenopus* Kaiso protein (XKaiso) shares 53% homology with murine Kaiso (mKaiso), and the XKaiso POZ and zinc finger domains share 87% and 90% homology, respectively with mKaiso (53). XKaiso also interacts with Xp120, and like its murine homologue, functions as a transcriptional repressor (53).

The first report on the functional consequences of XKaiso activity showed that it is required for proper gastrulation movements during later stage *Xenopus* embryo development (52). XKaiso depletion in *Xenopus* embryos resulted in a gastrulation defect phenotype characterized by an exposed endoderm, incomplete axis extension, and improper neural tube closure (52). Closer analysis of this striking phenotype revealed that XKaiso transcriptional repression of the non-canonical *xWnt11* gene contributed to the observed defect and as expected Xp120 was able to relieve this repression. Together these data demonstrated that XKaiso and Xp120 participate in the non-canonical Wnt signaling pathway to regulate gastrulation and convergent extension movements during *Xenopus* development (52).

XKaiso and Xp120 were subsequently shown to have a direct role in regulating canonical Wnt signaling. Studies revealed that XKaiso is a sequence-specific transcriptional repressor of several β-catenin/TCF target genes including *c-myc*, *cyclin D1*, and *c-Fos*, while Xp120 relieves this repression activity (88). These genes play key roles in *Xenopus* cell growth, differentiation and pattern formation during embryogenesis, but are also oncogenes associated with aberrant cell growth during tumour progression (81). Luciferase assays performed with the canonical Wnt signaling reporter TOPFLASH resulted in decreased luciferase expression upon XKaiso overexpression

(88), demonstrating that XKaiso inhibits β-catenin transcriptional activity. This led the researchers to question whether XKaiso inhibits Wnt signaling at the phenotypic level. In developing *Xenopus* embryos overexpression of β-catenin causes a prominent axis duplication phenotype in which embryos start to develop two heads; however, coexpression of XKaiso along with β-catenin rescued this phenotype (88). These exciting findings provide further credibility to the hypothesis that Kaiso and p120 converge on β-catenin target genes. The *Xenopus* studies outlined here provide strong evidence of the key role that Kaiso plays in amphibian development and expand upon the current body of knowledge on the interplay between Wnt and catenin signaling.

Various lines of evidence hint that Kaiso may be involved in cancer progression as well; (1) it is a transcriptional regulator of tumourigenesis-associated genes including *matrilysin* and *MTA2* (119, 142), (2) it is an inhibitor of canonical Wnt signaling (88, 119), and (3) it associates with p120 (20), which is often misexpressed in many human cancers (103). The first direct evidence that Kaiso may be involved in human tumour progression was recently published in a study that examined Kaiso subcellular localization in tumour tissues (118). A variety of normal and tumour paraffin-embedded tissues, such as liver, breast, testis, and colon, were probed with Kaiso antibody to visualize Kaiso subcellular localization in the context of the tumour microenviroment. In contrast to observations from cultured cells *in vitro*, most of the normal and tumour tissues displayed clear cytoplasmic Kaiso staining or in some cases very weak nuclear staining (118). This was a surprising finding since Kaiso is widely accepted as a nuclear protein, however in the tumour microenvironment these subcellular localization patterns

could point to a mechanism for Kaiso participation in tumour promoting cellular processes.

Interestingly, high levels of cytoplasmic p120 was often found in cells with cytoplasmic Kaiso, and no nuclear p120 was observed in any of the tissues examined (118). This suggested that perhaps p120 was responsible for sequestering Kaiso in the cytoplasm. Furthermore in a subset of tumours, a Kaiso subcellular localization gradient was observed in different tumour compartments. At the leading, invasive edge of the tumours, Kaiso showed a strong nuclear localization, which was accompanied by decreased E-cadherin and p120 staining at cell-cell contacts. In the central part of the tumours progressively less Kaiso was detected in the nucleus and eventually completely cytoplasmic staining was detected (118). This differential subcellular localization of Kaiso in different regions of the tumour tissue could indicate tumour microenvironment-directed regulation of Kaiso activity during different stages of tumour progression and could be the first step in unravelling the roles of Kaiso in tumourigenesis.

A second recently published study also directly investigated the role that Kaiso may play in tumour progression. In this study the *Kaiso* gene was disrupted in mice to assess the effects of *Kaiso* gene deletion in mammalian development and tumourigenesis. Unexpectedly the *Kaiso*-null mice were developmentally viable; they exhibited no obvious developmental abnormalities and did not display phenotypes related to those seen in *Xenopus* (95). Furthermore, analysis of specific Kaiso target genes such as *MTA2*, *Rapsyn*, and *Wnt11* in the *Kaiso*-null mice showed no difference in gene expression when compared to wild type mice (95). This was a surprising finding as

Kaiso depletion in *Xenopus* leads to such striking developmental phenotypes. Therefore, considering that *Kaiso* gene deletion had no obvious developmental effect in mice, investigators then decided to specifically investigate Kaiso roles in colorectal cancer since: (1) Kaiso's interaction with p120 attenuates its function and p120 is misexpressed in 25% of colorectal cancers, and (2) Kaiso is implicated in canonical Wnt signaling, which is perturbed in a large subset of colorectal and intestinal cancers. *Kaiso*-null mice were crossed with $Apc^{Min/+}$ mice, which are well characterized as a murine colorectal cancer model in which perturbed Wnt signaling contributes to tumour progression, reviewed in (89). The resulting *Kaiso*-null *Min* mice had a significantly increased survival rate and significantly smaller intestinal polyps than wild type control crossed mice (95). This study was the first to implicate Kaiso in colon tumourigenesis and suggests that it is an oncogene in this context.

Recent work from our lab suggests that Kaiso is involved in breast tumour progression as well. We probed a Cancer Profiling cDNA Array (Clontech) with Kaiso-specific radio-labelled probes to determine if Kaiso was misexpressed in any of the human cancers represented on the array. We noted that Kaiso was misexpressed in 42% of human breast cancers; it was underexpressed in 30% of breast tumours when compared to matched normal samples and overexpressed in 12% of the samples represented on the array (Daniel lab, unpublished data). This was an exciting finding because we simultaneously identified three sequence-specific Kaiso binding sites within the *cyclin D1* promoter, a gene that is perturbed in most human tumours, but is most often misexpressed in breast tumours (3, 15, 116). Furthermore, *cyclin D1* is a target gene of

the Wnt signaling pathway (126) implicating yet another canonical Wnt target as a putative Kaiso target gene.

1.12. cyclin D1: a putative Kaiso target gene

The aberrant expression and regulation of the cell cycle gene *cyclin D1* is thought to contribute significantly to the tumourigenic process. It was therefore quite exciting to discover that the human *cyclin D1* promoter contains one copy of the fully conserved KBS (TCCTGCnA) and two copies of the core KBS (CTGCnA). The *cyclin D1* gene product associates with **c**yclin-**d**ependent **k**inase **4** or **6** (CDK4/6) to function as the switch that regulates cell cycle progression from G1 (first growth phase) to S (DNA synthesis phase), reviewed in (120). Once a cell has passed this critical cell cycle checkpoint it is committed to completing the cell cycle through G2 (second growth phase) and M (mitosis and cytokinesis phase). Cyclin D1 expression is induced during the G1 phase of the cell cycle and is maintained until the beginning of the S phase where it is rapidly degraded (120) (Figure 7A).

Although *cyclin D1* is overexpresed in a variety of human cancers such as breast, esophageal, and B-cell lymphomas (44, 71, 83), its overexpression is most frequently observed in breast cancers (3, 15, 116). Cyclin D1 malfunction was first implicated in breast cancer when studies indicated that the *cyclin D1* gene localizes to chromosome 11q13, a region of the genome that is amplified in 15 - 20% of human breast cancers (15, 83, 123). Subsequent studies showed that the *cyclin D1* gene product is overexpressed at

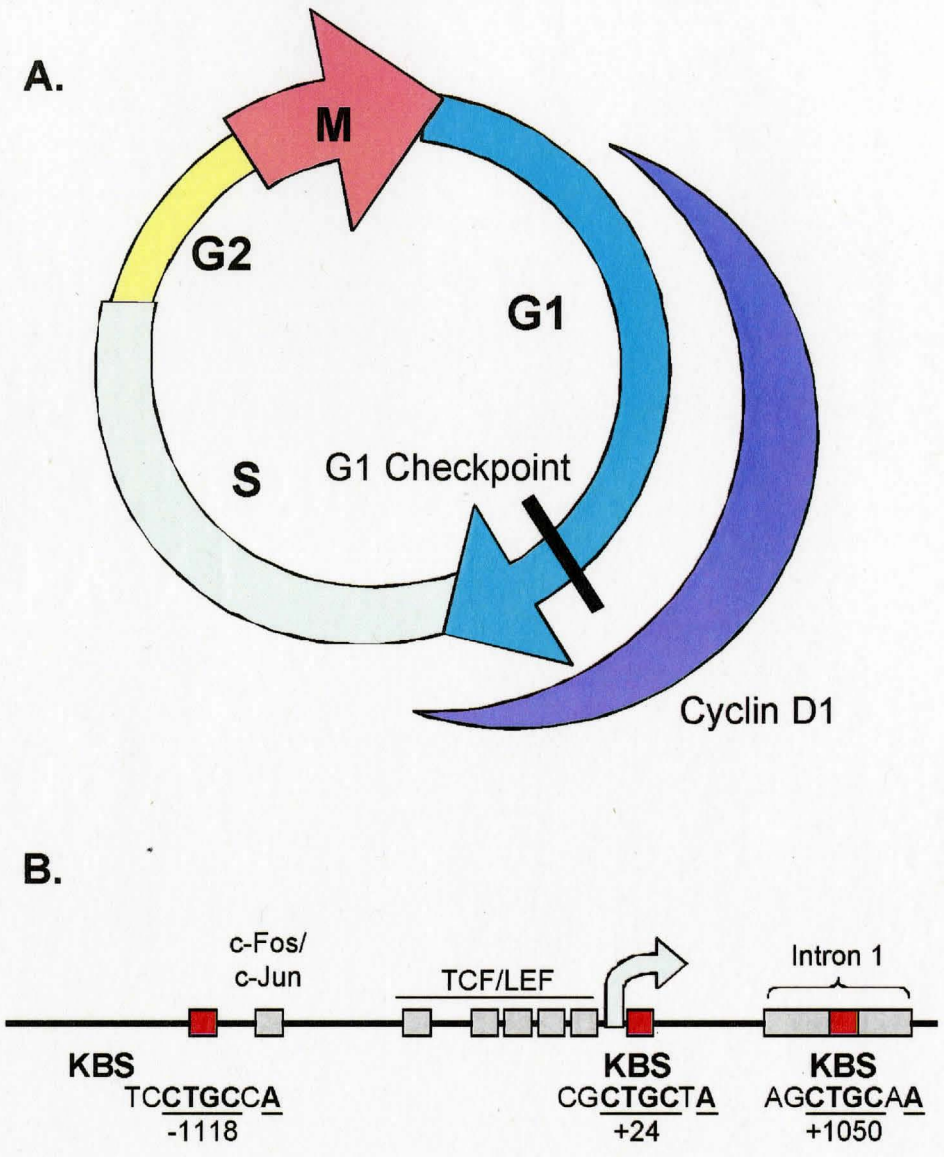


Figure 7: *Cyclin D1* is a putative Kaiso target gene. (A) Expression of the *cyclin D1* gene product is induced during the G1 phase of the cell cycle. Once Cyclin D1 reaches a critical level, it promotes the cell's entry into G2, committing the cell to proceed through the cell cycle. Once the G1 checkpoint has been crossed Cylin D1 is then rapidly degraded while other cyclins are produced to continue with the cell cycle. (B) The *cyclin D1* promoter contains three Kaiso binding sites (KBS) in areas upstream and downstream of the gene's transcriptional start site (arrow). The *cyclin D1* promoter also contains various sequence-specific transcription factor recognition sequences, of which the c-Fos/c-Jun and TCF/LEF recognition sites are diagrammed.

the mRNA and protein level in 50% of primary breast tumours (3, 15, 116), making *cyclin D1* one of the most commonly overexpressed oncogenes in breast cancer. Transgenic mice that overexpress Cyclin D1 from the MMTV promoter develop and die from breast cancer (131) and Cyclin D1 knockout mice are resistant to breast cancers that are induced by *ras* or *neu* overexpression (144), suggesting that the loss of cell cycle control is a major contributory factor to breast tumour formation and progression. Together these findings suggest that aberrant transcriptional regulation of *cyclin D1* expressison is responsible for contributing to the loss of cell cycle control in human tumours.

The *cyclin D1* promoter contains three copies of the KBS; one fully conserved sequence located at position -1118 and two core sequences located at positions +24 and +1050 (within intron 1), relative to the transcriptional start site (Figure 7B). Additionally, the *cyclin D1* promoter has 4 predicted methylatable CpG islands that may recruit methylation-specific transcription factors, such as Kaiso, to induce *cyclin D1* transcriptional repression. The β-catenin/TCF complex is a well-characterized transcriptional activator of *cyclin D1* (126), especially in colon tumours where β-catenin is often mutated (89). Our lab and others have reported a convergence of β-catenin/TCF and p120/Kaiso on Wnt target genes (88, 119), but no studies to date have been carried out on p120/Kaiso regulation of the Wnt target gene *cyclin D1* in the mammalian system. Our investigation of *cyclin D1* as a putative Kaiso target gene will expand upon our current knowledge of Kaiso's gene regulatory functions and its role in inhibiting canonical Wnt signaling. Furthermore this work will provide new opportunities for

studying Kaiso's role in tumourigenesis, and its potential role in breast tumour progression.

1.13. Rationale and Hypothesis

Kaiso is a sequence-specific and methylation-specific transcriptional repressor and has been implicated in regulating genes involved in cell growth and differentiation in development and tumour progression. Our lab previously reported that Kaiso is a sequence-specific repressor of the metastasis-associated *matrilysin* promoter and demonstrated that Kaiso could inhibit β-catenin-mediated activation of this promoter. We have since identified three sequence-specific Kaiso binding sites within the *cyclin D1* promoter, which is a prominent oncogene that is upregulated primarily in human breast tumours, and a gene target of the Wnt/β-catenin/TCF signaling pathway. Since preliminary data from our lab indicate that Kaiso is underexpressed in 30% of human breast tumours, the possibility exists that Kaiso is involved in regulating breast cell growth, proliferation, and tumourigenesis through its transcriptional regulation of *cyclin D1*. Together these observations led to the exciting possibility that Kaiso is a transcriptional regulator of the *cyclin D1* gene, where it may converge with β-catenin/TCF to co-ordinately regulate *cyclin D1* expression.

Hypothesis: Kaiso-mediated transcriptional regulation of *cyclin D1* plays a role in breast cell proliferation and tumourigenesis.

Materials and Methods

2.1. Cells and Tissue Culture

Human breast epithelial cell lines MCF-12A (non-tumour), MCF-7, MDA-468, and MDA-231 (tumour), and human cervical carcinoma HeLa cells were used in these studies and were purchased from ATCC (Manassas, VA). MCF-12A cells were propagated in DMEM:F12 medium (Hyclone) supplemented with 5% Horse Serum (Gibco/BRL), 10 μg/ml Insulin (Sigma), 5 μg/ml Hydrocortizone (Sigma), 20 ng/ml EGF (Invitrogen), and 100 ng/ml Cholera Toxin (Biomol). All other cells were propagated in Dulbecco's minimal essential medium (Hyclone), supplemented with 10% fetal bovine serum (Gibco/BRL), 100 units/ml penicillin, 100 μl/ml streptomycin (Invitrogen), and 0.5 μg/ml Fungizone (Invitrogen). All cells were incubated at 37°C in a 5% CO_2 humidified incubator.

2.2. Immunoprecipitation and Western Blot

Cultured cells were grown to 90% confluency on 100 mm tissue culture dishes. Each plate was washed once with 5 ml cold PBS and lysed on ice with 0.5% Nonidet P40 lysis buffer (0.5% NP40, 50 mM Tris, 150 mM NaCL, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, and 2.5 mM EDTA). The lysates were harvested using a cell scraper and cleared by centrifugation at 14,000 rpm for 5 minutes at 4°C. Protein concentration was quantitated using 1X Bradford Reagent (BioRad) and equal protein lysates were incubated with 4 μg of appropriate antibody at 4°C for one

hour. The following antibodies were used at 4 μg each for immunoprecipitation: anti-Kaiso 6F monoclonal antibody, anti-p120 15D2 monoclonal antibody, anti-β-catenin polyclonal antibody (Sigma), and anti-E-cadherin monoclonal antibody (Transduction Laboratories). Protein-A-Sepharose beads (Amersham Biosciences) were pre-incubated with rabbit anti-IgG (Jackson ImmunoResearch Laboratories), and 0.5% NP40 lysis buffer at 4°C for an hour, and added to each lysate sample. Immune complexes were captured for one hour after they were washed three times with 0.5% NP40 lysis buffer.

The complexes were then resuspended in 2X Laemmli Sample Buffer (0.1 M Tris pH 6.8, 2% SDS, 10% sucrose, 0.008% Bromophenol blue, 0.24 M β-mercaptoethanol), denatured by boiling, and loaded onto a 7% SDS-poly-acrylamide gel. The separated proteins were then transferred onto a nitrocellulose membrane (Fisher Scientific), incubated with 3% TBS-milk for 5 minutes, and incubated overnight at 4°C with the appropriate primary antibody diluted in 3% TBS-milk on a rocking platform. The following antibodies were used at the specified concentrations for Western blotting: anti-Kaiso polyclonal antibody (1:10,000), anti-p120 15D2 monoclonal antibody (1:2000, 1.0 μg/μl), anti-β-catenin polyclonal antibody (1:1000, 0.5 μg/μg), and anti-E-cadherin monoclonal antibody (1:500, 0.25 μg/μl). The next day the membranes were incubated for an additional 2 hours at room temperature before washing 5 times quickly with deionized water, and once with TBS (pH 7.4) for 5 minutes. Membranes were then incubated with the appropriate peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at a 1:40,000 dilution in 3% TBS-milk at room

temperature for 2 hours, washed as described above, and processed with ECL solution (Amersham) according to the manufacturer's protocol.

2.3. Immunofluorescence

Cells were grown to 60 - 80% confluency on 22 mm cover slips (Fisher Scientific) in 6-well dishes. The cells were washed once with 2 ml cold PBS and then permeabilized and fixed with 1 ml 100% cold methanol at -20°C for 10 minutes. After fixing, cells were washed twice with 2 ml cold PBS and then blocked with a 3% milk-PBS mixture at room temperature. The cells were then stained with the appropriate primary antibody diluted in 3% milk-PBS for 30 minutes at room temperature. The antibodies used were as follows: Kaiso 12G monoclonal antibody (1:200 dilution), p120 F1 rabbit polyclonal antibody (1:600 dilution), E-cadherin monoclonal antibody (1:200 dilution), or a negative control (3% milk-PBS alone). Following incubation in primary antibody the cells were washed 3 times with PBS and then quickly incubated with the 3% milk-PBS mixture. Cells were subsequently incubated with a secondary antibody-milk mixture for 30 minutes at room temperature in the dark. Alexa594-conjugated anti-mouse IgG (Molecular Probes) and Alexa488-conjugated anti-rabbit IgG (Molecular Probes) were used at a 1:300 dilution. 10 μg/ml Hoechst DNA staining reagent (Sigma) was also added to the secondary antibody-milk mixture at a 1:20 dilution. After incubation with secondary antibody the cells were washed as described above. The cover slips were then mounted on slides with Poly-Aquamount (PolyScience) and the cells were imaged using a Zeiss Axiovert fluorescent microscope.

2.4. Serum Stimulation and Starvation of Cells

Prior to chromatin immunoprecipitation, cells were subjected to conditions of serum stimulation or serum starvation. 24 hours prior to crosslinking, cells at ~70% to 80% confluency were washed once with PBS and then replenished with fresh media. For conditions of serum stimulation, the media contained all supplements including Fetal Bovine Serum or Horse Serum, and for conditions of serum starvation the media contained all supplements but lacked Fetal Bovine Serum or Horse Serum. The cells were then incubated at 37°C in a 5% CO_2 humidified incubator for 24 hours until the initiation of chromatin immunoprecipitation.

2.5. Chromatin Immunoprecipitation

MCF-12A, MDA-468, MCF-7 and MDA-231 cells were grown to 90% confluency on 100 mm tissue culture dishes. Cells were then incubated for 1 hour at room temperature in their growth medium with 1% formaldehyde to crosslink protein/DNA complexes. The crosslinking reaction was then stopped with 0.125 M glycine, after which cells were washed twice with cold 1X PBS and harvested into tubes in PBS-PMSF. The cells were pelleted at 5000 rpm at 4°C for 5 minutes, resuspended in Cell Lysis Solution (5 mM PIPES pH 8, 85 mM KCl, 0.5% NP40), and incubated on ice for 10 minutes. The nuclei were then pelletted at 5000 rpm for 5 minutes and the supernatant removed. The nuclear pellet was lysed in Nuclei Lysis Buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) on ice for ten minutes followed by sonication to break

chromatin into 600 bp fragments. The sonicated samples were centrifuged at 14000 rpm for 10 minutes at 4°C and the supernatants were precleared with equilibrated Protein-A-Sepharose beads. Precleared lysates were incubated overnight with 4 μg of each antibody at 4°C.

The next day immune complexes were captured by Rabbit-Anti-Mouse IgG-conjugated Protein-A-Sepharose beads for one hour and subsequently washed 3 times in IP Wash Buffer (100 mM Tris-Cl pH 8, 500 mM LiCl, 1% NP40, 1% Deoxycholic acid). The antibody-antigen-promoter complexes were eluted once by adding 30 μl of IP Elution Buffer (50 mM $NaHCO_3$, 1% SDS) to each sample, followed by light vortexing for 30 minutes. A second elution was performed using 30 μl of IP Elution Buffer and light vortexing for 15 minutes, and eluates were combined into one tube. Each sample was then treated with RNase A at 67°C for 5 hours to remove RNA and to reverse formaldehyde crosslinks, followed by ethanol precipitation. The precipitated products were treated with Proteinase K at 45°C for 2 hours followed by two rounds of phenol-chloroform extraction and ethanol precipitation. The DNA pellets were resuspended in 10 μl of dH_20 and 2 μl of this volume was used for PCR analysis. 10% of input from genomic DNA (not treated with antibody) was used as a positive control in the PCR reaction. The following primers specific for the region flanking the KBS at position -1118 within the human cyclin D1 promoter were used for PCR: 5'-TTT ACA TCT GCT TAA GTT TGC G-3' (forward) and 5'-TTA GAA TTT GCC CTG GGA CT-3' (reverse).

2.6. Plasmid Constructs

Glutathione-S-transferase (GST)-Kaiso fusion proteins were generated using conventional sub-cloning methods. Human Kaiso cDNA was amplified by PCR using Pfu polymerase or Pfu turbo (Fermentas) according to manufacturer's instructions. The primer sets used and resulting constructs are outlined in Table 1. PCR products were digested 5' with *BamHI* and 3' with *EcoRI* and ligated in-frame with the GST gene of the pGEX-5X-1 vector previously digested with the same restriction enzymes. All clones were verified by sequencing by the Mobix facility (McMaster University), after which they were transformed into chemically competent BL21 *E.coli* for subsequent protein expression induction and purification.

Table 1: Generation of GST-hKaiso constructs.

(A) All primers were 30 nucleotides long. *BamHI* recognition sites are depicted in red underlined font, while *EcoRI* recognition sites are depicted in blue underlined font.

Mobix ID	Primer Name	Sequence
ML13873	GST-hKaiso-for	5' CTA GTC GGG ATC CCC ATG GAG AGT AGA AAA 3'
ML13874	GST-hKaiso-rev	5' CTA GTC GAA TTC TTA GTA AGA CTC TGG TAT 3'
ML13875	GST-hΔPOZ-for	5' CTA GTC GGG ATC CCC GAG CTT GGT GTC CCA 3'
ML13876	GST-hΔZF-rev	5' CTA GTC GAA TTC TTA GAC ATA TGA CCT TTT 3'
ML13877	GST-hZF-for	5' CTA GTC GGG ATC CCC ATA GTA GAT GGA AGG 3'
ML13878	GST-hZF123-rev	5' CTA GTC GAA TTC TTA AAG CTT TGA GTC CCC 3'
ML14723	GST-ΔAR1-for	5' CTA GTC GGG ATC CCC GTC ATT TTT TGC TCC 3'
ML14724	GST-ΔAR2-for	5' CTA GTC GGG ATC CCC ACT ATT AGC TCC AGT 3'
ML15096	GST-hZF-for-2	5' CTA GTC GGG ATC CCC GGG GAG GCC AGA CTT 3'

(B) A panel of GST-hKaiso fusion constructs were generated using combinations of the above primers as outlined below.

Construct Name	Forward Primer	Reverse Primer	Nucleotides Amplified
GST-hKaiso	GST-hKaiso-for	GST-hKaiso-rev	1 – 2109 (2109 bp)
GST-hΔPOZ	GST-hΔPOZ-for	GST-hKaiso-rev	342 – 2019 (1677 bp)
GST-hΔPOZΔZF	GST-hΔPOZ-for	GST-hΔZF-rev	342 – 1512 (1170 bp)
GST-hcterm	GST-hZF-for	GST-hKaiso-rev	1458 – 2019 (561 bp)
GST-hZF123	GST-hZF-for	GST-hZF123-rev	1458 – 1749 (291 bp)
GST-hΔAR1	GST-ΔAR1-for	GST-hKaiso-rev	582 – 2019 (1437 bp)
GST-hΔAR2	GST-ΔAR2-for	GST-hKaiso-rev	1021 – 2019 (998 bp)
GST-hZF	GST-hZF-for-2	GST-hKaiso-rev	1362 – 2019 (657 bp)

2.7. Protein Expression Induction and Purification

BL21 bacteria containing various pGEX-5X-1 GST-Kaiso fusion constructs were incubated at 37°C for 16 hours overnight in 12 mL of LB media treated with Ampicillin (LB-Amp). The next morning 90 mL of LB-Amp media was inoculated with 10 mL of the overnight culture and incubated at 30°C on a shaking platform for 90 minutes. The cultures were then induced to express GST-Kaiso fusion protein using 0.1 mM IPTG, and were incubated for an additional 3 hours at 30°C. The bacteria were pelleted by centrifugation at 6000 rpm for 15 minutes at 4°C, and frozen overnight.

The next morning, the frozen bacterial pellets were thawed on ice and then resuspended in ice-cold PBS/0.1% NP-40. The cells were lysed by sonication for 3 minutes at 40% output and duty cycle 4. Sonicated lysates were cleared by centrifugation at 10,000 rpm for 20 minutes at 4°C, after which GST beads were added to the lysates and the mixture incubated with rotation at room temperature for one hour. The protein-bead complexes were captured and washed twice with 1X binding buffer (25 mM Hepes (pH 7.5), 100 mM KCL, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% NP-40, 5% glycerol, 1mM DTT). Proteins were eluted from the beads using 10 mM glutathione and quantitated prior to use in electrophoretic mobility shift assays (EMSA).

2.8. Electrophoretic Mobility Shift Assays

30-mer oligonucleotides derived from the *cyclin D1* promoter as outlined in Table 2, were annealed to create double-stranded probes and purified from a polyacrylamide gel. 50 ng of double-stranded oligonucleotides were radiolabelled by incubation with

1 μl of γ^{32}P dATP, 1μl of polynucleotide kinase buffer, 1 μl of polynucleotide kinase (New England Biolabs), and dH_20 to give a final volume of 10 μl. The reaction was incubated at 37°C for 45 minutes, terminated with 25 mM EDTA, and then purified through a Chromaspin TE-10 column (Clontech). For methylation-specific experiments, CpG-methylated probes were generated by first incubating the double-stranded oligonucleotides with 1 μl of Sss.I methylase, 1 μl of NEB buffer 2, and 160 mM S-adenosyl-methionine (SAM) at 37°C for 45 minutes. The enzyme was subsequently heat-inactivated at 65°C for 15 minutes prior to probe radiolabelling. 1 μl of labelled probe was mixed with 3 μg poly dI-DC, 5 μg BSA, 4.8% glycerol, 5 μl binding buffer (25 mM HEPES, pH 7.5, 100 mM KCL, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% NP-40, 5% glycerol, and 1 mM DTT), and 150 ng to 250 ng of purified GST-Kaiso protein. This binding reaction was incubated at 37°C for 25 minutes and then incubated on ice for 30 minutes. The samples were loaded onto a 4% non-denaturing poly-acrylamide gel (0.5X TBE, 2.5% glycerol, 4% acrylamide, 0.06% APS, and Temed), and electrophoresed at 200V for $2^1/_2$ hours. The gel was then dried at 80°C for 1 hour and visualized by autoradiography.

Table 2: *cyclin D1* promoter-derived oligomers used for electrophoretic mobility shift assays.

(A) *cyclin D1* promoter –1118 KBS: **K**aiso **b**inding **s**ites (KBS) are underlined, and mutant nucleotides are depicted in bold underlined font.

CD1-1118 KBS	**Corresponding *cyclin D1*-derived probes**
Wild type TCCTGCCA	Top strand (ML5102): 5' TTA TGC CGG C*TC CTG CCA* GCC CCC TCA CGC 3' Bottom strand (ML5103): 5' GCG TGA GGG GGC *TGG CAG GA*G CCG GCA TAA 3'
Mutant 1 TC**A**TGCCA	Top Strand (ML5104): 5' TTA TGC CGG C*TC ATG CCA* GCC CCC TCA CGC 3' Bottom strand (ML5105): 5' GCG TGA GGG GGC *TGG CAT GA*G CCG GCA TAA 3'
Mutant 2 TCCT**TT**CA	Top strand (ML9065): 5' TTA TGC CGG C*TC CTT TCA* GCC CCC TCA CGC 3' Bottom strand (ML9066): 5' GCG TGA GGG GGC *TGA AAG GA*G CCG GCA TAA 3'
Mutant 3 TC**A**AT**TA**CA	Top strand (ML14543): 5' TTA TGC CGG C*TC ATT ACA* GCC CCC TCA CGC 3' Bottom strand (ML14544): 5' GCG TGA GGG GGC *TGT AAT GA*G CCG GCA TAA 3'

Table 2: ***cyclin D1*** **promoter-derived oligomers used for electrophoretic mobility shift assays.**

(B) ***cyclin D1*** **promoter +24 KBS:** **K**aiso **b**inding **s**ites (KBS) are underlined, and mutant nucleotides are depicted in bold underlined font.

CD1+24 KBS	**Corresponding *cyclin D1*-derived probes**
Wild type CTGCTA	Top strand (ML7934): 5' CTG TCG GCG CAG *TAG CAG* CGA GCA GCA GAC 3' Bottom strand (ML7935): 5' GTC TGC TGC TCG *CTG CTA* CTG CTG CGC CGA CAG 3'
Mutant 1 **A**TGCTA	Top strand (ML7936): 5' CTG TCG GCG CAG *TAG CAT* CGA GCA GCA GAC 3' Bottom strand (ML7937): 5' GTC TGC TGC TCG *ATG CTA* CTG CTG CGC CGA CAG 3'
Mutant 2 CT**T**CTA	Top strand (ML8632): 5' CTG TCG GCG CAG *TAG AAG* CGA GCA GCA GAC 3' Bottom strand (ML8633): 5' GTC TGC TGC TCG *CTT CTA* CTG CTG CGC CGA CAG 3'
Mutant 3 CT**TT**TA	Top strand (ML9063): 5' CTG TCG GCG CAG *TAA AAG* CGA GCA GCA GAC 3' Bottom strand (ML9064): 5' GTC TGC TGC TCG *CTT TTA* CTG CTG CGC CGA CAG 3'
Mutant 4 **A**T**TT**TA	Top strand (ML14545): 5' CTG TCG GCG CAG *TAA AAT* CGA GCA GCA GAC 3' Bottom strand (ML14546): 5' GTC TGC TGC TCG *ATT TTA* CTG CTG CGC CGA CAG 3'

2.9. Site-directed mutagenesis

To generate *cyclin D1* promoter KBS point mutant constructs for use in artificial promoter assays the pGL3 Basic-1748CD1 construct (a kind gift of Dr. Osamu Tetsu, USCF) was used as a template for site-directed mutagenesis of the CD1–1118 KBS and the CD1+24 KBS, using the Quikchange site-directed mutagenesis kit according to manufacturer's protocol (Stratagene). The two KBS elements located in the 1748CD1 construct were mutated as outlined in Table 3. After verifying by sequencing that the mutations had been successfully incorporated, the mutant sites were subcloned back into the wild type backbone vector to generate single and double KBS point mutant constructs for subsequent use in artificial promoter assays, as described in Table 4.

Table 3: Site-directed mutagenesis of *cyclin D1* promoter KBS elements.

(A) CD1-1118 site directed mutagenesis primers: **K**aiso **b**inding **s**ites (KBS) are underlined, and mutant nucleotides are depicted in bold underlined font.

Mutation Desired	**Primers used to generate point mutation(s) in plasmid DNA**
TCCTGCCA ↓ TC**A**TGCCA	Forward primer (ML6694): 5' GAA TTA TGC CGG C*TC ATG CCA* GGCC CCC TCA CG 3' Reverse primer (ML6695): 5' CG TGA GGG GGC *TGG CAT GA*G CCG GCA TAA TTC 3'
TCCTGCCA ↓ TC**A**T**TA**CA	Forward primer (ML13784): 5' CTG AAT TAT GCC GGC *TCA TTA CA*G CCC CCT CAC GCT C 3' Reverse primer (ML13785): 5' GAG CGT GAG GGG GC*T GTA ATG A*GC CGG CAT AAT TCA G 3'

(B) CD1+24 site directed mutagenesis primers: **K**aiso **b**inding **s**ites (KBS) are underlined, and mutant nucleotides are depicted in bold underlined font.

Mutation Desired	**Primers used to generate point mutation(s) in plasmid DNA**
CTGCTA ↓ **A**TGCTA	Forward primer (ML6692): 5' GCT GTC GGC GCA G*TA GCA T*CG AGC AGC AGA GTC CG 3' Reverse primer (ML6693): 5' CG GAC TCT GCT GCT CG*A TGC TAC* TGC GCC GAC AGC 3'
CTGCTA ↓ **A**T**TT**TA	Forward primer (ML13786): 5' GCT GTC GGC GCA G*TA AAA T*CG AGC AGC AGA GTC CG 3' Reverse primer (ML 13787): 5' CGG ACT CTG CTG CTC G*AT TTT A*CT GCG CCG ACA GC 3'

2.10. Luciferase Assays

24 hours prior to transfection, cells were seeded onto 6-well dishes at 60% confluency, and incubated overnight (37°C, 5% CO_2). All transfections were performed using the ExGen-500 reagent (MBI Fermentas). For each transfection, plasmid DNA was diluted in 150 mM NaCl, ExGen-500 was added to the DNA-NaCl solution, and the mixture was gently vortexed. All transfections were performed using 0.2 μg of reporter construct DNA and up to 1 μg of effector DNA, as outlined in Table 4, with the addition of 3 μl of ExGen-500 per 1 μg of DNA. The mixture was incubated without agitation at RT for 10 minutes to allow reagent/DNA complex formation, before its addition to the cells in supplemented DMEM. The cells were incubated with the DNA complexes for 5 hours at 37°C, 5% CO_2, after which the transfection mixture was aspirated, and the cells were replenished with fresh media. The cells were then incubated for an additional 25 hours in supplemented DMEM medium before initiation of the assay.

30 hours post-transfection, each well was washed twice with PBS, treated with 350 μL of **P**assive **L**ysis **B**uffer (PLB) (Promega) and the plates were incubated at room temperature for 20 minutes with vigorous shaking. Lysates were resuspended by pipetting, after which 50 μL of lysate from each well was assayed using 100 μL of Luciferase Assay Reagent (Promega) for artificial promoter activity on a luminometer (Fisher Scientific). Each experimental condition was performed in triplicate, and the results displayed are representative of at least three separate experimental trials.

Table 4: Reporters and effectors used in artificial promoter assays.

(A) Reporters:

Construct Name	Characteristics
pGL3 Basic 1748CD1	~2kb fragment of the wild type human *cyclin D1* promoter containing nucleotides –1748 to +134 with respect to the transcriptional start site. Contains two KBS.
pGL3 Basic 962CD1	~1.1kb fragment of the wild type human *cyclin D1* promoter containing nucleotides –962 to +134 with respect to the transcriptional start site. Contains one KBS.
1MK2MK-CD1	A KBS double mutant construct derived from 1748CD1 in which both *cyclin D1* promoter KBS contain single nucleotide mutations. CD1-1118 (TCCTGCCA → TC**A**TGCCA), CD1+24 (CTGCTA → **A**TGCTA).
KBS(1,2)mt-CD1	A KBS double mutant construct derived from 1748CD1 in which both *cyclin D1* promoter KBS contain triple nucleotide mutations. CD1-1118 (TCCTGCCA → TC**A**T**TA**CA), CD1+24 (CTGCTA → **ATTT**TA).

(B) Effectors:

Effector Name	Purpose
pcDNA3-hKaiso	Overexpression of human Kaiso
pRc/RSV-p120 1A	Overexpression of murine p120^{ctn} isoform 1A
pCAN-ΔN89 β-catenin	Overexpression of constitutively active human β-catenin
pSilencer siRNA Kaiso 544	Kaiso mRNA depletion via nucleotides 544 to562

2.11. Semi-quantitative RT-PCR.

HeLa (cervical carcinoma) and MCF-7 (breast carcinoma) cells were used for these preliminary semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) experiments. Cells were seeded onto 100 mm tissue culture dishes 24 hours prior to transfection to reach a low confluency (30-40%) at the time of transfection. The cells were transfected with 6 μg of pcDNA3-hKaiso overexpression or siRNA Kaiso 544 depletion vectors incubated at 37°C for 6 hours before transfection complexes were replaced with fresh media. HeLa cells were transfected with the ExGen-500 transfection reagent, while MCF-7 cells were transfected with the Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer instructions. 24 hours post-transfection when the cells had reached 50-60% confluency, whole cell RNA was harvested from each plate of cells using the RNEasy Kit (Qiagen) according to manufacturer protocol. The isolated RNA was then utilized for one-step semi-quantitative RT-PCR to analyse the effects of Kaiso misexpression on *cyclin D1* transcript levels. Briefly, 0.25 to 1.0 μg of RNA was utilized in each experiment, in which specific primers amplified Kaiso, cyclin D1, and GAPDH first strand synthesis cDNA products.

The primer sequences used to amplify the mRNA products are as follows: Kaiso forward 5'-TGC CTA TTA TAA CAG AGT CTT T-3', Kaiso reverse 5'-AGT AGG TGT GAT ATT TGT TAA AG-3', cyclin D1 forward 5'TCA ATT GTG TGC AGA AGG AGG-3', cyclin D1 reverse 5'-GGA AGC GGT CCA GGT AGT TCA-3', GAPDH forward 5'-GAAGGTGAAGGTCGGAGT-3', GAPDH reverse

5'-GAAGATGGTGATGGGATTTC-3'. Kaiso overexpression and depletion were verified by immunoprecipitation and Western blot.

Results

3.1. Kaiso, p120, and E-cadherin are differentially expressed in breast cell lines.

Kaiso, p120, and E-cadherin expression have been characterized by us and other groups in various epithelial and fibroblast cell lines. However, our recent interest in Kaiso's potential role in breast tumourigenesis led us to further examine Kaiso, p120, and E-cadherin expression profiles specifically in breast epithelial tumour and non-tumour cell lines. Whole cell lysates from a panel of breast tumour (MCF-7, MDA-468, T47D, MDA-231, and SK-BR-3) and non-tumour (MCF-12A and MCF-10A) cell lines were examined for Kaiso, p120, and E-cadherin protein expression by immunoprecipitation and Western Blot analysis. Prior to immunoprecipitation all lysates were standardized to equal protein content.

Kaiso migrates as a 100 kDa phosphoprotein doublet in all the cell lines tested (Figure 8, top panel). Previous studies from our laboratory demonstrated that the slower migrating Kaiso band represents a phosphorylated form of Kaiso (Daniel lab, unpublished data). In MCF-7 and SK-BR-3 cells, Kaiso is detected at a level that is approximately 2-fold higher than the other cell lines tested, which generally express Kaiso at low levels. Interestingly, Kaiso expression was difficult to detect in MCF-12A cells.

To analyze the p120 expression profile, immunoprecipitation and Western blot analyses were performed using the p120-specific monoclonal antibody 15D2. This

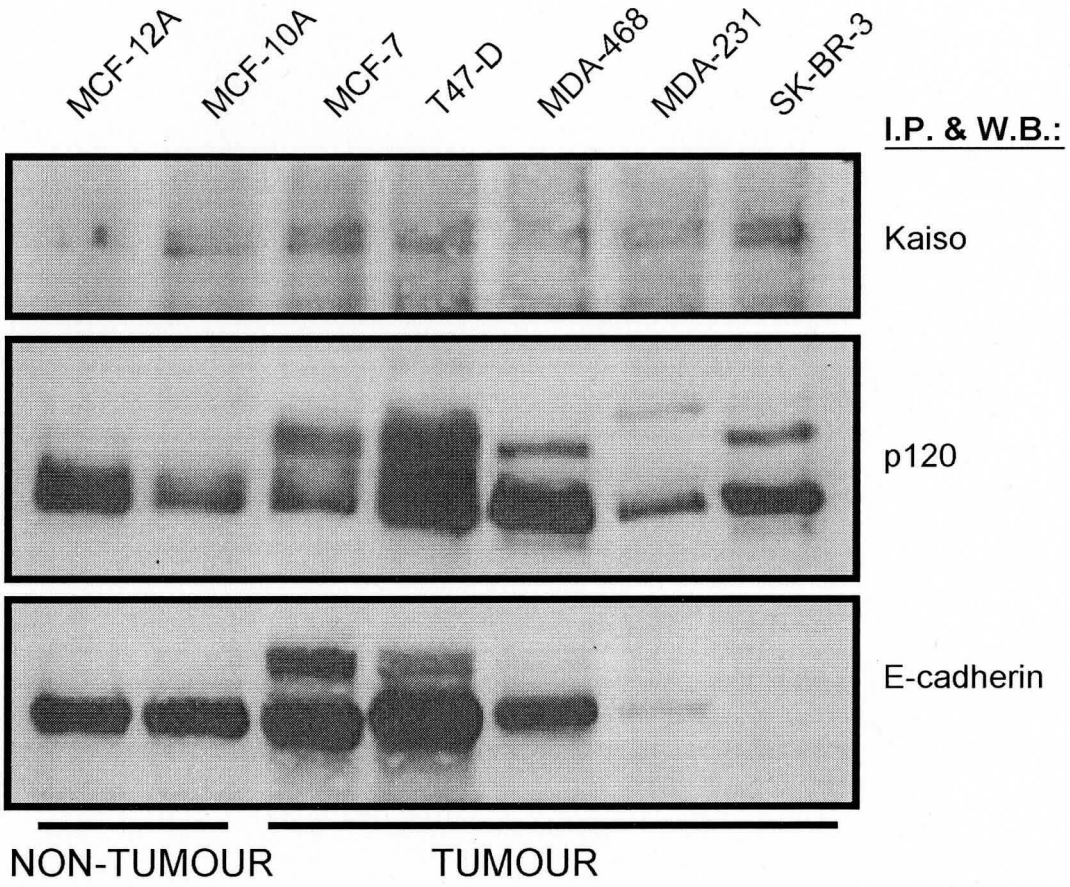


Figure 8: Characterization of Kaiso, p120, and E-cadherin expression in breast epithelial cell lines. Immunoprecipitation and Western Blot analyses were performed on whole cell lysates from a panel of breast tumour and non tumour cell lines to determine the protein expression profiles of Kaiso, p120, and E-cadherin. Kaiso migrates as a 100 kDa phosphoprotein doublet in all the cell lines tested. However, in the tumour cell lines, p120 is mainly expressed as two phosphorylated isoforms, isoform 1 (slowly migrating band) and isoform 3 (quickly migrating band), while the non-tumour cell lines express mainly isoform 3 of p120. E-cadherin is expressed in all of the tested cell lines except for MDA-231 and SK-BR-3.

monoclonal antibody recognizes all known isoforms of p120 (135), and is thus an ideal tool to assay for p120 expression. In the tumour cell lines tested, p120 is mainly expressed as two phosphorylated isoforms, isoform 1 (slowly migrating band in Figure 8, centre panel) and isoform 3 (faster migrating band in Figure 8, centre panel). The non-tumour cell lines, however, express mainly isoform 3 of p120. The thick, blurry banding observed with the majority of the p120 isoforms on SDS-PAGE has been previously demonstrated to be representative of p120 phosphorylation (127).

E-cadherin is expressed in all of the tested cell lines except for MDA-231 and SK-BR-3 (Figure 8, bottom panel). Interestingly, E-cadherin migrates as a doublet in MCF-7 and T47-D cells, which may be due to incompletely processed or glycosylated E-cadherin molecules.

3.2. Subcellular localization of Kaiso and p120 in different cell lines.

Immunofluoresence analysis was performed on the non-tumour MCF-12A and MCF-10A, and tumour-derived SK-BR-3 cells to investigate the subcellular compartments in which Kaiso and p120 are localized. In all three cell lines, Kaiso localized predominantly to nuclei (Figure 9), however some cytoplasmic Kaiso was also detected in SK-BR-3 cells (Figure 9C). In contrast, p120 demonstrated cell-line specific localization patterns. In MCF-12A cells, p120 localized predominantly to cell-cell junctions (Figure 9A). In MCF-10A cells, p120 was observed at cell-cell junctions as well as throughout the cytoplasm (Figure 9B), whereas p120 was observed mostly in the cytoplasm and somewhat in the nucleus in SK-BR-3 cells (Figure 9C).

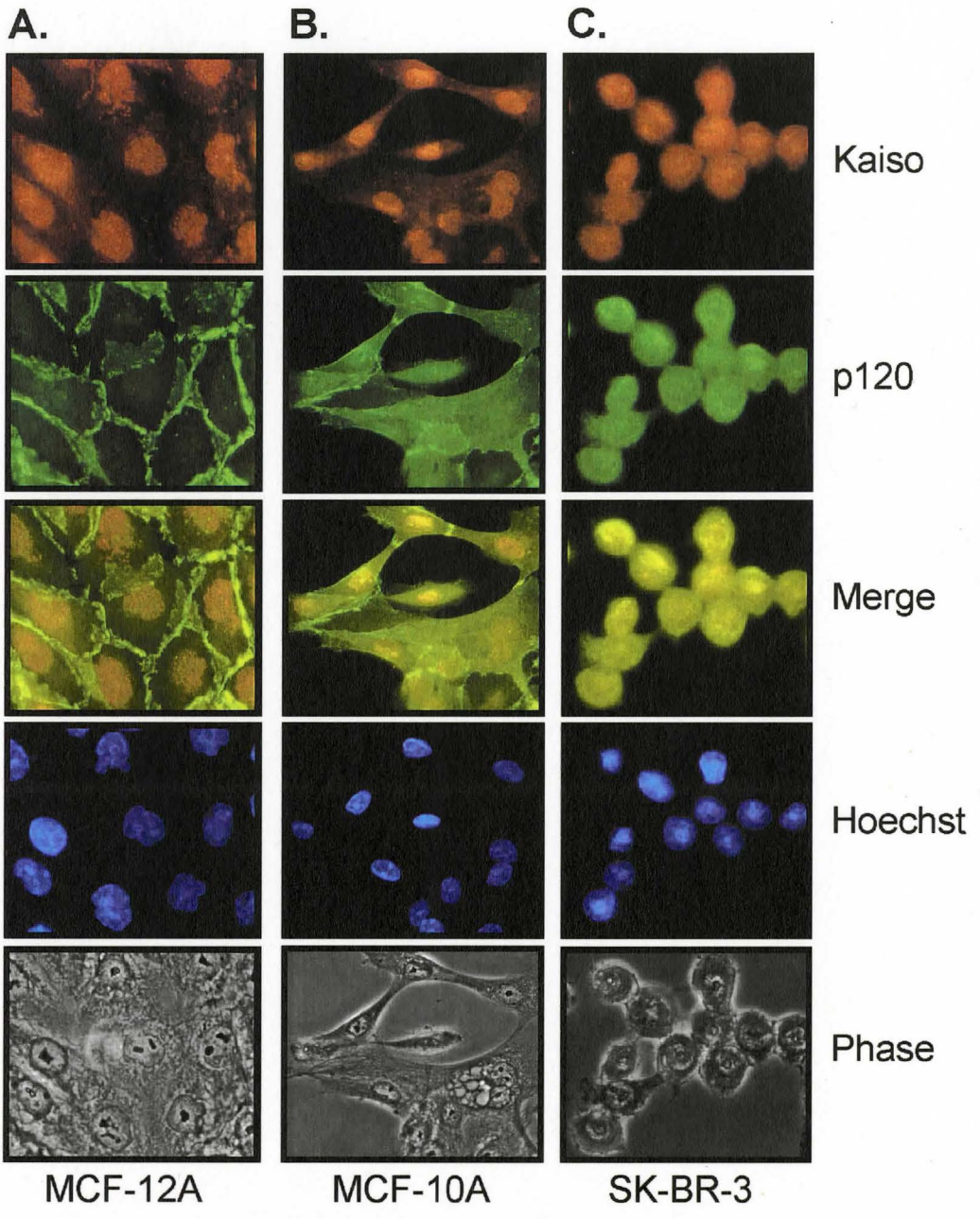


Figure 9: Kaiso and p120 sub-cellular localization. In all the cell lines tested, Kaiso localized predominantly to cell nuclei. p120, however, demonstrated cell-line specific localization patterns. In the MCF-12A cell line, p120 was observed to localize predominantly to cell-cell junctions. In MCF-10A cells, p120 was observed at cell-cell junctions as well as throughout the cytoplasm. In SK-BR-3 cells, p120 was observed mostly in the cytoplasm and somewhat in the nucleus.

3.3. Kaiso and p120 interact in breast cell lines independent of E-cadherin expression.

To determine if an interaction between Kaiso and p120 could be detected in our panel of breast cancer cell lines, co-immunoprecipitation and Western blot analyses were utilized. Using this methodology we also wanted to determine if a correlation existed between the p120/Kaiso interaction and E-cadherin expression. These analyses were performed only in MCF-12A, MCF-10A (non tumour) and SK-BR-3 (tumour) cells since similar analyses were previously done with the breast tumour cell lines (MCF-7, T47-D, MDA-468, and MDA-231), in which p120/Kaiso interaction was successfully detected (84). Kaiso protein was detected in p120 immunocomplexes from MCF-10A and SK-BR-3 cells (Figure 10, compare top and bottom panels) while Kaiso protein could not be detected in p120 immunocomplexes from MCF-12A cells (Figure 10, compare top and bottom panels). Moreover, in MCF-10A cells, it appeared that primarily unphosphorylated Kaiso coprecipitated with p120, but this was never further verified.

Interestingly, the p120/Kaiso interaction was observed irrespective of E-cadherin expression. An interaction was detected in MDA-231 and SK-BR-3 cells, which lack E-cadherin, but not in MCF-12A cells, which express E-cadherin. The other tumour cell lines in which we observed a p120/Kaiso interaction all express E-cadherin. The lack of correlation between the p120/Kaiso interaction and E-cadherin expression in these breast cell lines led us to search for other factors that may ultimately influence Kaiso-p120 interaction in these cell lines.

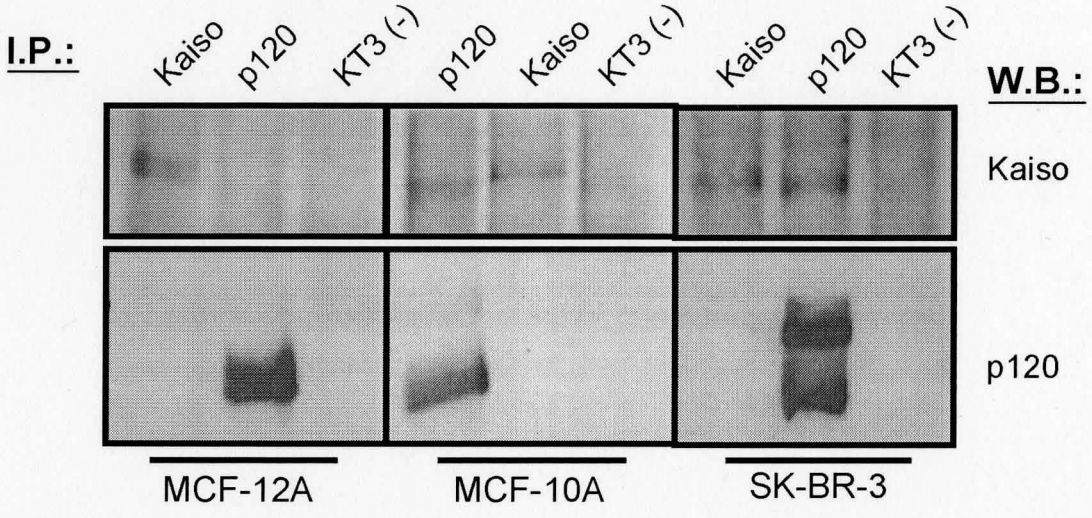


Figure 10: Characterization of Kaiso and p120 interaction by immunoprecipiation. The p120/Kaiso interaction is observed in two of the three cell lines tested. To test for specificity of observed co-precipitations, the KT3 unrelated antibody was used as a negative control during immunoprecipitation. Note that the interaction is only detected in one direction, i.e. p120 co-precipitates Kaiso but not vice-versa.

Through literature searches I discovered that cyclin D1 protein is expressed at normal levels in MCF-12A (non-tumour) and MCF-7 and MDA-231 (tumour cells) (Table 5). However cyclin D1 protein is expressed at low levels in MDA-468 tumour cells (Table 5). Kaiso, E-cadherin, and Cyclin D1 expression profiles, and tumourigenicty characteristics of the MCF-12A (non tumour) and MCF-7, MDA-468, and MDA-231 (tumour) cell lines are summarized in (Table 5); however, there does not appear to be any obvious correlation between the p120/Kaiso interaction and these parameters. Nevertheless, these four cell lines were selected to continue in further experiments to specifically investigate how Kaiso may affect *cyclin D1* regulation in the context of breast tumourigenesis.

Table 5: Comparison of E-cadherin and cyclin D1 expression in selected breast tumour and non-tumour epithelial cell lines. A literature search was carried out to determine the expression levels of cyclin D1 protein in selected breast cell lines. Cyclin D1 is expressed at normal levels in MCF-12A, MCF-7, and MDA-231 cells and is expressed at low levels in MDA-468 cells. This information was coupled with our Kaiso and E-cadherin expression data and cell line tumourigenicity information obtained from ATCC to create a profile for each cell line.

Name	Tumourigenicity	Kaiso Expression	E-cadherin Expression	Cyclin D1 expression
MCF-12A	Non Tumour	Low	Yes	Normal
MDA-468	Tumour	Normal	Yes	Low
MDA-231	Tumour	Normal	No	Normal
MCF-7	Tumour	High	Yes	Normal

3.4. Kaiso binds the cyclin D1 promoter in vivo in a growth factor-dependent manner.

MCF-12A (non tumour), MCF-7, MDA-468, and MDA-231 (tumour) breast epithelial cells were chosen to determine if Kaiso associates with the *cyclin D1* promoter *in vivo*. Chromatin immunoprecipitation (ChIP) with Kaiso-specific antibodies was performed, followed by PCR amplification of the *cyclin D1* promoter region flanking the CD1–1118 KBS located within the promoter. We initially chose to focus on this KBS because it contains the entire 8-nucleotide KBS sequence, and we hypothesized that this KBS might have a more significant role in *cyclin D1* regulation. The CD1+24 KBS contains only the core 6-nucleotide consensus, thus leading us to speculate that Kaiso may not bind as efficiently to this sequence.

Our analyses demonstrated that Kaiso specifically binds the *cyclin D1* promoter *in vivo* in all four cell lines tested, but only under specific conditions of serum stimulation or starvation (Figure 11). In MCF-12A and MDA-468 cells, Kaiso association with the *cyclin D1* promoter can only be detected if the cells have been serum starved for at least 24 hours. Conversely, in MCF-7 and MDA-231 cells, Kaiso association with the *cyclin D1* promoter can only be detected in cells that have been propagated in serum-supplemented media. The findings are summarized in Table 6 alongside each cell line profile. This result suggests that Kaiso's association with the *cyclin D1* promoter is dependent upon the presence and/or absence of mitogen stimulation, which in turn could be dependent upon unique molecular factors in each cell line. This effect may be direct or perhaps through Kaiso's interactions with other proteins, such as p120, which is phosphorylated upon growth factor stimulation (22, 26).

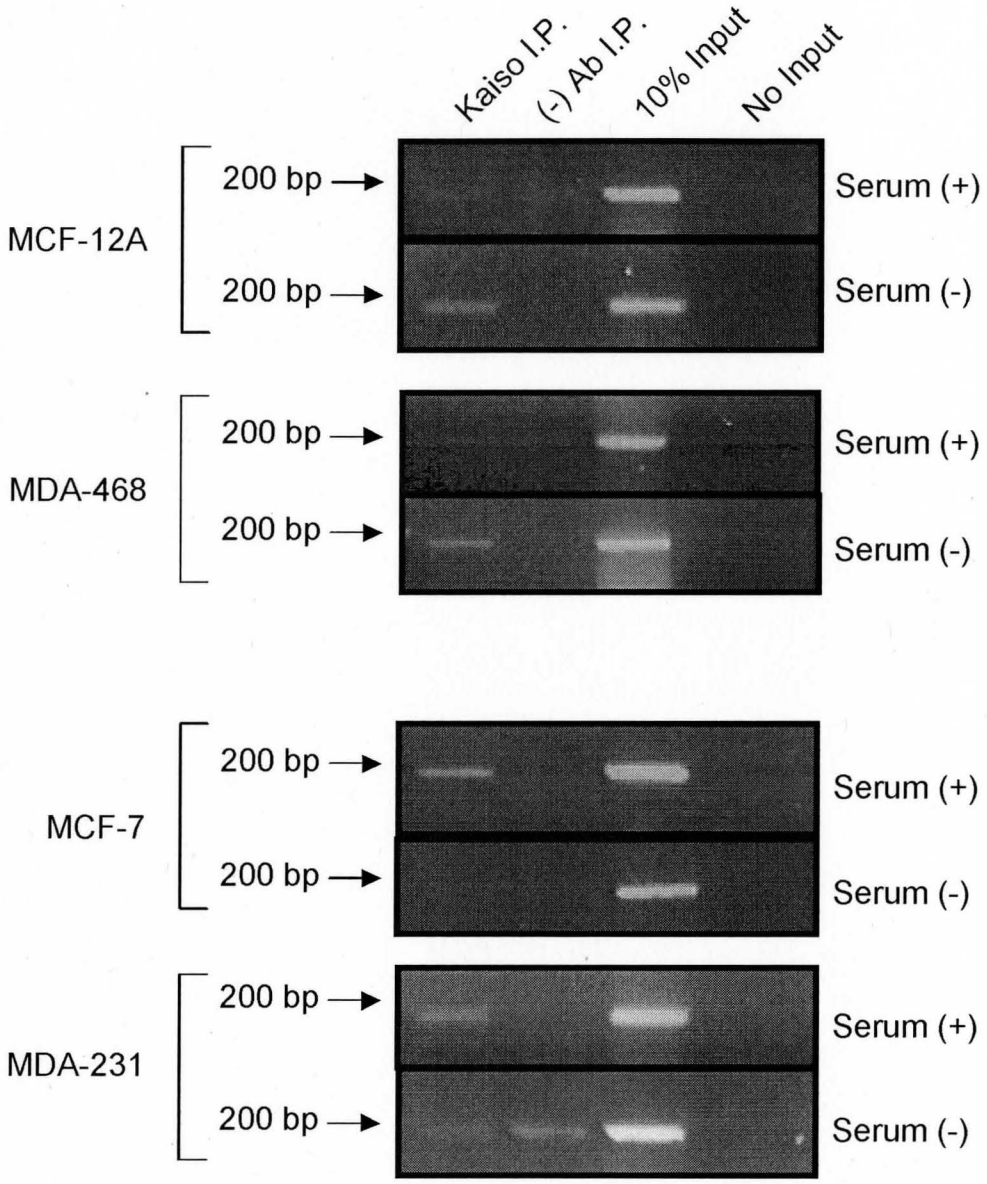


Figure 11: Kaiso associates with the *cyclin D1* promoter *in vivo*. Chromatin immunoprecipitation analysis revealed that *cyclin D1* promoter fragments (200 bp) can be amplified by PCR specifically from Kaiso immunocomplexes in breast tumour and non-tumour cell lines. Interestingly, Kaiso associates with the *cyclin D1* promoter only in conditions of serum-starvation in the MCF-12A (non tumour) and MDA-468 (tumour) cell lines. Conversely Kaiso associates with the *cyclin D1* promoter only in conditions of serum-stimulation in MCF-7 and MDA-231 tumour cell lines.

Table 6: Summary of serum-dependent Kaiso association with the *cyclin D1* promoter. ChIP analyses indicate that Kaiso associates with the *cyclin D1* promoter in selected breast tumour and non-tumour cells, however this is dependent on the presence or absence of serum in cell culture media.

Name	Serum Status (Kaiso promoter binding)	Tumourigenicity	Kaiso Expression	E-cadherin Expression	Cyclin D1 expression
MCF-12A	Serum absent	Non Tumour	Low	Yes	Normal
MDA-468	Serum absent	Tumour	Normal	Yes	Low
MDA-231	Serum present	Tumour	Normal	No	Normal
MCF-7	Serum present	Tumour	High	Yes	Normal

3.5. p120 associates with the cyclin D1 promoter in MCF-7 cells.

Our initial characterization of Kaiso and p120 expression and interaction in our panel of breast tumour and non-tumour cell lines demonstrated a clear and reproducible interaction between Kaiso and p120 in MCF-7 cells (84). I thus proceeded to determine if p120 could also be detected in association with the *cyclin D1* promoter via ChIP assays using the MCF-7 cell line. Although previous experiments from our lab showed that p120 inhibits Kaiso's DNA binding *in vitro*, we predicted that the ChIP initial crosslinking step would preserve any p120/Kaiso complexes that may associate with the *cyclin D1* promoter *in vivo*. The ChIP assays were performed in serum stimulated conditions, as those were the conditions in which Kaiso association with the *cyclin D1* promoter could be detected in MCF-7 cells. We were able to efficiently detect *cyclin D1* promoter fragments from immunocomplexes isolated with the p120-specific antibody 15D2 (Figure 12), indicating that p120, like Kaiso, associates with the *cyclin D1* promoter. To determine if p120 association with the *cyclin D1* promoter was through its interaction with Kaiso, cells were depleted of Kaiso by **s**hort **i**nterfering **RNA** (siRNA) methodologies and subsequently assayed by ChIP for p120 association with the *cyclin D1* promoter. However, these experiments were inconclusive and difficult to interpret (data not shown) therefore additional experiments are required to further characterize p120 association with the *cyclin D1* promoter.

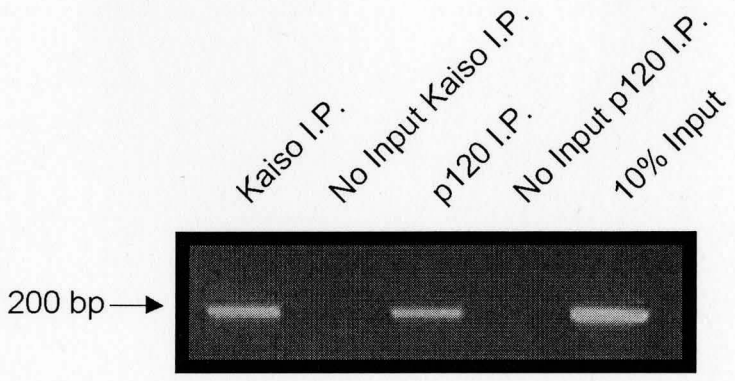


Figure 12: p120 associates with the *cyclin D1* promoter *in vivo*. Chromatin immunoprecipitation experiments were performed in MCF-7 breast tumour cells using p120 and Kaiso-specific antibodies. *Cyclin D1* promoter fragments were efficiently purified and amplified from p120 immunocomplexes, in a similar manner to Kaiso.

3.6. Kaiso binds cyclin D1 promoter-derived probes in vitro.

Following our *in vivo* characterization of Kaiso binding to the *cyclin D1* promoter in breast tumour and non-tumour cell lines, we initiated experiments to further characterize and test the specificity of this binding *in vitro*. Using **e**lectrophoretic **m**obility **s**hift **a**ssays (EMSA) we analyzed the ability for human Kaiso to bind 30-mer oligos derived from the CD1-1118 KBS. We also tested the ability of Kaiso to bind oligos derived from the CD1+24 KBS to determine if this KBS contributed to Kaiso association with the *cyclin D1* promoter. Our analyses were carried out using GST-Kaiso fusion proteins. We chose to utilize POZ deletion mutant proteins in these assays because full-length POZ proteins are rarely used for *in vitro* DNA binding assays due to POZ domain inhibition of *in vitro* protein-DNA interactions (8). The GST-Kaiso fusion proteins that were prepared and purified for use in these assays are as follows: GST-Kaiso-ΔPOZ (hΔPOZ) lacking the N-terminus portion of Kaiso that includes the POZ domain; GST-Kaiso-ΔAR1 and GST-Kaiso-ΔAR2 (hΔAR1 and hΔAR2) which lack the N-terminal portion of Kaiso up to and including the first and second highly conserved **a**cidic **r**egions (AR) respectively; and GST-Kaiso-ZF (hZF) which consists of the C-terminal portion of Kaiso containing the three DNA-binding zinc fingers (Figure 14). GST-only and GST-Kaiso-ΔPOZΔZF (hΔPOZΔZF) proteins were used as negative controls. GST does not possess any intrinsic DNA-binding capabilities and hΔPOZΔZF lacks the POZ and zinc finger domains, which are the two characterized Kaiso functional domains (Figure 14).

Repeated experiments determined that the various human GST-Kaiso fusion proteins bind the fully conserved CD1-1118 KBS, albeit with differing affinities (Figure 15, left panel). The hZF protein exhibited the strongest DNA binding capability while the hΔPOZ protein exhibited the weakest DNA binding ability. A single base pair point mutation in the fully conserved KBS (TCCTGCCA → TCATGCCA) completely abolished GST-Kaiso protein binding to the *cyclin D1* promoter probe (Figure 15). Surprisingly, human Kaiso did not bind the CD1+24 KBS (Figure 16), which only possesses the core consensus sequence. A single base pair point mutation introduced into the KBS of this probe (CTGCTA → ATGCTA) had no additive or diminutive effect to this lack of binding (Figure 16).

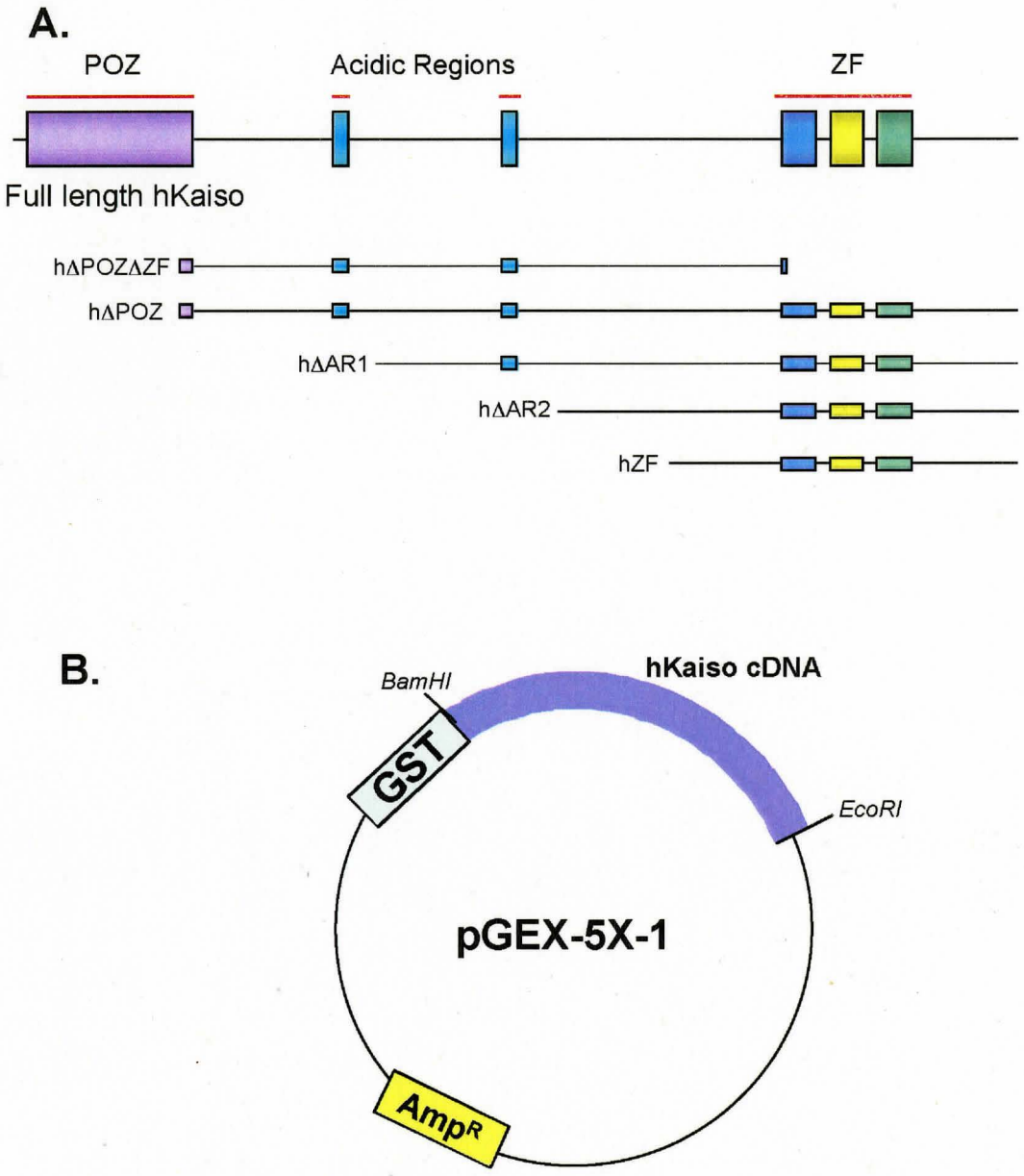


Figure 13: A schematic representation of the human Kaiso deletion mutants fused to GST. (A) A schematic diagram of the human Kaiso cDNA constructs generated depict the Kaiso POZ domain in purple, acidic regions in teal, and the zinc fingers in blue, yellow, and green. (B) All Kaiso fusion constructs were designed to be fused in frame to the C-terminus of GST. The lactose promoter upstream of the GST cDNA allows for efficient protein induction in bacteria following treatment of bacterial cultures with IPTG (a lactose homologue).

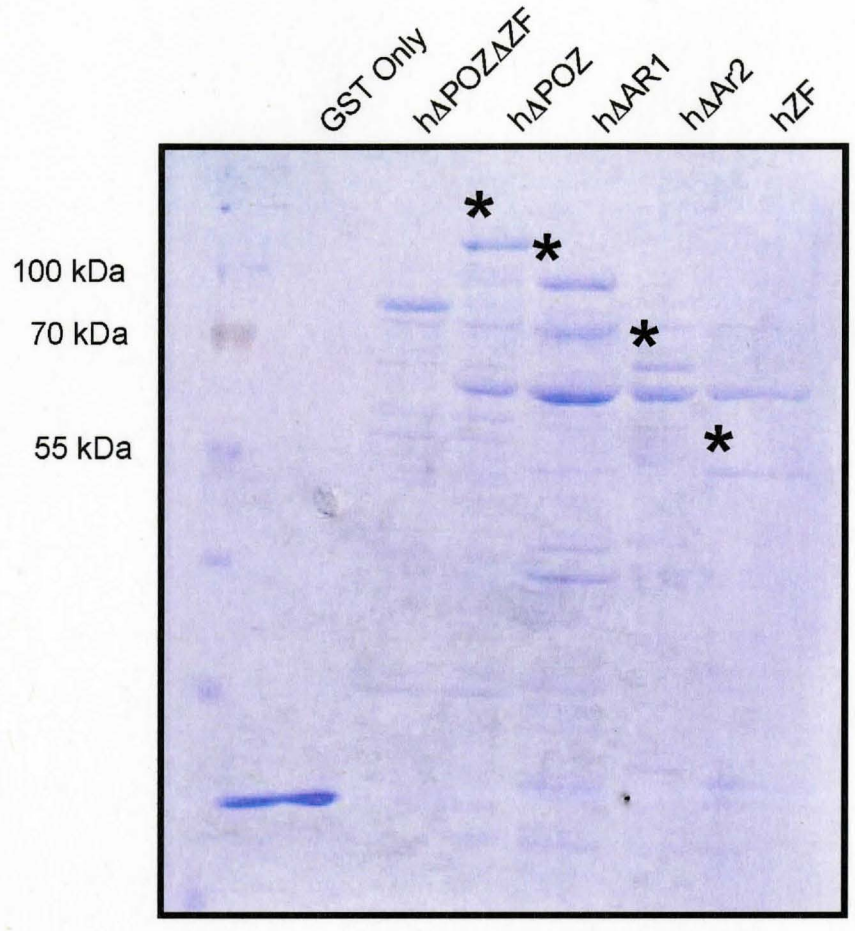


Figure 14: Preparation and purification of GST-hKaiso fusion proteins. GST-hKaiso cDNA constructs, and a GST-only cDNA construct, were induced to express protein in BL21 bacteria cells. The proteins were then purified and used in electrophoretic mobility shift assays. Bands of interest are denoted by an asterisk (*) in lanes where more than one strongly stained band was observed.

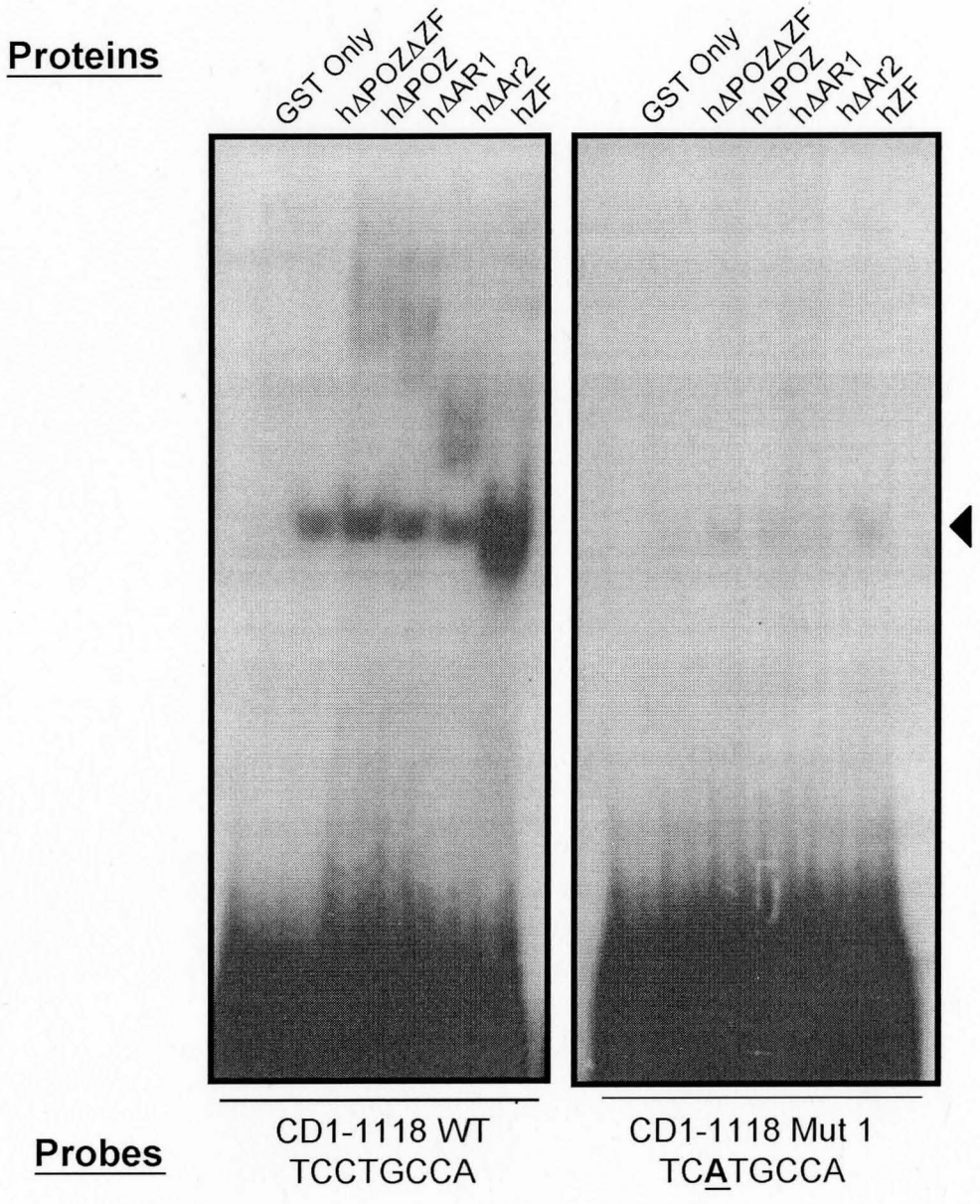


Figure 15: Kaiso specifically binds the *cyclin D1* promoter -1118 KBS *in vitro*. Purified GST-hKaiso fusion proteins were incubated with radiolabeled oligonucleotides generated from the *cyclin D1* promoter and subjected to electrophoresis and autoradiography. The GST-Kaiso fusion protein constructs bind the wild type CD1-1118 KBS with varying affinities, with hZF showing the highest affinity for DNA binding. A single base pair mutation (TCCTGCCA → TCATGCCA) in the KBS completely abolishes all binding. Non-specific bands are indiciated by the arrowhead.

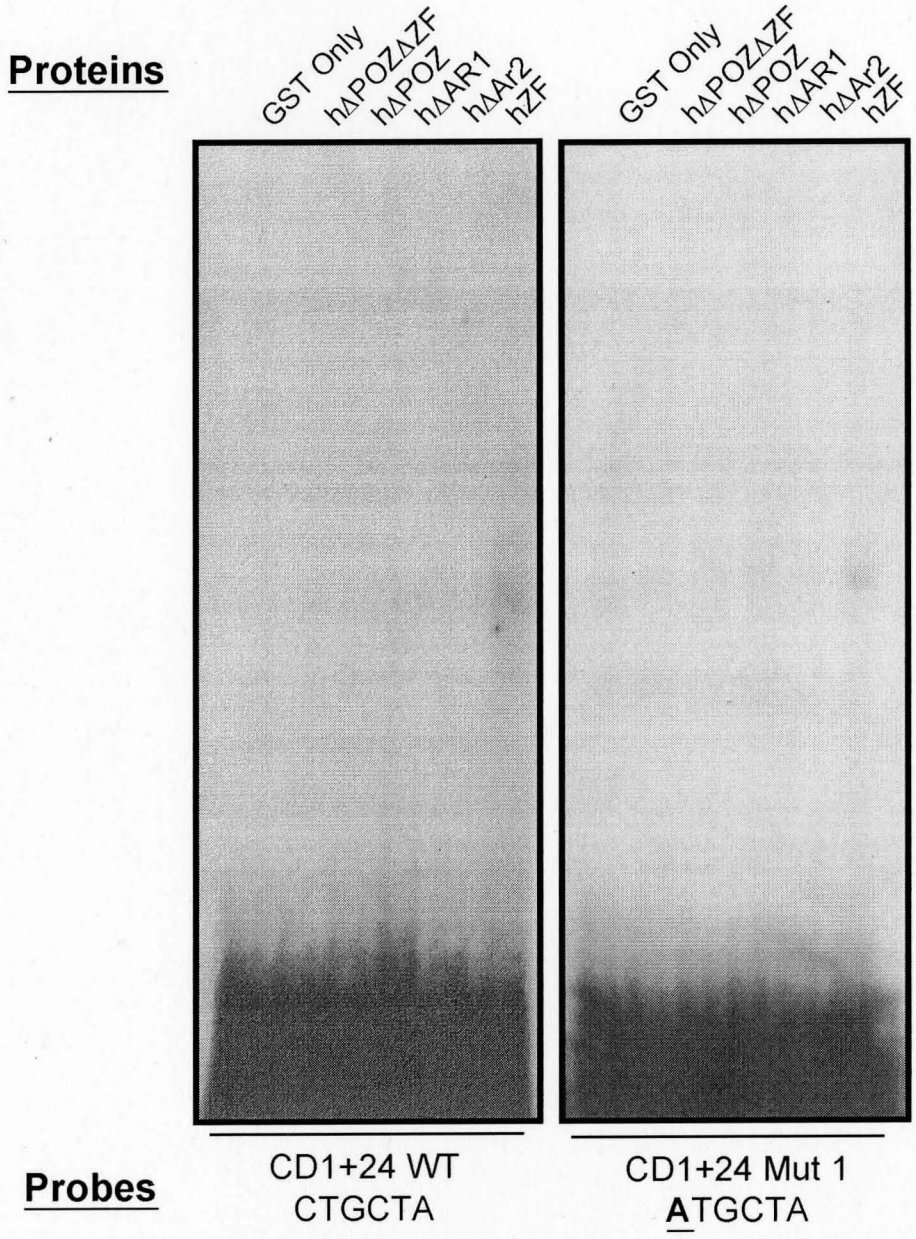


Figure 16: Kaiso does not bind the *cyclin D1* promoter +24 KBS *in vitro*. Purified GST-hKaiso proteins were incubated with radiolabelled *cyclin D1* promoter +24 KBS-derived probes. Kaiso does not bind the wild type CD1+24 KBS. A single base pair mutation (CTGCTA → ATGCTA) in the KBS did not affect binding.

3.7. Kaiso represses the cyclin D1 promoter in a dose-dependent manner.

Once we established that Kaiso binds the *cyclin D1* promoter *in vivo* and *in vitro*, we next examined Kaiso's transcriptional effect on this promoter using the luciferase assay system. HeLa cells were chosen for use in luciferase assays due to their high transfection efficiency and their endogenous p120/Kaiso interaction. The 1748CD1 wild type *cyclin D1* promoter luciferase reporter contains two of the three KBS elements within the human *cyclin D1* promoter region (Figure 17). Cells were transfected with 0.2 μg of 1748CD1 reporter plasmid and incremental amounts of pcDNA3-Kaiso effector plasmid up to 1.0 μg, and were incubated for 30 hours. After 30 hours cells were lysed, assayed, and the results analyzed. Our initial experiments clearly demonstrated that co-transfection of the 1748CD1 construct and human Kaiso in HeLa cells resulted in the repression of luciferase expression in a dose-dependent manner to a saturation level of 2-fold repression (Figure 18).

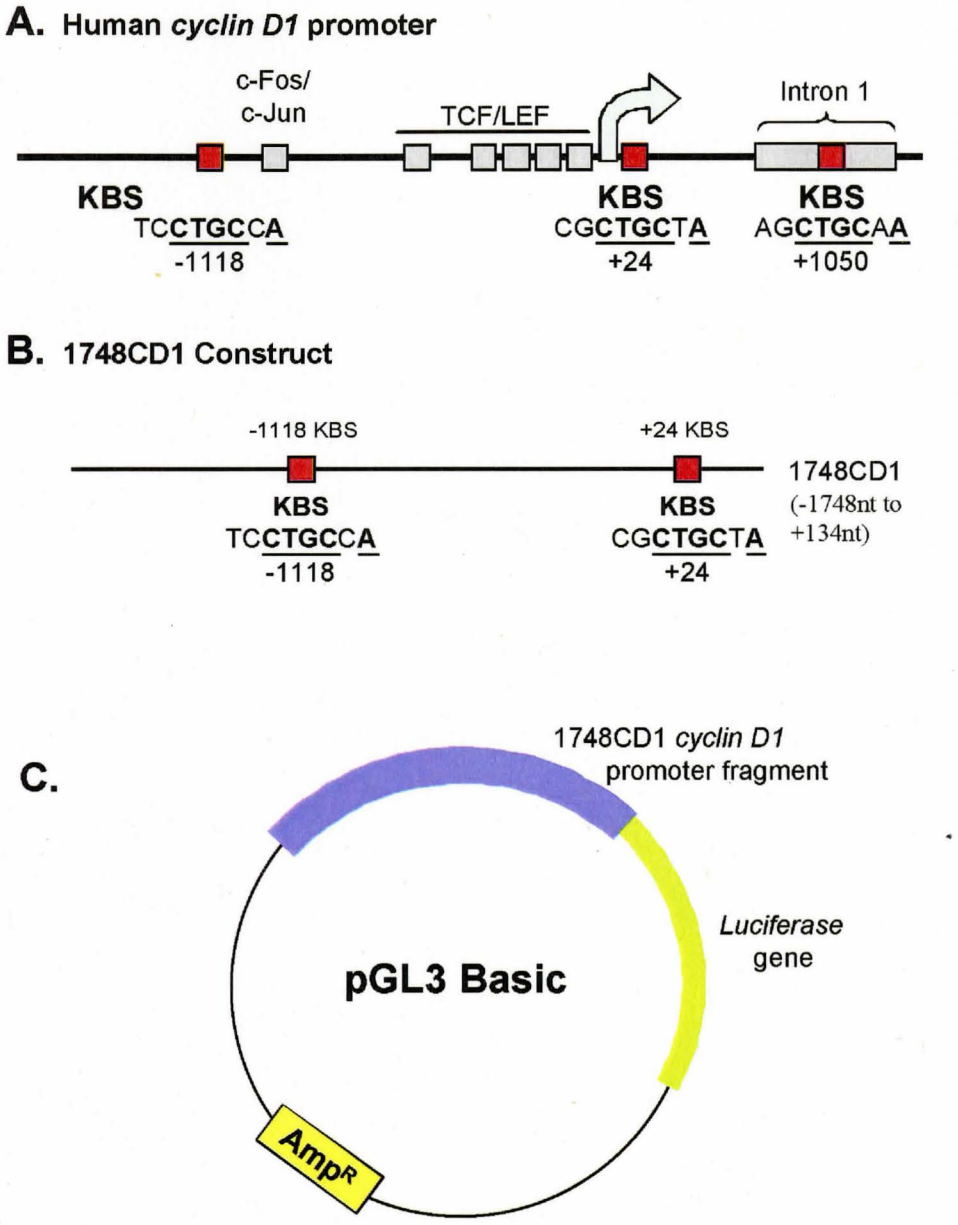


Figure 17: Schematic diagram of the 1748CD1 artificial promoter construct. The pGL3 Basic 1748CD1 artificial promoter construct contains a partial human *cyclin D1* promoter sequence (1748CD1), spanning nucleotides -1748 to +134 relative to the transcriptional start site (compare A to B). (B) The 1748CD1 construct contains two of the three *cyclin D1* promoter KBS elements. (C) The 1748CD1 *cyclin D1* promoter fragment was cloned into the pGL3 Basic luciferase assay reporter backbone vector. In this system the *cyclin D1* minimal promoter controls the luciferase-reporter gene expression.

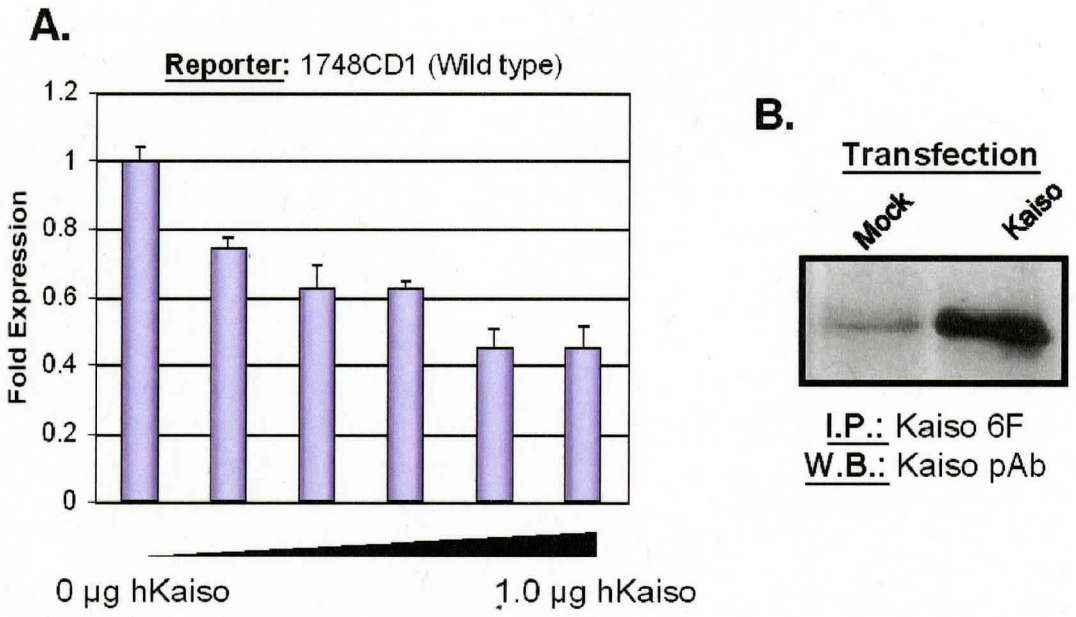


Figure 18: Kaiso represses transcription from the *cyclin D1* promoter in a dose-dependent manner. (A) The 1748CD1 minimal *cyclin D1* promoter construct was co-transfected with increasing amounts of human Kaiso expression vector, up to 1.0 μg, in HeLa cells. Kaiso overexpression in this system results in an enhanced repression of luciferase as Kaiso expression increases to achieve a maximum 2-fold repression. (B) Immunoprecipitation and Western Blot demonstrate that Kaiso is efficiently overexpressed in HeLa cells.

3.8. p120 activates the cyclin D1 promoter.

As a next step in characterizing Kaiso's transcriptional regulation of the *cyclin D1* promoter, I investigated p120's ability to modulate this function. Previous studies from our lab have indicated that p120 inhibits Kaiso's sequence-specific DNA-binding and relieves Kaiso transcriptional repression of the 4xKBS artificial promoter (119). I initially tested p120's ability to regulate the *cyclin D1* promoter by co-transfecting the 1748CD1 reporter construct along with a p120 expression construct. Indeed ectopic p120 activated the *cyclin D1* promoter approximately 1.5-fold (Figure 19A,B), providing our first indication that p120 overexpression had an effect on luciferase expression from the *cyclin D1* promoter. Next, to determine if this activation occured through the p120/Kaiso interaction, Kaiso and p120 expression plasmid vectors were co-transfected with the 1748CD1 *cyclin D1* promoter reporter vector and the cells were subsequently assayed for luciferase expression. Contrary to our predictions, p120 was unable to relieve Kaiso-mediated repression of the *cyclin D1* promoter (Figure 19C). Specifically, co-transfection of p120 along with Kaiso did not have any effect on luciferase repression observed when Kaiso was transfected in the absence of p120 (Figure 19C), suggesting that p120 is not involved in modulating Kaiso transcriptional repression of the *cyclin D1* promoter.

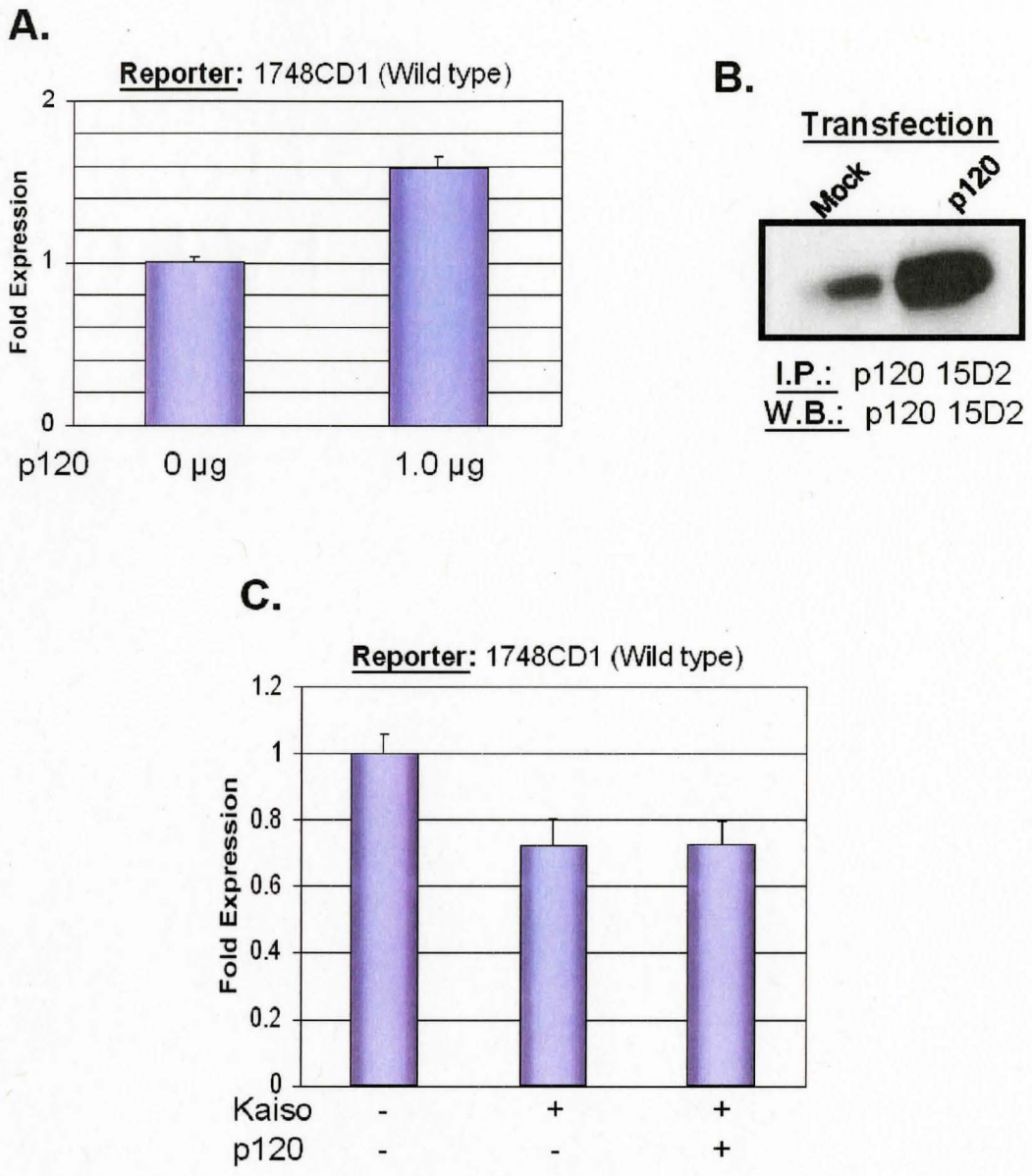


Figure 19: p120 activates the *cyclin D1* promoter. (A) The Kaiso binding partner p120 was utilized in luciferase assay experiments to determine if it also regulates *cyclin D1* promoter activity. As expected 1.0 μg of p120 introduced into a luciferse assay activates the cyclin D1 promoter 1.5-fold. (B) p120 protein expression levels increase significantly upon transfection of p120 expression plasmid into HeLa cells. (C) To determine if the observed p120 activation of the *cyclin D1* promoter was a consequence of its interaction with Kaiso, p120 expression plasmid was co-transfected with Kaiso in luciferase assays. Surprisingly, despite p120 co-expression with Kaiso, luciferase expression remained at levels concomitant with just Kaiso overexpression, suggesting that p120 is not able to relieve Kaiso-mediated repression of the *cyclin D1* promoter.

3.9. Kaiso represses cyclin D1 promoter reporter constructs lacking the KBS.

Our initial observations that Kaiso represses *luciferase* expression via the *cyclin D1* promoter strengthened our hypothesis that *cyclin D1* is a Kaiso target gene. We next sought to determine if this repression occurred via either of the 2 KBS elements located within the 1748CD1 construct, as was suggested by our *in vitro* DNA-binding assays. Site-directed mutagenesis was utilized to create single base-pair mutations in each of the KBS located within the 1748CD1 *cyclin D1* promoter fragment. The CD1-1118 KBS was mutated as follows (TCCTGCCA → TC**A**TGCCA) and the CD1+24 KBS was similarly mutated (CTGCTA → **A**TGCTA). A double KBS 1748CD1 mutant construct was generated from these site-directed mutagenesis products to create the 1MK2MK-CD1 luciferase reporter construct (Figure 20B), which was subsequently used in luciferase assays. As with previously described luciferase assays, 0.2 μg of KBS mutant reporter plasmid was co-transfected along with 1.0 μg of Kaiso effector plasmid and the cells were subsequently assayed for luciferase expression.

To our surprise, Kaiso was able to repress luciferase expression from the double KBS mutant 1MK2MK-CD1 *cyclin D1* promoter reporter construct, at the same potency (2-fold) as with the wild type 1748CD1 construct (Figure 21A). This was an unforeseen result, as EMSA experiments had indicated to us that Kaiso did not interact with oligonucleotdies bearing single mutations in either KBS. We therefore anticipated that a minimal *cyclin D1* promoter with single point mutations within each KBS element would not be bound or transcriptionally repressed by Kaiso. Our luciferase assay promoter-reporter data thus raised the possibility that contrary to our *in vitro* DNA-binding

findings, a single nucleotide change within the KBS was not sufficient to inhibit Kaiso's DNA-binding or regulation of the *cyclin D1* promoter *in vivo*. The presence of co-repressors and other Kaiso interacting proteins could very well stabilize Kaiso's interaction with the mutated *cyclin D1* KBS.

To address this possibility I created triple base pair mutations in each of the KBS located within the 1748CD1 *cyclin D1* partial promoter to completely obliterate any potential Kaiso binding capabilities to each site. The CD1-1118 KBS was mutated as follows (TCCTGCCA → TC**A**T**TA**CA) and the CD1+24 KBS was similarly mutated (CTGCTA → **A**T**TT**TA). The CD1+24 KBS was given a C → T mutation at the 4 th nucleotide within the sequene to avoid the creation of a TATA binding site, which might cause unwanted TATA protein binding to the construct due to the proximity of the CD1+24 KBS to the transcriptional start site. A second double-KBS mutant *cyclin D1* promoter reporter construct was generated from these site-directed mutagenesis products and named KBS(1,2)mt-CD1 (Figure 20B) to differentiate from the first double mutant construct, 1MK2MK-CD1.

The KBS(1,2)mt-CD1 construct was utilized in luciferase assays as previously described, but yet again to our surprise Kaiso was still able to repress luciferase expression from this KBS mutant promoter construct 2-fold (Figure 21B). This result firmly demonstrated that Kaiso was mediating *cyclin D1* repression by an alternate mechanism that did not involve the sequence-specific KBS elements. We therefore questioned whether Kaiso's methylation-specific DNA-binding capabilities may explain its transcriptional repression of the mutated *cyclin D1* promoter.

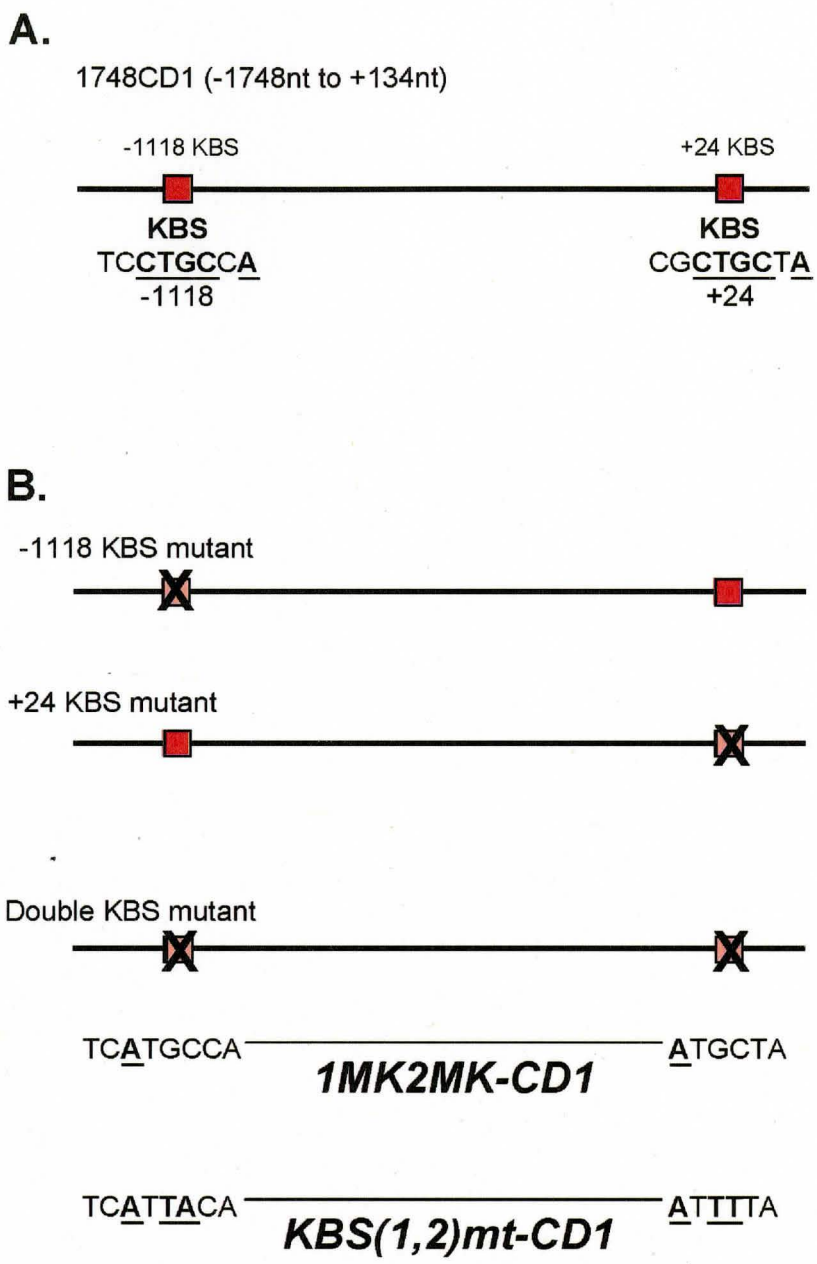


Figure 20: Schematic diagram of the *cyclin D1* promoter KBS mutant constructs generated. (A) The 1748CD1 construct was used as a template to introduce point mutations into each of its two KBS. (B) Single or triple point mutations (described in detail in the materials and methods, Table 3) were created in each of the two 1748CD1 KBS by site-directed mutagenesis. The mutagenesis products were then utilized to create double KBS mutant minimal promoter constructs which were named 1MK2MK-CD1 and KBS(1,2)mt-CD1.

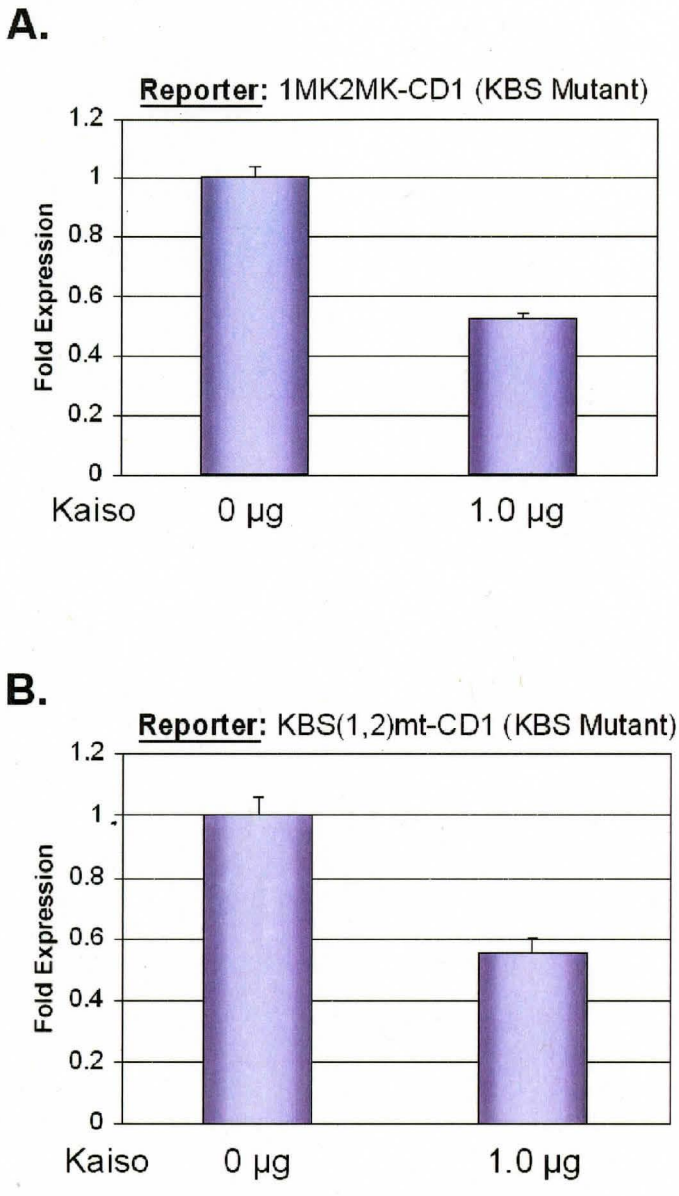


Figure 21: Kaiso represses luciferase expression from KBS point mutant *cyclin D1* promoter constructs. The 1MK2MK-CD1 and KBS(1,2)mt-CD1 KBS mutant promoter reporter constructs were utilized in luciferase assays to determine whether mutation of the KBS would inhibit Kaiso repression of the *cyclin D1* promoter. Contrary to our predictions, mutation of the KBS elements did not prevent Kaiso-mediated repression of the *cyclin D1* promoter. (A, B) Kaiso repressed luciferase expression 2-fold from each *cylin D1* promoter KBS mutant reporter construct.

3.10. The cyclin D1 promoter may be regulated by CpG methylation.

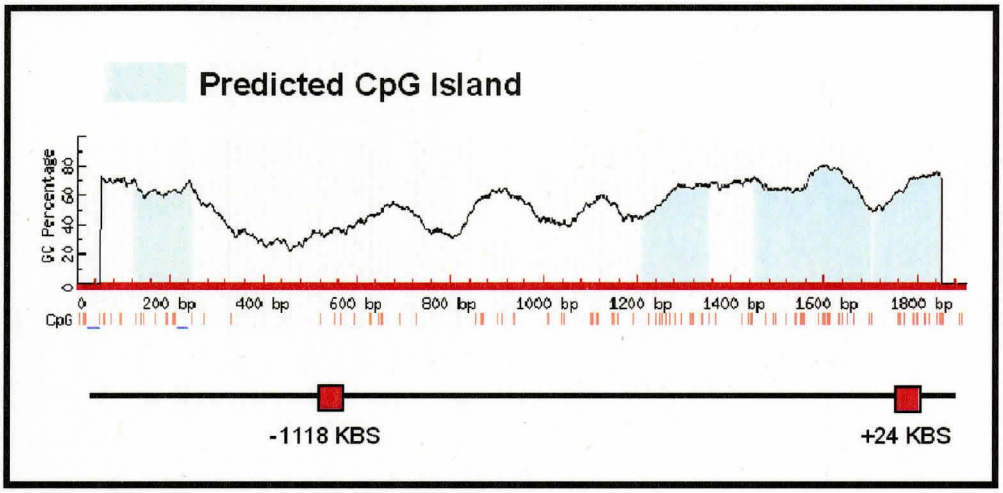
Our unexpected finding that Kaiso could still repress luciferase expression from KBS mutant *cyclin D1* promoter reporter constructs led us to investigate whether Kaiso regulation of the *cyclin D1* promoter may be due to its methylation-specific rather than sequence-specific binding. As a first step in investigating Kaiso's potential to bind the *cyclin D1* promoter in a methylation specific manner, the 1748CD1 partial *cyclin D1* promoter was analyzed for putative methylation sites. Anaysis of the 2000 bp 1748CD1 sequence using the methylation prediction software Methprimer (www.urogene.org) indicated that the *cyclin D1* promoter does in fact contain putative CpG islands (Figure 22A). Interestingly, the CD1+24 KBS is located within one of these putative CpG islands, but the CD1-1118 KBS is not located within or near any putative CpG islands. This discovery prompted a preliminary investigation of how these potential CpG methylation sites might affect Kaiso's transcriptional regulation of the *cyclin D1* promoter.

When we first obtained the 1748CD1 construct we were also provided with a second construct, 962CD1, which contains a truncated *cyclin D1* promoter fragment spanning nucleotides –962 to +134 relative to the transcriptional start site. During our initial analysis of Kaiso transcriptional activities in the context of the *cyclin D1* promoter, this construct was not used in our experiments because it did not contain the CD1-1118 KBS, which at that time was of primary interest to us. However, in light of our recent findings, the 962CD1 construct became a useful tool for studying Kaiso's transcriptional repression of the *cyclin D1* promoter in the absence of the CD1-1118 KBS and in the

presence of predicted CpG islands and the CD1+24 KBS. We thus performed luciferase assays using the 962CD1 partial *cyclin D1* promoter reporter construct and Kaiso expression vectors as described earlier for the 1748CD1 minimal promoter.

Interestingly, increasing amounts of Kaiso expression vector caused a dose-dependent repression of *luciferase* expression from the 962CD1 construct in an almost identical manner to what was observed with 1748CD1 (Compare Figure 22B and 18A). Since removal of the CD1-1118 KBS alone had little to no effect on Kaiso's transcriptional repression of the *cyclin D1* promoter, this indicated that the CD1-1118 KBS was not the primary Kaiso regulatory element on the *cyclin D1* promoter as we had thought. Together our findings that Kaiso could repress luciferase expression from a promoter construct lacking the CD1-1118 KBS and did not bind the CD1+24 KBS *in vitro* hinted that perhaps Kaiso was functioning as a methylation-dependent rather than consensus sequence-specific repressor of the *cyclin D1* promoter.

A.



B.

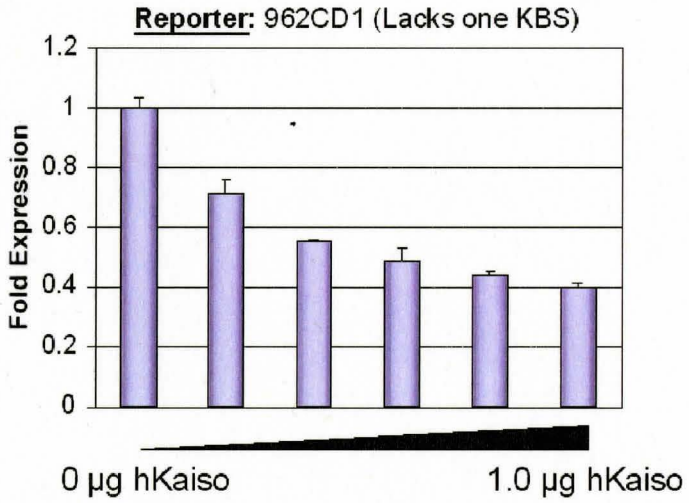


Figure 22: The *cyclin D1* promoter contains methylatable DNA sequences. (A) The 1748CD1 promter was analyzed to determine if it contained any putative CpG islands. Four potential CpG islands were predicted to be located in this fragment of the *cyclin D1* promoter, one of which contains the CD1+24 KBS. (B) The 962CD1 construct, which lacks the CD1-1118 KBS but contains the CD1+24 KBS and 3 of the 4 predicted CpG islands, was co-transfected into HeLa cells along with Kaiso. Interestingly Kaiso repressed luciferase expression from this partial *cyclin D1* promoter fragment 2-fold, similar to its effects on the 1748CD1 construct.

3.11. Kaiso binds methylated cyclin D1 promoter fragments in vitro.

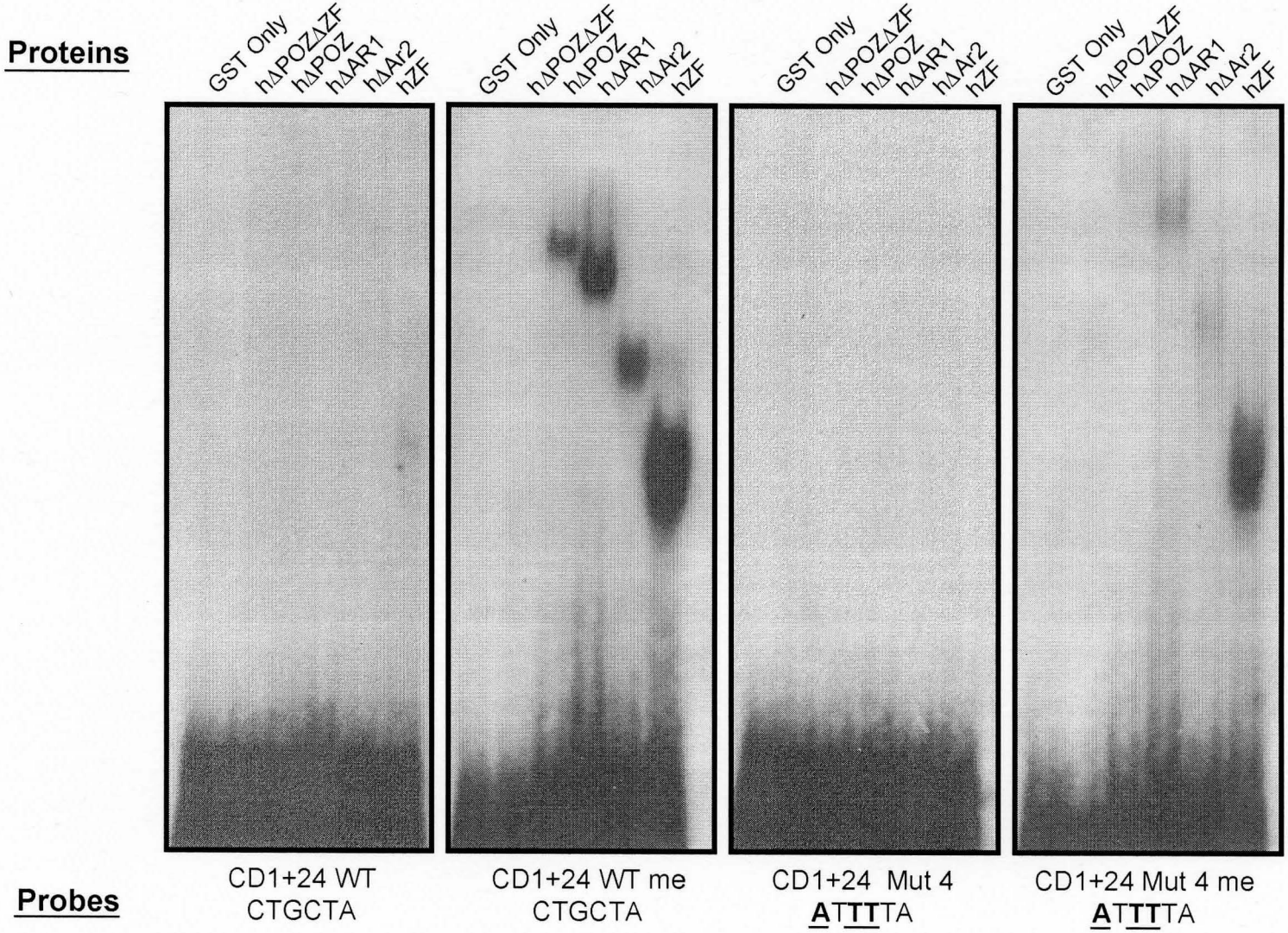
The online Methprimer CpG methylation prediction software indicated that the CD1+24 KBS was situated within a predicted CpG island. Fortuitously our CD1+24 KBS EMSA oligonucleotide probe that was generated for previous experiments harboured some of these putative methyl-CpG sites. The CD1+24 probe has a GCGC sequence just upstream of the KBS, in addition to other CpG di-nucleotides. Since Kaiso has been characterized to bind double CpG methylation sequences rather than single sequences, this made the CD1+24 probe a likely candidate for methylation-specific Kaiso DNA-binding. Moreover the CD1-1118 derived oligonucleotide probe, although not predicted to be located within a CpG island, also possesses several CpG dinucleotids.

The CD1-1118 and CD1+24 probes were methylated *in vitro* prior to radiolabeling and incubation with our panel of purified mutant GST-Kaiso fusion proteins. Although our Kaiso protein panel did not bind the wild type CD1+24 KBS probe in the unmethylated condition, they were able to efficiently bind the CD1+24 KBS probe once it had been methylated (Figure 23). This was an exciting finding as it provided the first indication that Kaiso may associate with the *cyclin D1* promoter in a methylation-dependent manner. We next investigated the effects that point mutations of the KBS within this promoter fragment would have on Kaiso's ability to bind the CD1+24 probe *in vitro*. The Kaiso protein panel was able to bind a methylated probe containing a triple nucleotide mutation within the KBS (CTGCTA → **A**T**TT**TA) (Figure 23), although it was unable to bind to the unmethylated mutant probe. This mutation was identical to that introduced into the CD1+24 KBS to create the mutant reporter

KBS(1,2)mt-CD1. It is noteworthy that the affinity of Kaiso binding to the methylated KBS-mutant CD1+24 probe was significantly lower than Kaiso binding to the methylated wild type CD1+24 probe (Figure 23).

The same sets of experiments were performed with the CD1-1118 probe. The Kaiso mutant proteins bound to the wild type unmethylated and methylated CD1-1118 probe (Figure 24), however a triple nucleotide mutation within the KBS identical to the KBS(1,2)mt-CD1 reporter CD1-1118 KBS mutation (TCCTGCCA → TC**A**T**TA**CA) abrogated Kaiso binding to both the unmethylated and methylated CD1-1118 probe. These results indicated that Kaiso binding to the CD1-1118 probe was KBS-specific, and not attributed to Kaiso binding the various methylated CpG dinucleotides contained near the KBS within the probe. Our findings using methylated CD1+24 and CD1-1118 probes demonstrated that Kaiso has the ability to bind methylated *cyclin D1* promoter probes *in vitro* in specific CpG methylatable regions in the absence of an intact KBS. This possibly explains why Kaiso is still able to repress the KBS-mutated minimal *cyclin D1* promoter in luciferase reporter assays.

Figure 23: Kaiso binds the CD1+24 probe in a methylation-dependent manner. CD1+24 oligos were methylated *in vitro*, radiolabelled and incubated with GST-Kaiso fusion proteins. The Kaiso proteins bind efficiently to the methylated wild type CD1+24 probe, with hZF and hΔAR1 showing the strongest DNA-binding affinity. Methylated KBS mutant probes (CTGCTA → **A**T**TT**TA) were also incubated with Kaiso proteins, which were able to bind the oligos, albeit at a lesser affinity than their binding to methylated wild type probe. Interestingly, Kaiso was not able to bind to the unmethylated wild type or KBS mutant CD1+24 probe.


Proteins
GST Only
hΔPOZΔZF
hΔPOZ
hΔAR1
hΔAr2
hZF
GST Only
hΔPOZΔZF
hΔPOZ
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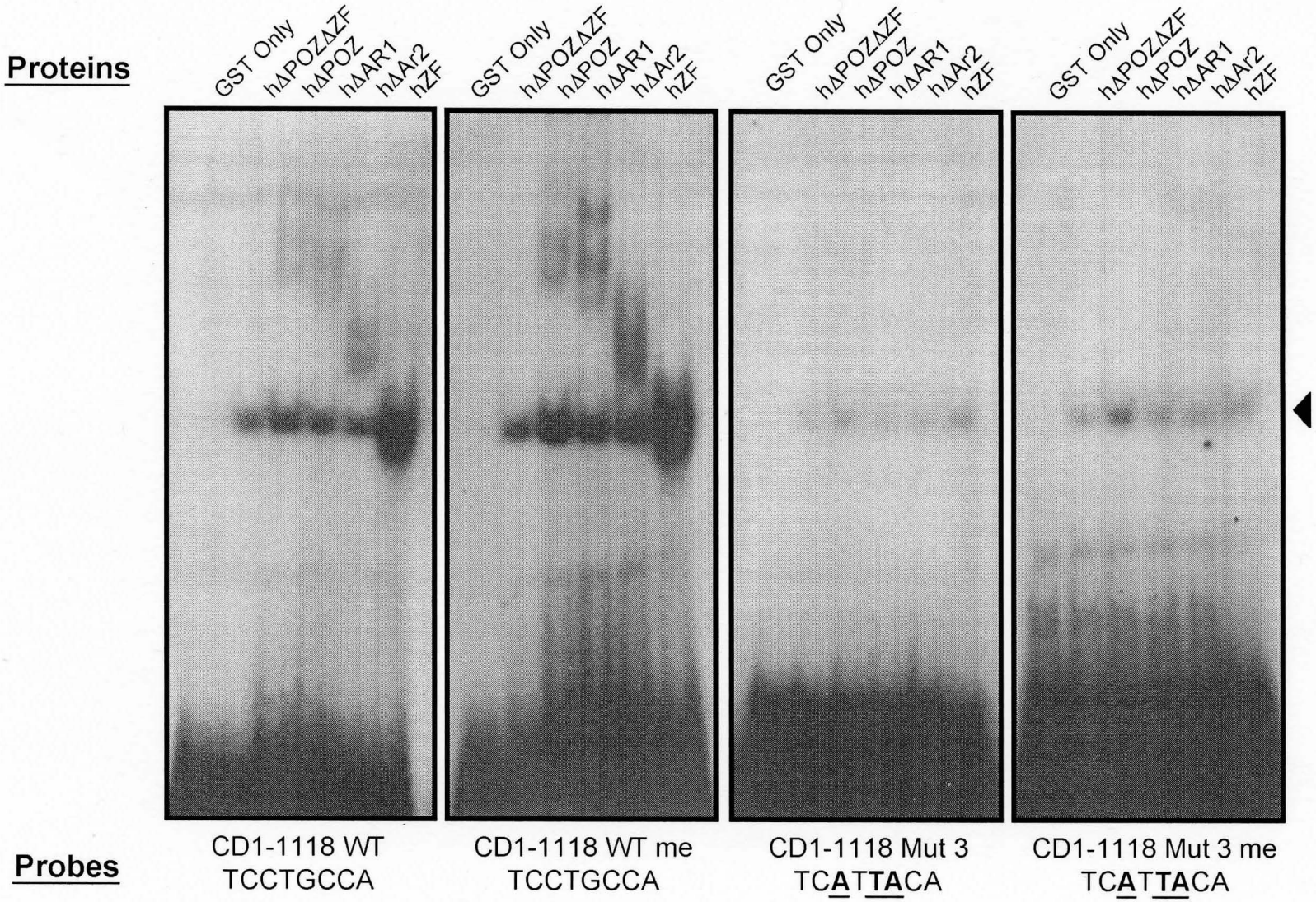
hΔAr2
hZF
GST Only
hΔPOZΔZF
hΔPOZ
hΔAR1
hΔAr2
hZF
GST Only
hΔPOZΔZF
hΔPOZ
hΔAR1
hΔAr2
hZF
Probes
CD1+24 WT
CTGCTA
CD1+24 WT me
CTGCTA
CD1+24 Mut 4
ATTTTA
CD1+24 Mut 4 me
ATTTTA

Figure 24: Kaiso does not bind the CD1-1118 probe in a methylation-dependent manner. GST-Kaiso fusion proteins were incubated with methylated wild type and KBS mutant (TCCTGCCA → TC**A**T**TA**CA) CD1-1118 probes. The Kaiso proteins showed efficient binding to the unmethylated as well as the methylated wild type probe. However, upon mutation of the KBS, Kaiso binding to the probe was abrogated in both the unmethylated and methylated probes. Non-specific bands are indicated by the arrowhead.


Proteins
GST Only
hΔPOZΔZF
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hΔAR1
hΔAr2
hZF
GST Only
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hΔPOZ
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hΔAr2
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hΔPOZΔZF
hΔPOZ
hΔAR1
hΔAr2
hZF
GST Only
hΔPOZΔZF
hΔPOZ
hΔAR1
hΔAr2
hZF
Probes
CD1-1118 WT
TCCTGCCA
CD1-1118 WT me
TCCTGCCA
CD1-1118 Mut 3
TCATTACA
CD1-1118 Mut 3 me
TCATTACA

3.12. Kaiso and p120 modulate β-catenin transcriptional activation of the cyclin D1 promoter.

Since our initial identification of Kaiso binding sites in the *cyclin D1* promoter resulted from a visual search of β-catenin target gene promoters, we next investigated the possibility that Kaiso and p120 may have an effect on β-catenin mediated activation of this promoter. Using luciferase assays and consistent with published studies (88, 119) we observed that β-catenin activates luciferase expression from the *cyclin D1* promoter 5-fold. More importantly however, we found that Kaiso completely inhibits β-catenin-mediated *cyclin D1* activation (Figure 25A). Moreover, p120 enhances β-catenin activation of the promoter 2-fold, presumably independent of its interaction with Kaiso (Figure 25B). These results are quite exciting as they support our hypothesis that Kaiso and p120 modulate canonical Wnt signaling and corroborate data published by our collaborator, Dr. Pierre McCrea (MD Anderson Cancer Centre, Houston, TX), who demonstrated that Kaiso antagonizes canonical Wnt/β-catenin signaling in the *Xenopus laevis* model system (88, 119).

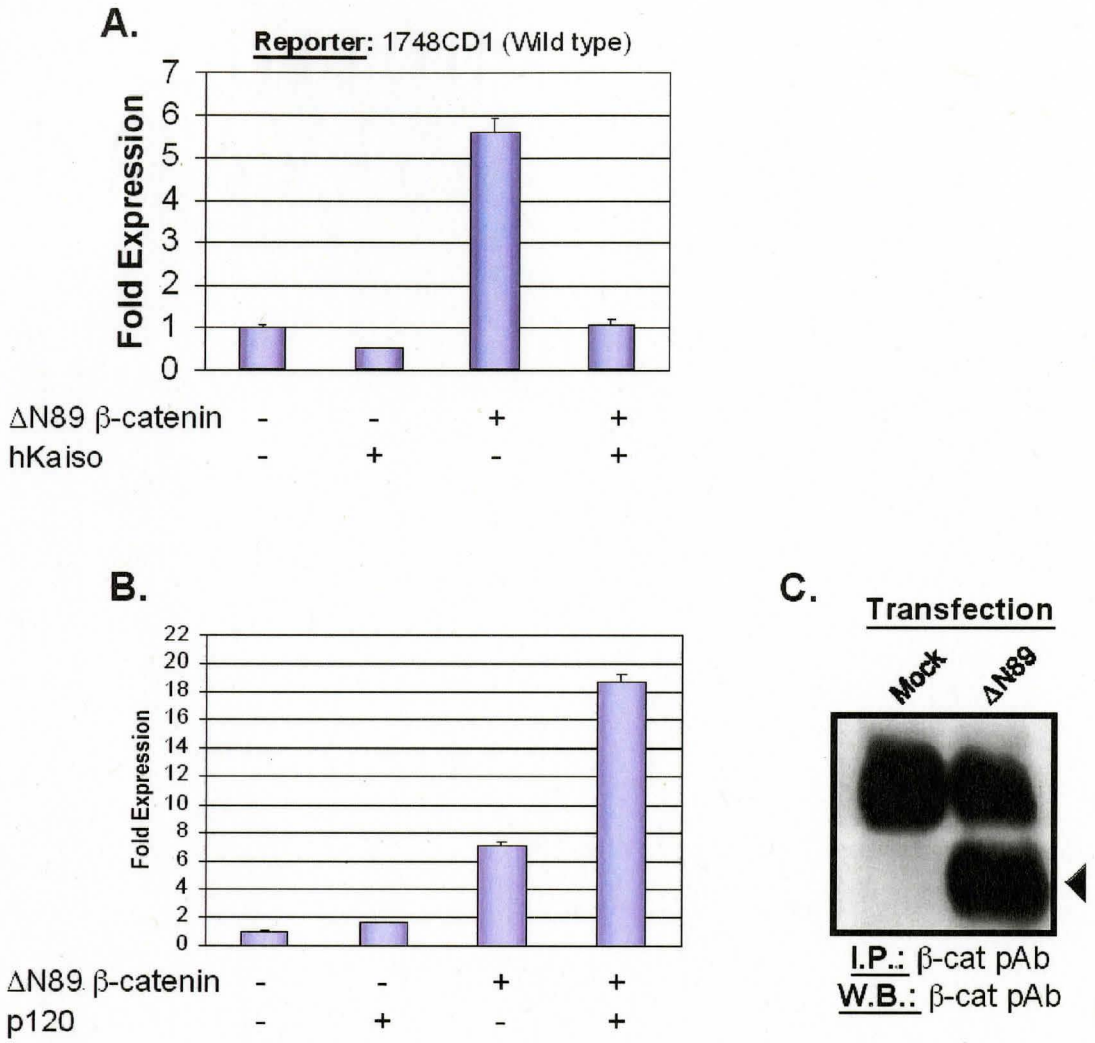


Figure 25: **Kaiso and p120 modulate β-catenin mediated activation of the *cyclin D1* promoter.** The similarity between the p120/Kaiso interaction and the β-catenin/TCF interaction led us to question whether Kaiso and p120 may synergize or antagonize β-catenin/TCF regulation of *cyclin D1*. To investigate this possibility the wild type 1748CD1 partial *cyclin D1* promoter reporter construct was co-transfected with Kaiso, p120, and an N-terminally truncated and constitutively active β-catenin expression construct, ΔN89. (A) β-catenin activated the *cyclin D1* promoter 5-6-fold, however Kaiso inhibited this activation and reduced luciferase expression back to basal levels. (B) Conversely, p120 enhanced β-catenin activation to twice its normal level. (C) The ΔN89 β-catenin protein is efficiently expressed using standard transient transfection methods, which is indicated by the arrowhead.

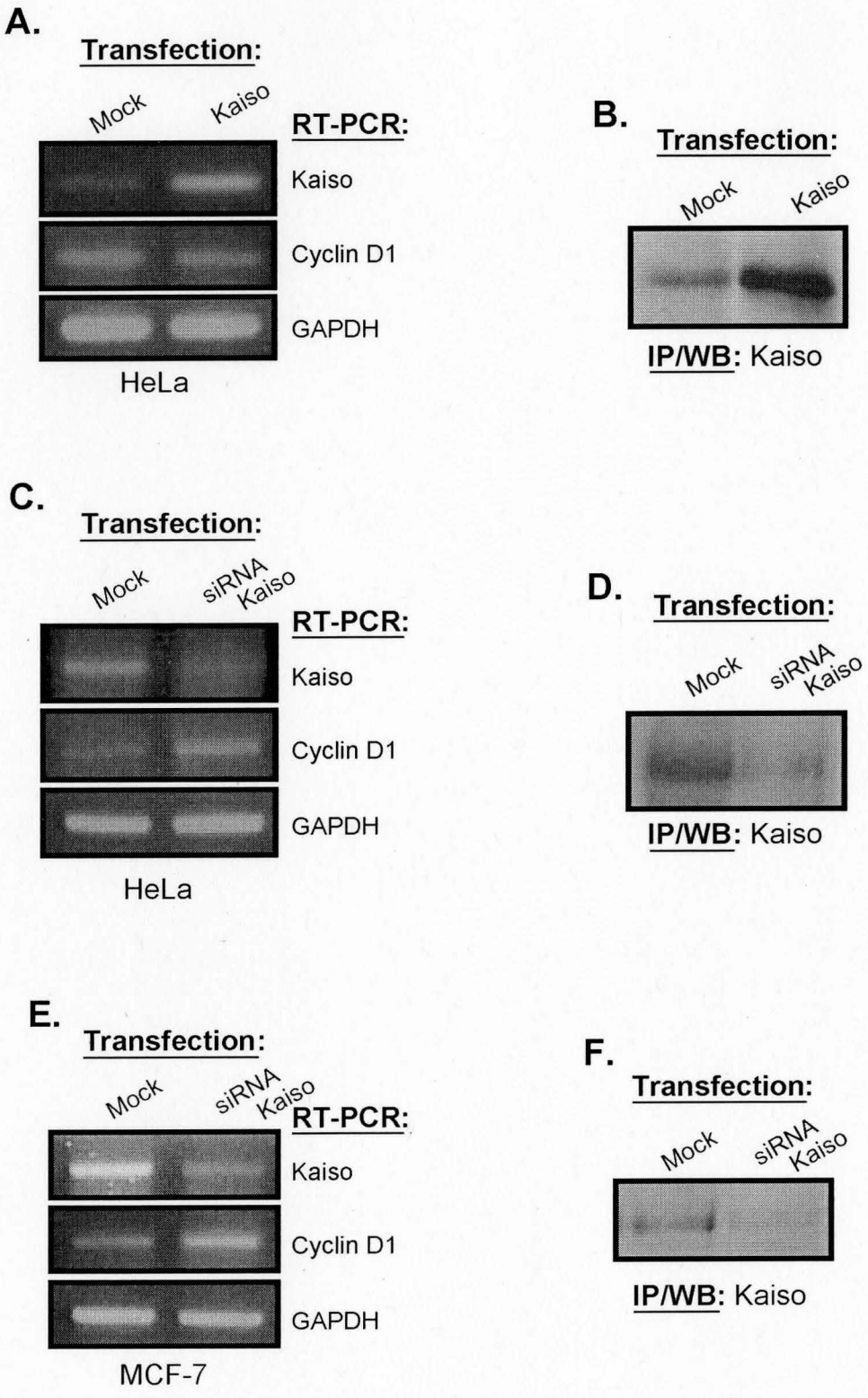
3.13. Kaiso misexpression regulates cyclin D1 transcript levels.

As a first step in determining if Kaiso misexpression could sufficiently modulate *cyclin D1* mRNA expression, we employed transient transfection methods to misexpress Kaiso in HeLa and MCF-7 cells. Our initial Kaiso overexpression experiments in HeLa cells followed by RT-PCR amplification of Kaiso, cyclin D1, and GAPDH mRNA did not yield any significant results (Figure 26A,B). Although Kaiso overexpression could be detected at the mRNA and protein level (Figure 26A,B), we could not detect any change in cyclin D1 transcript levels (Figure 26A). Changes in experimental conditions (e.g. manipulation of starting RNA concentration and number of PCR cycles) did not yield any differences in the results obtained. Based on our *cyclin D1* minimal promoter assay experiments, we had predicted that Kaiso overexpression would repress *cyclin D1*. However it is possible that in an *in vivo* context with numerous regulatory factors at play, this Kaiso-mediated repression of *cyclin D1* would be difficult to detect. To circumvent this problem, we turned to analysing the effects of Kaiso depletion on *cyclin D1* transcript levels.

HeLa cells were transfected with Kaiso-specific siRNA and subsequent RT-PCR of harvested RNA demonstrated a significant, but not complete knock down of Kaiso mRNA (Figure 26C). More importantly however, we efficiently detected an approximate 3-fold upregulation of *cyclin D1* mRNA in the Kaiso-depleted samples (Figure 26C,D). This experiment was reproduced with similar results in MCF-7 cells (Figure 26E,F). This exciting finding is consistent with our *cyclin D1* minimal promoter assay data in which Kaiso overexpression causes a repression of luciferase expression under control of

the *cyclin D1* promoter. Furthermore this finding provides the first direct *in vivo* evidence that Kaiso regulates *cyclin D1* gene expression, and suggests that Kaiso is a tumour suppressor in the context of its regulation of *cyclin D1*.

Figure 26: Effects of Kaiso misexpression on *cyclin D1* transcript expression levels. Semi-quantitative real time PCR experiments were performed on HeLa cells transiently transfected with Kaiso siRNA or overexpression constructs. We examined *Kaiso*, *cyclin D1*, and *GAPDH* transcript levels after Kaiso depletion or overexpression by subjecting equal amounts of PCR products to agarose gel electrophoresis. (A) No significant change in *cyclin D1* transcript levels was observed upon Kaiso overexpression in HeLa cells. Kaiso overexpression was verified at the mRNA (A) and protein levels (B). Conversely, Kaiso depletion by RNAi in HeLa and MCF-7 cells resulted in a 3-fold increase of cyclin D1 mRNA(C, E). Immunoprecipitation and Western blot experiments verified Kaiso depletion at the protein level in both cell lines (D, F).


A.
Transfection:
Mock
Kaiso
RT-PCR:
Kaiso
Cyclin D1
GAPDH
HeLa
B.
Transfection:
Mock
Kaiso
IP/WB: Kaiso
C.
Transfection:
Mock
siRNA
Kaiso
RT-PCR:
Kaiso
Cyclin D1
GAPDH
HeLa
D.
Transfection:
Mock
siRNA
Kaiso
IP/WB: Kaiso
E.
Transfection:
Mock
siRNA
Kaiso
RT-PCR:
Kaiso
Cyclin D1
GAPDH
MCF-7
F.
Transfection:
Mock
siRNA
Kaiso
IP/WB: Kaiso

Discussion

4.1. Kaiso, p120 and E-cadherin expression in breast cell lines.

Kaiso is a member of the POZ-ZF family of transcription factors with roles in development and tumourigenesis (2, 8, 20). Hence it was not surprising when preliminary experiments in our lab suggested that Kaiso may be involved in breast tumourigenesis. A cancer profiling array indicated that Kaiso was misexpressed in 42% of the breast tumours represented on the array; Kaiso was underexpressed in 30% of the samples and overexpressed in 12% of the samples (Daniel lab, unpublished data). This suggested that Kaiso may have dual functions as a tumour suppressor and an oncoprotein depending on the context. Indeed the E2F1 transcription factor, a downstream effector of cyclin D1 activity in the G1 phase of the cell cycle, has been shown to exhibit both tumour suppressor and oncoprotein properties. In rodent fibroblasts, E2F1 overexpression leads to S phase cell cycle entry and DNA synthesis based on its capacity to activate genes involved in cell proliferation (45, 129). However E2F1 can conversely induce apoptosis in cooperation with p53 in rodent fibroblasts, based on its regulation of apoptosis-associated genes (60, 96, 137), and can also induce apoptosis in several human tumour cell lines independent of p53 (29, 40, 77, 92). In light of these findings and our identification of Kaiso binding sites in the *cyclin D1* promoter, an oncogene that is frequently overexpressed in breast tumours (3, 15, 116), we hypothesized that Kaiso may play a role in breast tumour progression via its mis-regulation of *cyclin D1* expression.

As a first step in exploring this hypothesis we examined Kaiso, p120 and E-cadherin expression, subcellular localization and co-precipiation in a panel of breast tumour (MCF-7, T47-D, MDA-468, MDA-231) and non-tumour (MCF-12A, MCF10A) cell lines. In all seven cell lines, Kaiso was observed as a 100 kDa phosphoprotein doublet with the highest expression in MCF-7 and SK-BR-3 tumour cells, and the lowest expression in MCF-12A non-tumour cells (Figure 8). These expression profiles suggest that Kaiso is expressed at higher levels in breast tumour than non-tumour cells and would implicate Kaiso as an oncoprotein. However, the fact that a cancer profiling array displayed increased Kaiso protein levels in 12% of breast tumour samples and decreased levels in 30% of the samples indicates that depending on the cell type or tissue origin Kaiso may display oncogenic or tumour suppressor characteristics. Breast tumours of different tissue origins have very specific characteristics with respect to etiology, pathology, treatment, and prognosis (111), and as such it is possible that Kaiso has very specific roles in these different types of breast tumours at the cellular level. Future experiments such as a breast tissue microarray screen, colony formation assays, and analysis of tumours derived from injection of Kaiso-overexpressing or –depleted cells in nude mice will help to definitively determine whether Kaiso acts as a tumour suppressor or oncoprotein in breast tumourigenesis.

In contrast to Kaiso, the p120 expression profile was unique to each cell line. In the tumour cell lines (MCF-7, T47-D, MDA-468, MDA-231, and SK-BR-3), pl20 was mainly observed as two isoforms, 1 and 3. Although isoform 3 was more abundant than isoform 1 in all cell lines, the ratio of isoform 3: isoform 1 varied with each cell line

(Figure 8). In the MCF-12A and MCF-10A non-tumour cells, only p120 isoform 3 was observed (Figure 8). p120 isoform 3 is preferentially expressed in epithelial cells and the presence of isoform 1 in tumour cell lines suggests that isoform-1 may be a contributing factor to the tumourigenic properties of these cells. The presence of p120 isoform 1 in the breast non-tumour cell lines may hint at a difference in the ability for p120 to interact with Kaiso in tumour cells compared to non-tumour cells. Although Kaiso preferentially interacts with the smaller p120 isoform 3 (Daniel lab, unpublished data), it is also able to interact with isoform 1 (Daniel lab, unpublished data). The combined presence of p120 isoform 1 and 3 in the tumour cell lines may confer an increased ability to observe a p120/Kaiso interaction in tumour cells, which may contribute to the tumourigenic process.

The E-cadherin expression profile has been well documented in many cell lines and our findings are consistent with earlier studies showing that E-cadherin is expressed in all the breast cell lines we tested, except for MDA-231 and SK-BR-3 (Figure 8) (37, 110). Lack of E-cadherin expression is attributed to promoter hypermethylation in MDA-231 cells (110) and homozygous gene deletion in SK-BR-3 cells (37). Interestingly, E-cadherin is expressed as a doublet in MCF-7 and T47-D tumour cells. The slowly migrating band may be comprised of incompletely processed E-cadherin molecules and/or E-caderin molecules that have been aberrantly modified post-translationally (143).

Together these experiments indicate that Kaiso, p120, and E-cadherin have unique expression profiles in each of the seven breast epithelial cell lines tested but no obvious

pattern or correlation was detected. The non-tumour MCF-12A and MCF-10A share almost identical Kaiso, p120, and E-cadherin expression profiles; the MCF-7 and T47-D tumour cells share similar profiles; and the MDA-468, MDA-231, and SK-BR-3 tumour cells have different profiles. MCF-12A and MCF-10A cells are most often used as normal controls in studies using breast tumour cell lines and are not thoroughly characterized for tumour suppressor or oncogene expression. MCF-7 and T47-D tumour cells express both the c-ErbB-4 receptor and estrogen receptor, while MDA-231 and SK-BR-3 tumour cells do not (122). When activated by epidermal growth factor-like ligands, the ErbB family of receptor tyrosine kinases transduce signals that lead to the activation of various genes involved in cell growth and transformation (6). The estrogen receptor is a transcription factor that activates genes involved in cell proliferation in response to the presence of estrogen in breast cells, reviewed in (41). It is clear that the MCF-7 and T47-D cell lines share similar characteristics that affect breast tumour progression. Therefore the fact that they share similar Kaiso, p120, and E-cadherin expression profiles may also hint that these proteins may function in a similar manner in these cells to contribute to breast tumourigenesis. Future experiments in MCF-7 and T47-D versus MDA-231 and SK-BR-3 cells such as growth curve analyses and colony formation assays using Kaiso-overexpressing or –depleted cells, may help elucidate the signaling pathway(s) involved in Kaiso effects on cell growth and transformation. These cell lines have the potential to become elegant system in which to examine if tumour-associated genes such as the ErbB and estrogen receptors contribute to Kaiso's involvement in breast tumourigenesis.

4.2. Kaiso and p120 interaction in breast cell lines.

Our investigation of the Kaiso-p120 interaction in a panel of breast cell lines revealed that Kaiso and p120 co-localize in nuclear and peri-nuclear compartments, and co-precipitate in most breast tumour cell lines (SK-BR-3, MCF-7, T47-D, MDA-468, and MDA-231) but not in the MCF-12A non-tumour cell line. Immunoflourescence studies indicated that Kaiso and p120 have different subcellular and colocalization patterns in the non-tumour MCF-12A and MCF-10A, and tumour-derived SK-BR-3 cell lines. Kaiso was predominantly expressed as a nuclear protein in all three cell lines (Figure 9), but also displayed some cytoplasmic staining in the SK-BR-3 cell line (Figure 9B). p120, on the other hand, displayed cell-type specific subcellular localization patterns; p120 localized to cell-cell junctions in MCF-12A cells (Figure 9A), to cell-cell junctions and the cytoplasm in MCF-10A cells (Figure 9B), and throughout the cytoplasm and somewhat to the nucleus in SK-BR-3 cells (Figure 9C). Kaiso and p120 co-localized to the same subcellular compartments in SK-BR-3 and MCF-10A cells, providing our first clue that they may interact in these cells (Figure 10).

The p120/Kaiso interaction has thus far been only observed in epithelial cells that express p120 isoform 3 ((20, 50) and Daniel lab, unpublished data), as experiments using fibroblast cells that primarily express p120 isoform 1 have not demonstrated a p120/Kaiso interaction. It was therefore an interesting finding that the p120/Kaiso interaction could not be detected in the MCF-12A non-tumour cell line of epithelial origin. Our observation that Kaiso and p120 localize to different subcellular compartments in MCF-12A cells (nucleus and cell membrane, respectively) may explain

why an interaction was not detected in an epithelial cell line between the two proteins in our experiments. Restriction of p120 to the cell membrane may indicate a cell-adhesion specific role for this protein and specific factors may inhibit its nuclear localization or accumulation to interact with Kaiso.

4.3. Kaiso association with the cyclin D1 promoter in vivo.

In human breast tumours, one of the most frequently upregulated oncogenes is *cyclin D1* which encodes a cell cycle regulator and is a target gene of the Wnt/β-catenin signaling pathway that is often perturbed in breast tumours (14). Since preliminary evidence had indicated Kaiso was misexpressed in breast tumours and we recently foound that Kaiso converged on a subset of Wnt target genes, we decided to determine whether Kaiso could be contribute to tumorigenesis through misregulation of *cyclin D1*. ChIP experiments were carried out using breast epithelial non-tumour (MCF-12A) and tumour, (MCF-7, MDA-468, and MDA-231) cell lines. We detected 200 bp *cyclin D1* promoter fragments in Kaiso immunocomplexes from all four cell lines but interestingly this was dependent on the presence or absence of serum (Figure 11). Specifically, MDA-468 and MCF-12A cells required serum-starvation before *cyclin D1* promoter fragments could be detected in Kaiso immunocomplexes. Meanwhile Kaiso association with the *cyclin D1* promoter was easily detected in MCF-7 and MDA-231 cells propagated in serum-supplemented media. This strongly suggests that growth factor signaling pathways and hence Kaiso and/or pl20 phosphorylation status is a crucial factor in directing Kaiso association with the *cyclin D1* promoter. Alternatively, cell-type or

tumour-specific factors may contribute to the Kaiso/*cyclin D1* promoter association since the different breast cell lines are of different tumour origins.

Previous studies from our laboratory determined that Kaiso is a serine/threonine phosphoprotein (Daniel lab, unpublished data), however the specific kinases that direct its phosphorylation are currently unknown. It is therefore possible that Kaiso function and association with the *cyclin D1 promoter* may be affected by its phosphorylation status upon growth factor signaling from serum-supplemented cell culture media. Bcl-6, a POZ-ZF transcription factor associated with B-cell lymphomas (2, 8), is phosphorylated at serine and proline clusters by **m**itogen-**a**ctivated **p**rotein **k**inase (MAPK) signaling upon receptor activation (70, 78). This phosphorylation leads to decreased Bcl-6 transcriptional activity, and decreased protein stability due to rapid Bcl-6 degradation by the ubiquitin-dependent proteosomal pathway (78). Additionally, the *Drosophila* POZ-ZF transcription factor Tramtrack, which was among the first identified BTB/POZ proteins, is also targeted for degradation via MAPK phosphorylation (61, 125). Hence it is possible that Kaiso, like its family members, is also phosphorylated by MAPK signaling, or signaling through other kinases linked to receptor tyrosine kinases. These signaling events may target Kaiso for degradation or regulate Kaiso subcellular localization, DNA-binding and/or function in another manner, thus limiting its association with the *cyclin D1* promoter. The opposing Kaiso/*cyclin D1* promoter interactions observed in MCF-7 and MDA-231 cells compared to MCF-12A and MDA-468 cells may be due to the effects of Kaiso phosphorylation events that are controlled by divergent cell type specific components.

Our ChIP experiments also indicated that p120 associates with the *cyclin D1* promoter in MCF-7 cells (Figure 12). We presume that this association is via p120's direct interaction with Kaiso, which in turn actually binds the DNA. Although our lab has shown that p120 inhibits Kaiso DNA-binding *in vitro*, the crosslinking step of the ChIP protocol may be sufficient to preserve any transient p120/Kaiso complexes located in proximity to the *cyclin D1* promoter. This would allow for precipitation of *cyclin D1* promoter fragments from p120-specific immunocomplexes. However, further experiments must be performed to determine if p120 association with the *cyclin D1* promoter occurs through (1) the p120/Kaiso interaction, (2) an unknown intrinsic ability for p120 to bind DNA, or (3) an interaction with another transcription factor or nuclear protein. One experiment to resolve these options is to use siRNA methodologies to deplete Kaiso from the cells, and then test p120-specific immunocomplexes for the presence or absence of *cyclin D1* promoter fragments. If p120 associates with the *cyclin D1* promoter indirectly through its interaction with Kaiso, then Kaiso depletion would result in reduced p120/*cyclin D1* promoter association. Conversely if p120 associates with the promoter directly or via some other currently unknown nucler protein, then its association with *cyclin D1* would not be affected by Kaiso siRNA treatment.

4.4. Kaiso association with the cyclin D1 promoter in vitro.

Having established that Kasio does indeed associate with the *cyclin D1* promoter *in vivo*, we next investigated whether Kaiso binds the *cyclin D1* promoter via its sequence-specific sites (CD1-1118 KBS and CD1+24 KBS). Using EMSA analyses we

determined that Kaiso specifically binds the CD1-1118 KBS (Figure 15), but not the CD1+24 KBS (Figure 16). These results confirmed our prediction that the CD1-1118 KBS is the more relevant binding site for Kaiso within the *cyclin D1* promoter due to its fully conserved KBS (TCCTGCCA), in contrast to the CD1+24 KBS which contains only the core KBS (CTGCTA). However, it was surprising that Kaiso did not bind the CD1+24 KBS, since Kaiso should still exhibit some DNA-binding with the core KBS. Interestingly EMSA experiments performed using murine Kaiso-GST fusion proteins (Appendix B) revealed that unlike human Kaiso-GST fusion proteins, murine Kaiso binds both the CD1-1118 KBS and CD1+24 KBS elements. This suggests that murine Kaiso has a higher affinity for the core KBS than human Kaiso. Additionally, the presence of stabilizing or destabilizing nucleotides surrounding the KBS in the CD1-1118 and CD1+24 EMSA probes may influence the specificity of murine versus human Kaiso binding to the KBS.

An alignment of the human and murine Kaiso zinc finger domains shows that they differ slightly in the amino acid content of **z**inc **f**ingers (ZF) 2 and 3 (Figure 27). Human Kaiso contains uncharged and hydrophobic residues in proximity to the ZF2 and ZF3 cysteine residues respectively, while murine Kaiso contains polar resides in the same region. Since ZF2 and ZF3 are necessary for Kaiso DNA-binding, the amino acid differences that occur in proximity to the cysteine residues that define the C_2H_2 characteristic of each zinc finger most likely bestow slightly different DNA-binding affinities or specificities to murine and human Kaiso. While it

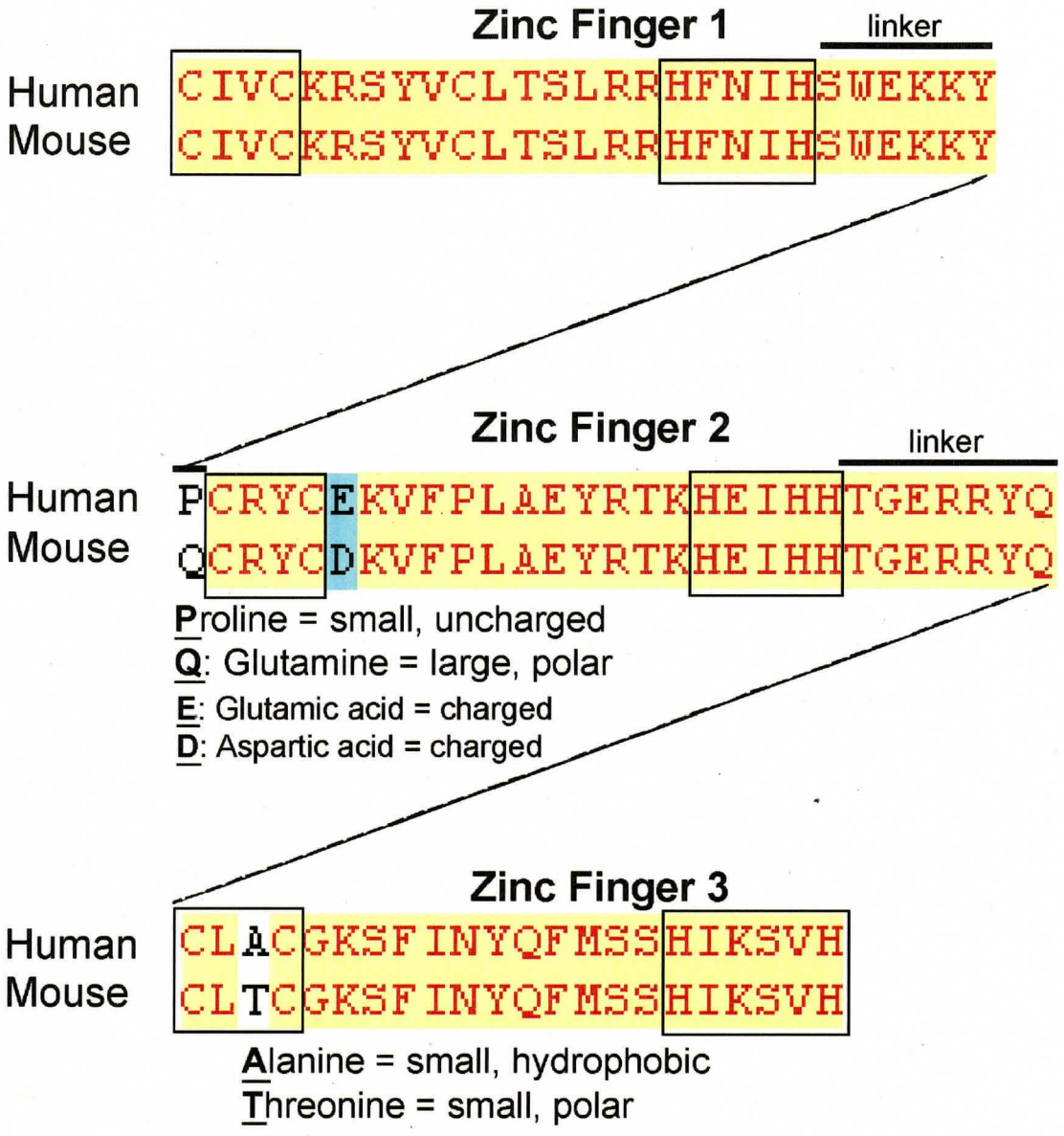


Figure 27: Comparison of human and murine Kaiso zinc finger domains. An alignment of the human and mouse zinc finger domains reveals that zinc fingers (ZF) 2 and 3 differ to a small extent in their amino acid content. The human ZFs contain uncharged and hydrophobic residues in proximity to ZF 2 and 3 respectively, while the murine ZFs contain polar resides in the same region. ZF2 and ZF3 are necessary for Kaiso DNA binding therefore these slight differences in amino acid content may confer moderately different DNA-binding affinities to human and murine Kaiso.

has been established that DNA sequence recognition is achieved from the interaction between the DNA bases and the side chains of the amino acids comprising the zinc finger (134), it is believed that these interactions are stabilized by the "linkers" or amino acid residues between the zinc fingers (66). Therefore although the amino acid differences between human and murine Kaiso are quite minor, they may be sufficient to confer slightly different DNA-binding properties to the two proteins, and thus explain why human Kaiso, in contrast to murine Kaiso, does not have a high affinity for the core KBS.

However, another explanation for the differences in KBS binding affinity exhibited by human and murine Kaiso is that the protein purification protocol utilized for GST-fusion protein expression and purification may not be optimal for the induction and purification of human GST-Kaiso fusion proteins. This may ultimately affect the ability of the human GST-Kaiso fusion proteins purified in this manner to bind DNA in EMSA experiments. Many degradation products were observed in human GST-Kaiso fusion protein preparations (Figure 14), whereas murine Kaiso GST-fusion proteins were non-degraded (Figure B-3,A). It is therefore possible that in EMSA experiments, these human GST-Kaiso degradation products may have interfered with DNA-protein interactions to confound the results obtained.

4.5. Kaiso and p120 regulation of the cyclin D1 promoter.

Once we had established that Kaiso associates with the *cyclin D1* promoter, luciferase assays were carried out to determine the effects that Kaiso and its binding partner p120 would have on luciferase expression under control of the 1748CD1 partial

cyclin D1 promoter. Kaiso repressed the promoter in a dose-dependent manner (Figure 18), while p120 activated the promoter (Figure 19). These initial results were consistent with previous findings from our lab indicating that Kaiso is a transcriptional repressor and p120 de-represses (activates) Kaiso target genes (119). However, further investigation of p120 and Kaiso-mediated repression of the *cyclin D1* promoter demonstrated that p120 was unable to derepress Kaiso transcriptional activity (Figure 19C). These results suggested that p120 activation of the *cyclin D1* promoter is independent of its interaction with Kaiso and may be a result of other signaling pathway(s) that involve p120. For example, p120 increases the activity of the Rho GTPase Rac1, which can induce DNA synthesis and cell cycle progression (82, 97) via activation of NF_Kb and ATF-2, transcription factors which in turn bind the *cyclin D1* promoter to activate gene expression (31, 46). This implicates p120 as an indirect regulator of *cyclin D1* through its activation of Rac1.

Next, to determine if Kaiso regulation of the *cyclin D1* promoter occurred via its interaction with the KBS elements in the promoter, we utilized KBS-mutated *cyclin D1* promoter-reporter constructs in luciferase assays. The mutated promoters contained point mutations within each of the KBS elements located in the 1748CD1 *cyclin D1* partial promoter and were generated to ensure that the KBS elements were completely abolished. Surprisingly Kaiso was still able to repress luciferase expression from KBS-mutant *cyclin D1* promoter reporters at the same potency as the wild type promoter reporter (Figure 21). This suggested that the Kaiso-mediated repression of the wild type *cyclin D1* promoter reporter 1748CD1 was not due to Kaiso association with the KBS

elements of the *cyclin D1* promoter. These new findings led us to investigate the possibility that Kaiso may regulate the *cyclin D1* promoter through its methylation-specific DNA-binding abilities. Kaiso is dual-specificity DNA-binding protein that binds a sequence specific consensus as well as methyl-CpG dinucleotides (23), and as such, these findings pointed to the possibility that Kaiso methylation-directed DNA binding may play a role in its regulation of *cyclin D1*.

4.6. Kaiso association with methylated sequences within the cyclin D1 promoter.

Methylation prediction software indicated that the 1748CD1 *cyclin D1* partial promoter used in our studies contains four putative CpG islands, one of which contains the CD1+24 KBS (Figure 22A). Luciferase assays with the 962CD1 *cyclin D1* partial promoter reporter that lacks the CD1-1118 KBS but contains three of the four putative CpG islands as well as the CD1+24 KBS, demonstrated that Kaiso represses this reporter in a dose-dependent manner as potently as the 1748CD1 promoter reporter (Figure 22B, Compare with Figure 18A). This indicated that the CD1-1118 KBS is not essential for Kaiso-mediated repression of the *cyclin D1* promoter and further suggested that Kaiso-mediated *cyclin D1* repression was perhaps primarily via methyl-CpG sites.

EMSA experiments carried out using the CD1+24 probe provided further support for this notion as Kaiso was able to bind methylated wild type and KBS-mutant CD1+24 probes (Figure 23), whereas is was not able to bind unmethylated CD1+24 probes (Figure 16). Furthermore, *in vitro* methylation of the CD1-1118 probe did not enhance Kaiso binding (Figure 24). Kaiso binds CGCG methylated sequences, one of which is

contained within the CD1+24 probe sequence (GCGC) along with several single CpG-dinucleotide pairs. The presence of the GCGC sequence within the CD1+24 probe may possibly explain why Kaiso was able to bind to this probe. Moreover, the CD1-1118 probe contains several single CpG-dinucleotide pairs and also a GCCG sequence, however, Kaiso was not able to bind to this probe in a methylation-specific manner, verifying the specificity of the Kaiso methylated DNA binding sequence (CGCG). These findings provide a plausible explanation for our observations that Kaiso is still able to repress KBS mutated *cyclin D1* promoter reporter constructs since Kaiso may associate with methylated CpG-islands in the *cyclin D1* promoter. The results obtained with the methylated CD1-1118 and CD1+24 EMSA probes warrant a more thorough analysis of Kaiso binding to probes derived from other portions of predicted CpG-islands within the *cyclin D1* promoter.

Another exciting possibility raised by these findings is that Kaiso regulation of the *cyclin D1* promoter in the CD1+24 region may be dually directed by Kaiso's sequence-specific and methylation-dependent DNA-binding abilities. EMSA experiments showed that although Kaiso can bind to methylated wild type and methylated KBS-mutant CD1+24 probes, stronger binding occurs with the methylated wild type probe (Figure 23). Within the cell nucleus Kaiso may be recruited to the CD1+24 region by both the KBS and methylated CpG di-nucleotides to direct dual-specificity *cyclin D1* transcriptional repression. Kaiso's sequence-specific regulation of the *cyclin D1* promoter has been previously reported in the *Xenopus* system by our collaborator Dr. Pierre McCrea (MD Anderson Cancer Centre, Houston, TX) (88). Moreover, Kaiso

regulation of *cyclin D1* is currently under investigation in another *Xenopus* lab that studies methylation-dependent Kaiso transcriptional regulation (Personal communication, Richard Meehan). Collectively these findings strongly support the idea that Kaiso utilizes a bi-modal mechanism of transcriptional regulation of *cyclin D1* that is conserved across species.

Several lines of evidence hint that promoter methylation may play a key role in regulating *cyclin D1* gene expression. The rat *cyclin D1* promoter contains two CpG islands and their methylation status is crucial for maintaining steady state *cyclin D1* expression levels in rat leukemic cell lines (56). In human B-cell lymphoma cell lines, the *cyclin D1* promoter was observed to be hypomethylated in cyclin D1 expressing cell lines and methylated in non-expressing cell lines (62). Lastly the methyl-CpG binding proteins MeCp1 and MeCp2 have been observed in association with the *cyclin D1* promoter in MDA-231 breast tumour cells (99). Methyl-CpG associated Kaiso is observed in a multiprotein complex involving MeCp2 (94, 142) and together these two proteins may bind the *cyclin D1* promoter to direct *cyclin D1* transcriptional repression. Since *cyclin D1* is the master switch for the cell cycle, Kaiso's putative involvement in maintaining steady state *cyclin D1* expression levels through a methylation-dependent repression mechanism suggests that Kaiso is essential for the tight regulation of cell cycle dynamics.

4.7. Kaiso misexpresion effects on cyclin D1 mRNA.

To verify our luciferase assay results indicating that Kaiso represses the *cyclin D1* promoter, RT-PCR experiments were performed to investigate Kaiso misexpression effects on *cyclin D1* transcript levels. Our initial Kaiso-overexpressing experiments did not appear to have any effects on *cyclin D1* transcript levels (Figure 26A), suggesting that perhaps the promoter is already maximally repressed or occupied. The *cyclin D1* gene product is highly regulated throughout the cell cycle; therefore due to this careful control, further reduction of its low steady-state expression levels may be detrimental to the cells. *Cyclin D1* promoter methylation has been shown to maintain steady state gene expression levels (56), and our current findings suggest that Kaiso may regulate *cyclin D1* in a methylation-dependent manner. Therefore it is possible that Kaiso is involved in maintaining steady-state *cyclin D1* expression, and as such its overexpression may not enhance its activity.

To address this possibility we next investigated the consequences of Kaiso depletion on *cyclin D1* transcript levels. Indeed Kaiso depletion resulted in an approximate 3-fold increase in *cyclin D1* mRNA, and was readily observed in HeLa and MCF-7 cells (Figure 26C,E). These results corroborated the luciferase assay experiments and provided our first *in vivo* evidence that Kaiso functions to negatively regulate *cyclin D1* expression. Immunoprecipitation and Western blot experiments must be carried out to determine if this Kaiso depletion effect on *cyclin D1* transcript expression translates into a 3-fold increase at the protein level as well.

4.8. Kaiso and p120 modulation of β-catenin-mediated activation of the cyclin D1 promoter.

Cyclin D1 is a well-documented gene target of the Wnt/β-catenin/TCF signaling pathway; therefore while investigating Kaiso and p120 transcriptional regulation of the *cyclin D1* promoter, we also examined their effects on β-catenin-mediated activation of *cyclin D1*. As predicted, Kaiso inhibited and p120 enhanced β-catenin-mediated activation of the *cyclin D1* promoter (Figure 25). However, due to our previous observations that p120 is unable to relieve Kaiso-mediated repression of the *cyclin D1* promoter, it is plausible that the p120-induced enhancement of β-catenin-mediated activation of the *cyclin D1* promoter occurs independently of Kaiso. It remains to be determined whether p120 directly binds the *cyclin D1* promoter or whether the p120 effect is via some other nuclear p120-binding protein or through Rac1-mediated *cyclin D1* activation, as previously discussed.

The Kaiso-mediated inhibition of β-catenin transcriptional activity in the context of the *cyclin D1* promoter is consistent with published reports that Kaiso converges on a subset of Wnt/β-catenin target genes such as *matrilysin* and *siamois* (88, 119). This Kaiso effect most likely involves recruitment of co-repressors that inhibit the effects of β-catenin activation, or Kaiso-initiated DNA conformational changes that dissociate β-catenin from the promoter. Disruption of the Wnt/β-catenin/TCF signaling pathway contributes to the progression of many human tumours (14, 63, 81), and as such our recent findings that Kaiso inhibits the activation of Wnt target genes hints at a general role for Kaiso in counteracting Wnt-induced tumour progression. Future experiments

investigating the upstream signaling events that lead to Kaiso repression of Wnt target genes and inhibition of β-catenin-mediated activation of these genes will significantly advance our understanding of how Wnt signaling and the as yet undefined Kaiso signaling pathway(s) collectively contribute to tumourigenesis and cancer progression.

4.9. Putative mechanisms of Kaiso-mediated cyclin D1 repression.

Together our data demonstrate that Kaiso represses *cyclin D1* expression, however, we have yet to clearly define the mechanism(s) directing this repression. Our experiments to date strongly suggest that Kaiso repression of the *cyclin D1* gene occurs through its dual-specificity association with closely situated sequence-specific DNA elements and methyl-CpG di-nucleotides within the promoter (Figure 28). Although Kaiso has been characterized as a bi-modal transcription factor, it has been studied almost exclusively as either a sequence-specific or methylation-dependent DNA binding protein. However, our findings provide the first experimental evidence of a gene, *cyclin D1*, which is possibly regulated by Kaiso's simultaneous binding to the consensus KBS as well as methyl-CpG di-nucleotides (Figure 28). These findings warrant a more in-depth investigation into the possibility that Kaiso regulates genes through its ability to bind sequence-specific and methylated DNA within the same promoter.

However the possibility also exists that Kaiso's cytoplasmic roles may indirectly contribute to *cyclin D1* transcriptional regulation through upstream signaling events. Luciferase assays carried out using the Kaiso-NLS mutant that harbours minimal point mutations within the NLS, demonstrated that NLS-mutant Kaiso was able to repress the

cyclin D1 promoter at the same strength as wild type Kaiso (Appendix A, Figure A-2,B). This suggested that the *cyclin D1* repression effects observed in luciferase assay and RT-PCR experiments were due to cytoplasmic Kaiso activity, resulting in *cyclin D1* expression through signaling events (Figure 29). Recent findings demonstrating that Kaiso is a predominantly cytoplasmic protein in tumour tissue samples (118), our detection of some cytoplasmic Kaiso in breast tumour cells (Figure 9C, (84)), coupled with our minimal promoter reporter studies demonstrating that NLS-mutant Kaiso is still able to repress the *cyclin D1* promoter support the possibility that Kaiso may indirectly regulate *cyclin D1* expression via a currently unknown role for Kaiso in the cytoplasm (Figure 29). Our findings have laid the groundwork for future studies to effectively elucidate the mechanism by which Kaiso regulates *cyclin D1* and determine the physiological relevance of this repression.

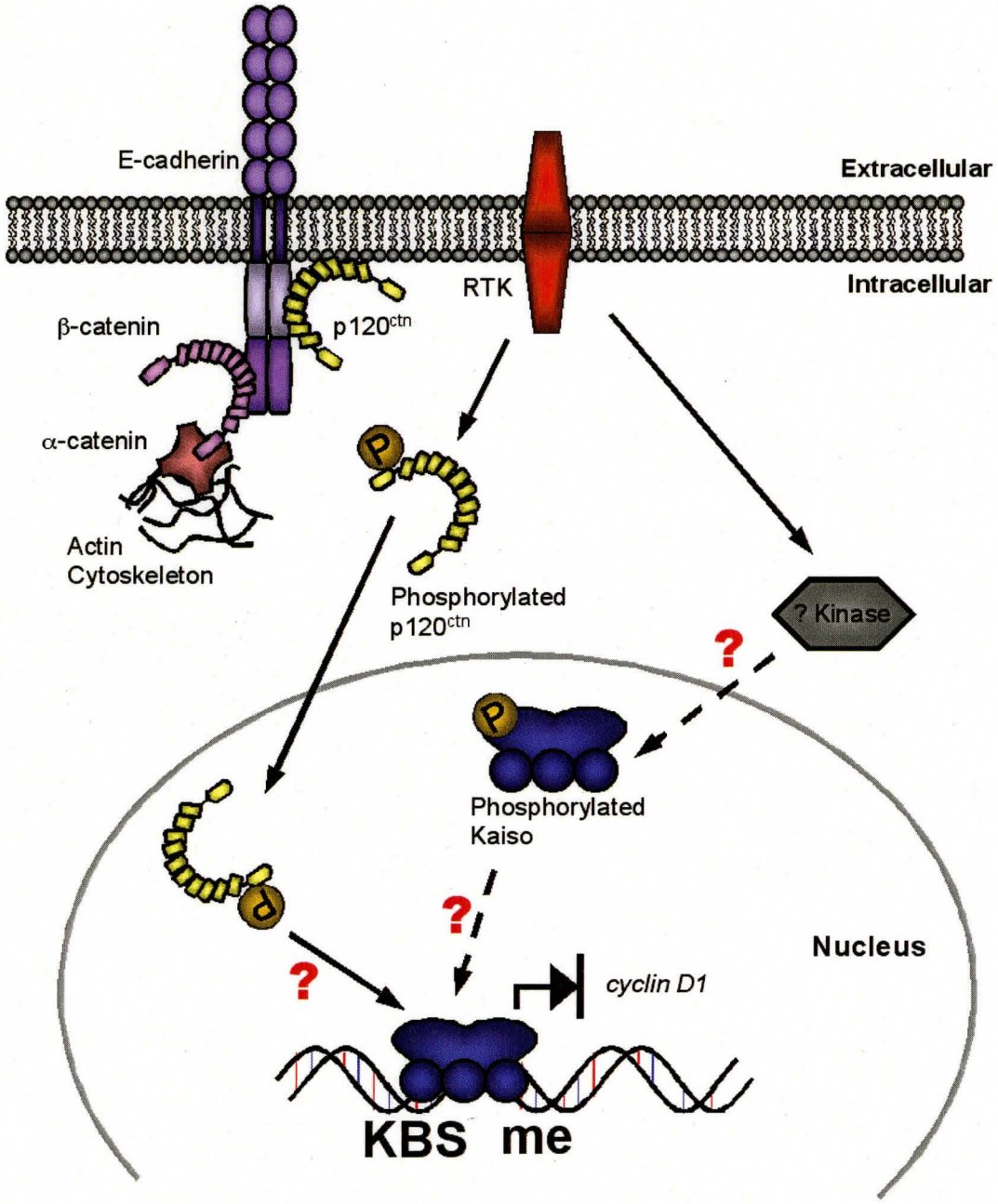


Figure 28: Bimodal DNA-binding-directed *cyclin D1* repression by Kaiso. Our experiments indicate that Kaiso associates with the *cyclin D1* promoter *in vivo* and associates with promoter-derived KBS elements and methylated CpG dinucleotides *in vitro*. This suggests that Kaiso repression of *cyclin D1* is directed by both its sequence-specific and methlyated DNA-binding abilities. Kaiso phosphorylation status may play a role in its ability to bind these DNA elements, and its association with p120 may not affect its regulation of *cyclin D1*. Dashed lines depict putative signaling events.

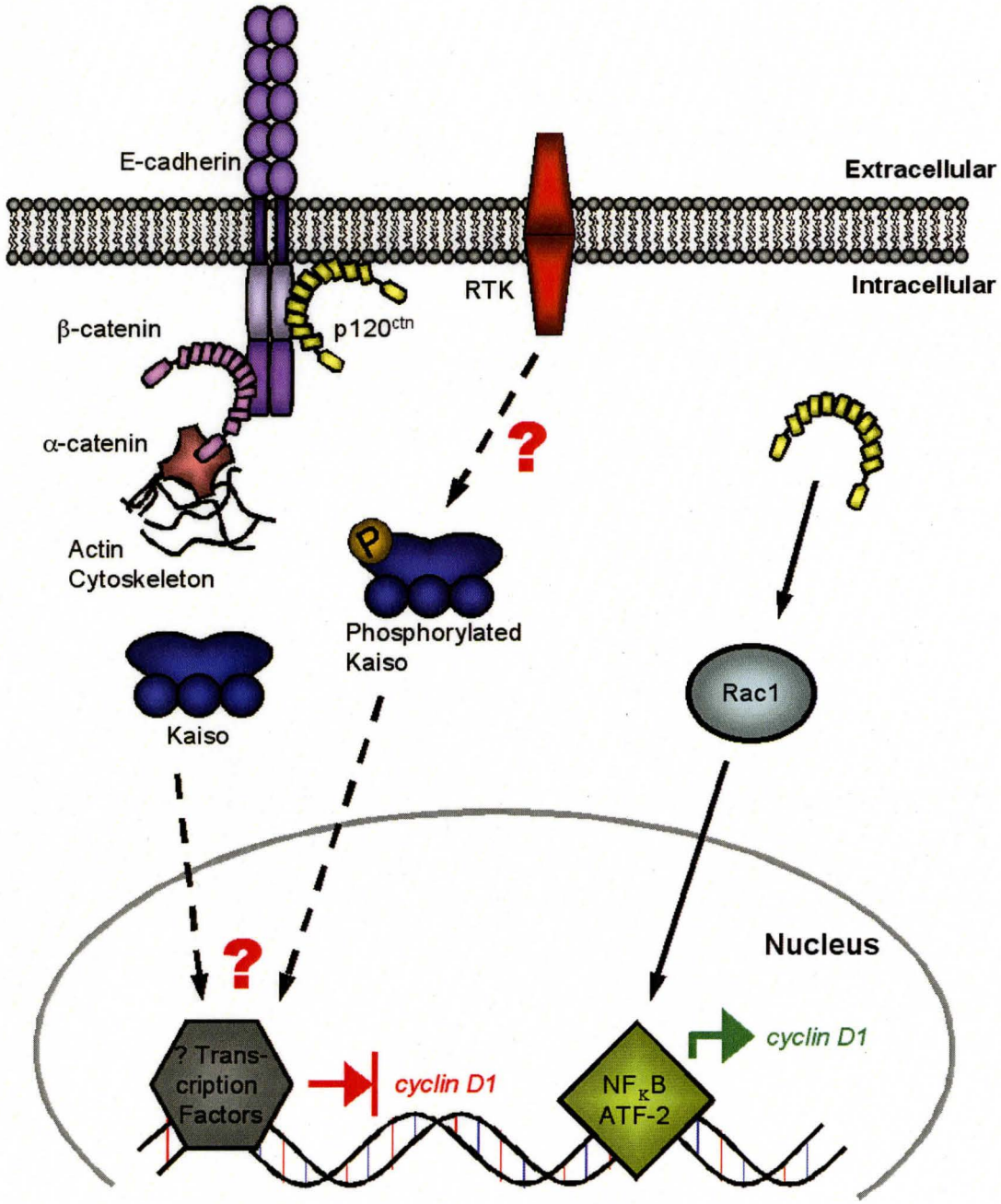


Figure 29: Potential cytoplasmic roles for Kaiso in directing *cyclin D1* repression. Luciferase assays performed with an NLS-mutant Kaiso expression construct demonstrate that cytoplasmic-restricted Kaiso still represses the *cyclin D1* promoter. This raises the possibility that cytosolic pools of Kaiso may be involved in upstream signaling events that result in *cyclin D1* repression through unknown transcription factors. p120 regulates the Rho GTPases, which activate *cyclin D1* through signaling events to transcription factors. Dashed lines depict putative signaling events.

4.10. A potential role for Kaiso in breast tumourigenesis.

Our results demonstrating that Kaiso associates with the *cyclin D1* promoter in a panel of breast cell lines and represses *cyclin D1* mRNA expression in MCF-7 breast tumour cells strengthens our hypothesis that Kaiso plays a role in breast tumourigenesis. This hypothesis first evolved after a Cancer Profiling Array revealed that Kaiso was misexpressed in approximately 40% of human breast tumours. The *cyclin D1* oncogene is overexpressed most often in breast tumours (3, 15, 116), and Kaiso's regulation of its expression provides another link between Kaiso and breast tumorigenesis. Our data suggest that Kaiso functions as a tumour suppressor in the context of its regulation of *cyclin D1* to inhibit breast tumour proliferation. As observed in these studies, aberrant Kaiso expression, specifically depletion, leads to increased *cyclin D1* transcript levels, which in turn may lead to increased cell proliferation. Alternatively, aberrant Kaiso phosphorylation may lead to *cyclin D1* activation if phosphorylation events render Kaiso unable to bind and repress the promoter. Furthermore the possibility exists that in tumour cells,abnormal *cyclin D1* promoter methylation may affect Kaiso's ability to bind and repress the promoter, thus perturbing Kaiso's tumour suppressor role and further promoting tumour progression.

Moreover, luciferase assays indicating that Kaiso inhibits β-catenin-mediated activation of the *cyclin D1* promoter further implicates Kaiso function in breast tumourigenesis. The Wnt signaling pathway is often perturbed in breast tumours (14), and Kaiso's repression of the Wnt target gene *cyclin D1* and its inhibition of β-catenin mediated activation of *cyclin D1* suggests that Kaiso may be involved in counteracting

breast tumour progression. This places Kaiso at a crucial point as a negative regulator of Wnt signaling and may involve Kaiso regulation of other Wnt target genes during breast tumourigenesis. Moreover our findings place Kaiso as a key factor in regulating cell cycle dynamics by maintaining steady *cyclin D1* expression levels. This process may be disrupted during tumour progression by aberrant Kaiso phosphorylation events or abnormal *cyclin D1* promoter methylation to enhance breast cell proliferation, and further promote tumour progression.

4.11. Conclusion and Significance.

In this study, we have characterized *cyclin D1* as a Kaiso target gene and effectively demonstrated that Kaiso represses *cyclinl D1*, and important cell cycle regulator. Furthermore we uncovered two putative mechanisms that may direct Kaiso-mediated *cyclin D1* repression; we discovered the exciting possibility that Kaiso represses *cyclin D1* by its dual-specificity DNA-binding ability (sequence-specific and methyl-CpG binding), as well as the possibility that this repressioin occurs via upstream cytoplasmic Kaiso functions. Lastly our data indicate that Kaiso and p120 modulate β-catenin-mediated activation of *cyclin D1*, and provide yet another example of Kaiso and p120 involvement modulating the outcomes of canonical Wnt signaling. Together these data strengthen our hypothesis that **Kaiso-mediated transcriptional regulation of *cyclin D1* plays a role in cell proliferation and breast tumourigenesis.**

These experiments are the first to investigate *cyclin D1* as a Kaiso target gene in mammalian cells, and add *cyclin D1* to the growing list of Kaiso target genes involved in cell growth and proliferation. Kaiso repression of *cyclin D1* places it at a key position as a regulator of cell cycle dynamics, and further investigation of this regulation and the resulting functional consequences will enhance our understanding of the molecular factors involved in tumour progression. Our continued investigations of the dual-specificity POZ-ZF transcription factor Kaiso and its associated co-factors will aid in clarifying the roles that Kaiso plays in normal cell growth, development and tumourigenesis.

Appendix A: Control Experiments

The data presented here represent control experiments that were carried out to clarify luciferase assay results indicating that Kaiso is a transcriptional repressor of *cyclin D1*.

Appendix A: Control Experiments

Materials and Methods

A.1. Luciferase Assays.

Luciferase assays were carried out as previously described. 0.2 μg of reporter construct and up to 1.0 μg of effector DNA were co-tranfected into HeLa cells using the Exgen-500 transfection reagent (MBI Fermentas). The effectors used in these experiments were as follows: pcDNA3-hKaiso, pcDNA3-hΔPOZ, pcDNA3-hKaiso-NLSmut, pcDNA3-Bcl6, siRNA Kaiso 3-1, and siRNA Kaiso 544. 30 hours post-transfection the cells were lysed and assayed for luciferase activity.

Results and Discussion

A.2. Kaiso does not repress transcription from the pGL3 Basic backbone vector.

Luciferase assay experiments using the 1748CD1 construct suggested that Kaiso is a transcriptional repressor of *cyclin D1* promoter. However, the possibility existed that the effects seen in the luciferase assays were due to the pGL3 Basic backbone vector and not the 1748CD1 partial *cyclin D1* promoter insert. To address this possibility Kaiso expression vector was co-transfected with the pGL3 Basic backbone vector into HeLa cells, which were assayed for luciferase expression 30 hours later. Luciferase expression levels did not change when Kaiso was co-transfected with pGL3 Basic, indicating that the

results seen with the 1748CD1 construct were due to the presence of the 1748CD1 insert (Figure A-1).

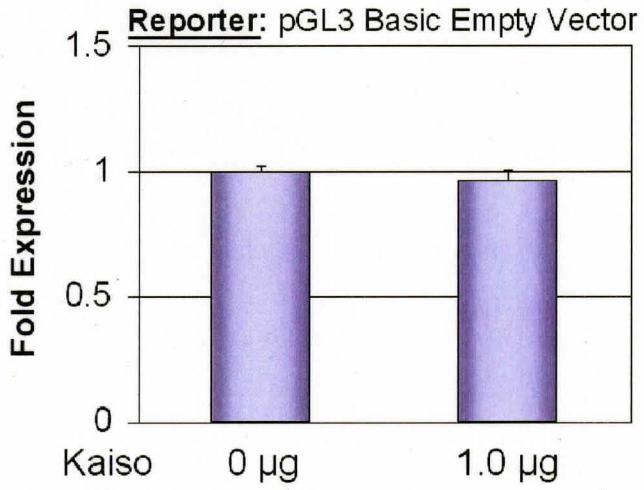


Figure A-1: Kaiso overexpression has no effect on the empty pGL3 Basic vector. Kaiso expression vector was co-transfected along with the pGL3 Basic empty backbone vector to determine if Kaiso expression had any effect on luciferase expression in the absence of the 1748CD1 *cyclin D1* promoter. Transfection of 1.0 µg of Kaiso did not alter luciferase expression.

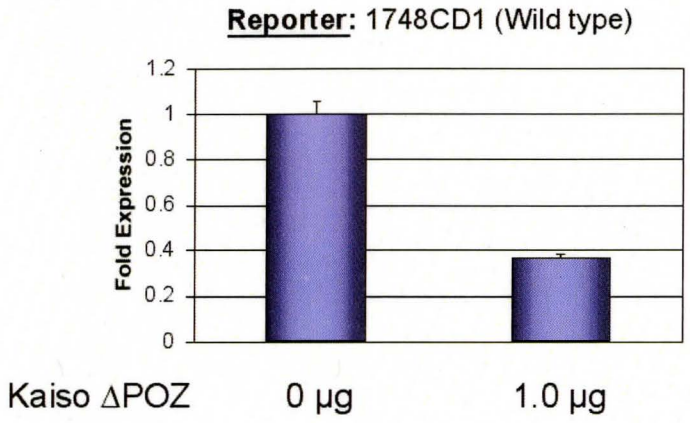
A.3. Kaiso mutant proteins repress the cyclin D1 promoter.

Kaiso mutant expression vectors were transfected into HeLa cells along with the 1748CD1 construct and luciferase expression levels were assessed. First pcDNA3-hΔPOZ was ectopically expressed along with the *cyclin D1* promoter reporter, with the prediction that little to no change would be observed in luciferase expression levels. The POZ domain is known as a site where transcriptional co-repressors often bind, therefore we predicted that removal of the Kaiso POZ domain would inhibit Kaiso transcriptional repression of *cyclin D1*. Unexpectedly, deletion of the POZ domain did not inhibit Kaiso transcriptional repression of *cyclin D1*, but rather repressed the *cyclin D1* promoter 2-fold, at the same strength as wild type Kaiso (Figure A-2,A). This observation, although highly unexpected indicated that perhaps there are other repression domains located within the Kaiso sequence that are responsible for its transcriptional repression activities.

We next tested what effects of the Kaiso NLS mutant (hNLSm) on the *cyclin D1* promoter. Again we predicted that ectopic expression of this Kaiso mutant would have no effect on the promoter, and again our results were contrary to our predictions. 1.0 μg of hNLSm repressed the *cyclin D1* promoter 2-fold (Figure A-2,B), as potently as wild type Kaiso. This result suggested that perhaps Kaiso's effects on the *cyclin D1* promoter is due to upstream cytoplasmic signaling that is then propagated in the nucleus. However, it is also plausible that the Kaiso NLS mutant protein is not completely restricted to the cytosol. Although the Kaiso NLS was extensively characterized previously (49), this was done with murine Kaiso, and current follow-up experiments

characterizing the human Kaiso NLS have not differentiated between endogenous and exogenous Kaiso.

A.



B.

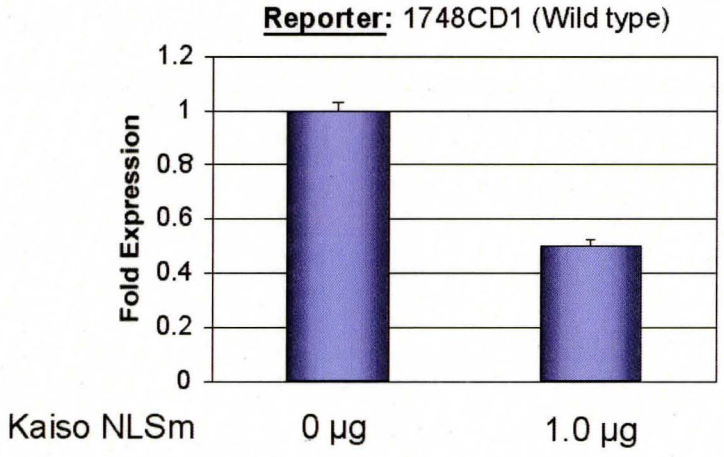


Figure A-2: Kaiso mutant proteins repress the *cyclin D1* promoter. Mutant Kaiso expression vectors were utilized along with the 1748CD1 *cyclin D1* promoter reporter construct in control luciferase assay experiments. (A) A Kaiso mutant lacking the POZ repression domain repressed expression of the *cyclin D1* promoter reporter 2-fold. (B) A second experiment carried out with the Kaiso NLS mutant, which localizes primarily to the cytoplasm, also repressed expression of the *cyclin D1* promoter reporter 2-fold.

A.4. Kaiso siRNA constructs have opposite effects on the cyclin D1 promoter.

Since the effects of Kaiso overexpression on the *cyclin D1* promoter were difficult to verify by conventional mutant protein overexpression control experiments, we decided to investigate the effects of Kaiso depletion using siRNA methodologies. We first utilized the plasmid-based siRNA Kaiso 3-1 construct, which targets Kaiso at nucleotides 1294-1012. Luciferase assays using this construct were very promising, showing a 2-fold de-repression of the *cyclin D1* promoter upon siRNA transfection (Figure A-3,A). However, immunoprecipitation and Western blot experiments could not demonstrate any depletion of Kaiso at the protein level using this construct (Figure A-3,B). The siRNA Kaiso 3-1 construct was previously utilized in experiments in Cos-1 monkey fibrolasts (119), so the possibility existed that it may function in the context of monkey cells but not human cells.

We then generated a new siRNA Kaiso construct that targeted nucleotides 544-562 of human Kaiso; previous studies had determined that this siRNA efficiently depleted Kaiso levels (142). Immunoprecipitation and Western blot experiments were first carried out to verify the efficacy of the siRNA Kaiso 544 plasmid in knocking down Kaiso levels. This plasmid-based system, induced efficient Kaiso depletion (Figure A-3,D), so luciferase assays were subsequently carried out. To our surprise, however, the siRNA Kaiso 544 construct *repressed* luciferase expression under control of the partial *cyclin D1* promoter almost 2-fold (Figure A-3,C). It is possible that in the luciferase assay system, Kaiso depletion by the siRNA Kaiso 544 construct is inefficient, disrupting Kaiso homodimers to enhance Kaiso repression activity. The opposite effects observed

with these two different siRNA constructs is difficult to interpret or explain, and poses many questions about how siRNA depletion functions and whether it is a good tool for use in luciferase assays.

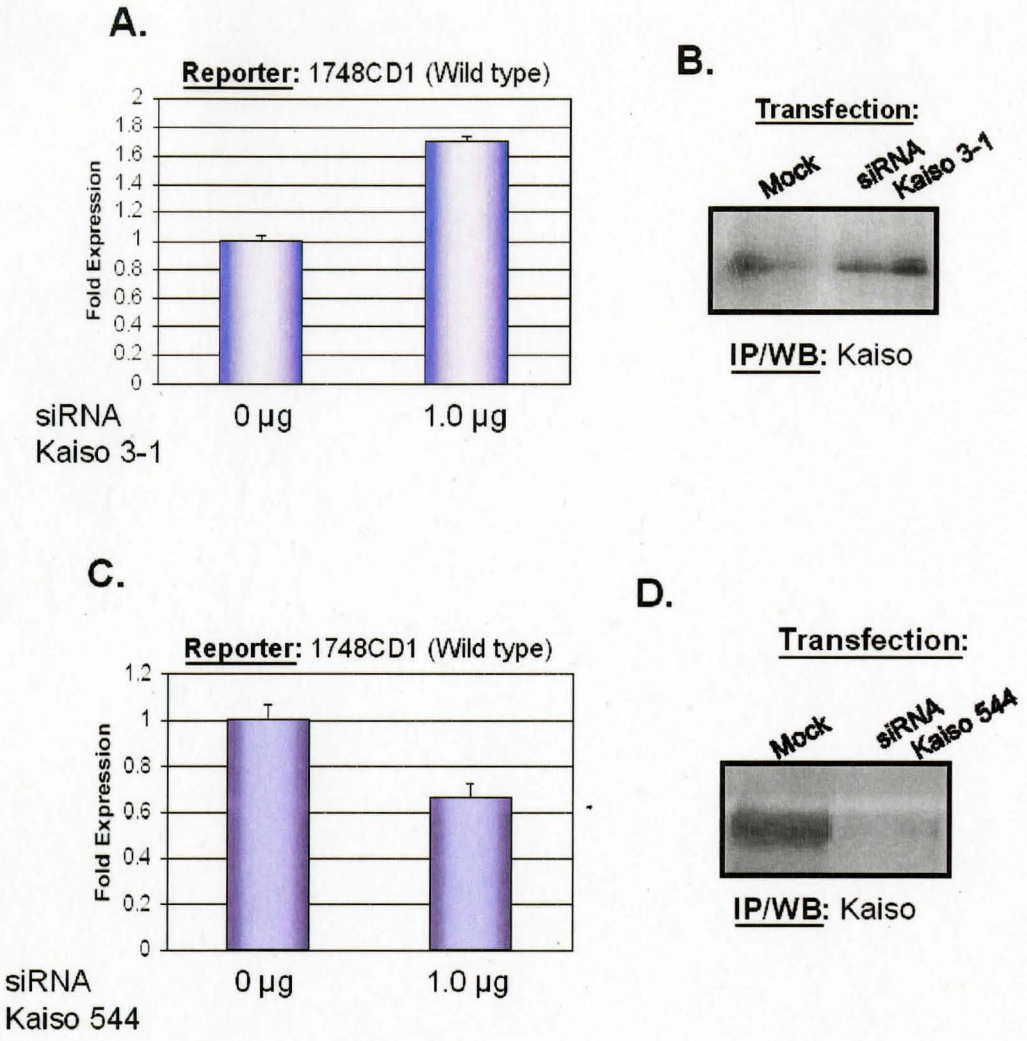


Figure A-3: SiRNA Kaiso constructs have opposing effects on luciferase expression. Plasmid vectors containing two different Kaiso siRNA target sequences were analyzed for their effects on the *cyclin D1* promoter. Co-transfection of the siRNA Kaiso 3-1 vector, targeting Kaiso nucleotides 1294-1012, along with the 1748CD1 *cyclin D1* promoter reporter resulted in a 2-fold derepression of the *cyclin D1* promoter as predicted (A). However, immunoprecipitation and Western blot analysis did not demonstrate any decrease in Kaiso expression upon siRNA Kaiso 3-1 vector transfection (B). A second Kaiso siRNA sequence, targeting Kaiso nucleotides 544-562, was inserted into a plasmid vector and utilized in luciferase assays. Transfection of this Kaiso siRNA vector along with 1748CD1 resulted in a 70% *repression* of the *cyclin D1* promoter (C). Kaiso depletion at the protein level was verified by Immunoprecipitation and Western blot (D).

Appendix B: Supplemental Data

The data presented here are a summary of results obtained during the course of this thesis project that are beyond the scope of the project. However, these results may provide a basis for future research into Kaiso function.

Appendix B: Supplemental Data

Materials and Methods

B.1. Luciferase Assays.

At the beginning of this project the pcDNA3-Kaiso expression vector in house was of murine origin, and was used for luciferase assays as previously described. 0.2 μg of reporter construct and up to 1.0 μg of pcDNA3-Kaiso effector DNA were co-tranfected into HeLa cells using the ExGen-500 transfection reagent (MBI Fermentas). 30 hours post-transfection the cells were lysed and assayed for luciferase activity.

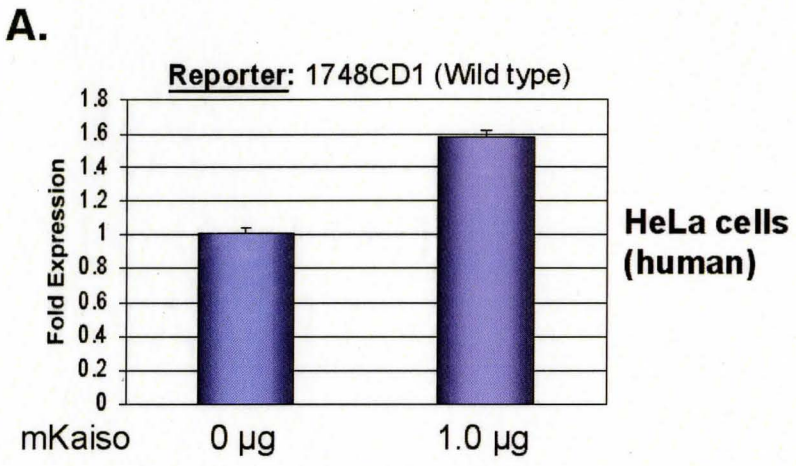
B.2. Electrophoretic Mobility Shift Assays.

Murine GST-fusion Kaiso proteins were initially utilized for the EMSA experiments prior to our generation of human GST-fusion Kaiso proteins. The experiments described here utilize the murine-specific GST-ΔPOZ, GST-ZF123, and GST-ΔPOZΔZF proteins, which are homologous in DNA-binding ability to their counterparts of the human protein. The remainder of the experimental procedure is the same as previously described for those carried out with the human GST-fusion proteins. Briefly 250 ng of purified GST-fusion murine Kaiso proteins were incubated at 37°C for 25 minutes and then incubated on ice for 30 minutes with radiolabelled *cyclin D1* promoter-derived probes. The samples were separated by polyacyrlamide gel electrophoresis and visualized by autoradiography.

Results and Discussion

B.3. Murine Kaiso activates cyclin D1 expression in HeLa cells

Our initial analyses of Kaiso effects on the *cyclin D1* promoter indicated that Kaiso was a transcriptional activator of this promoter (Figure B-1,A). This was an unexpected result since we had predicted that Kaiso would repress this gene; however, this was not an unprecedented result since Kaiso was recently established as an activator of the *rapsyn* gene in neuromuscular cells (108). However, a deeper investigation of our result demonstrated that it was specific to murine Kaiso overexpression in human HeLa cells. In this human cell line murine Kaiso proved to be an activator of the 1748CD1 *cyclin D1* promoter reporter, up to 1.5-fold (Figure B-1,A). However, when expressed in the murine fibroblast cell line, NIH 3T3, murine Kaiso exhibited transcriptional repression effects on the 1748CD1 *cyclin D1* promoter reporter (Figure B-1,B). Moreover, when expressed in HeLa cells and co-tranfected with the 1MK2MK-CD1 KBS mutant partial *cyclin D1* promoter reporter construct, murine Kaiso was still able to activate the *cyclin D1* promoter (Figure B-2).



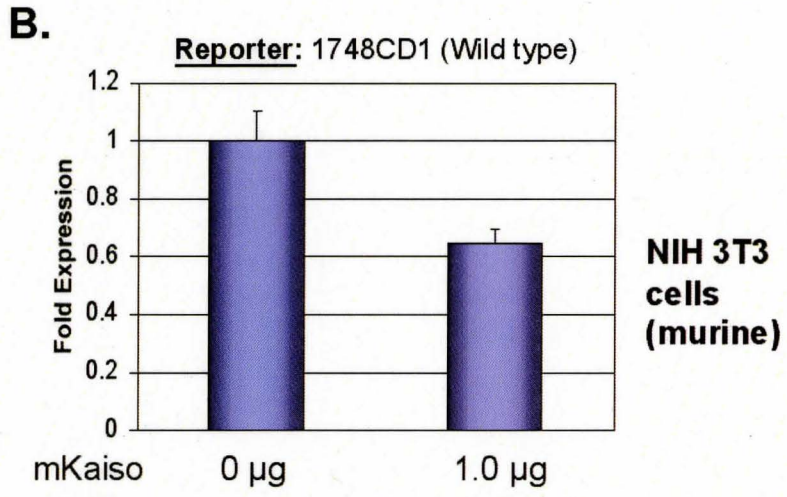


Figure B-1: Murine Kaiso exhibits different transcriptional activity in human vs. murine cell lines. The HeLa cell line was chosen for utlization in luciferase assasys due to its high transfection efficiency. Murine Kaiso was co-transfected along with the 1748CD1 cyclin D1 promoter reporter construct and the HeLa cells were later assayed for luciferase expression. Murine Kaiso activated the cyclin D1 promoter 1.5-fold, contrary to our preditions that it would repress the promoter (A). To investigage this result, we next co-transfected murine Kaiso and 1748CD1 in the murine fibroblast cell line NIH 3T3. In this mouse cell line murine Kaiso repressed the cyclin D1 promoter almost 2-fold (B).

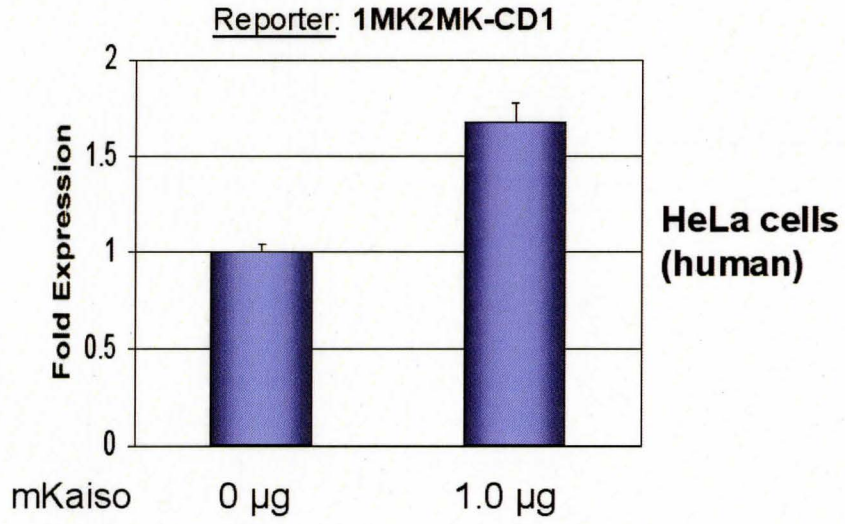


Figure B-2: Mutation of the KBS does not affect murine Kaiso regulation of the *cyclin D1* promoter. The murine Kaiso expression construct was used in initial luciferase assays characterizing the 1MK2MK-CD1 KBS mutant *cyclin D1* promoter reporter construct. These experiments reproducibly showed that murine Kaiso was still able to activate expression 1.6-fold from this construct although point mutations had been introduced into the two KBS.

At the same time of these intriguing findings another member of our lab was comparing the POZ domains of human and murine Kaiso. The POZ domains of POZ-ZF proteins are usually the site at which transcriptional co-repressors interact (69); these interactions are primarily controlled by specific amino acid residues within the POZ domain (69). Comparison of the human and murine POZ domains revealed that they differed at two key amino acid residues within the domain that have been established to direct co-repressor binding in other POZ proteins (Kevin Kelly, unpublished data and (69)). Specifically murine Kaiso contains an arginine residue at amino acid position 47 (R47), located within the POZ domain while human Kaiso contains a lysine residue at the same position (K47) (Kevin Kelly, unpublished data and (69)). Studies on the PLZF POZ domain established that R49 located within the POZ domain is key for directing its interaction with transcriptional co-repressors and determining the strength of its transcriptional repressor activities (69). Furthermore the Bcl-6 POZ domain contains a K47 residue, and was found to be a stronger transcriptional repressor than PLZF (69), suggesting that a similar phenomenon might be occurring with murine and human Kaiso.

This finding could explain our superfluous luciferase assay results utilizing the murine Kaiso expression construct. The murine POZ domain contains the R47 residue, suggesting that it is a less potent transcriptional repressor than human Kaiso, which contains the K47 residue. In the context of its expression in human cell lines, murine Kaiso may very well exhibit transcriptional activator characteristics due to a lack murine-specific transcriptional co-repressors or an inability to recruit human-specific co-

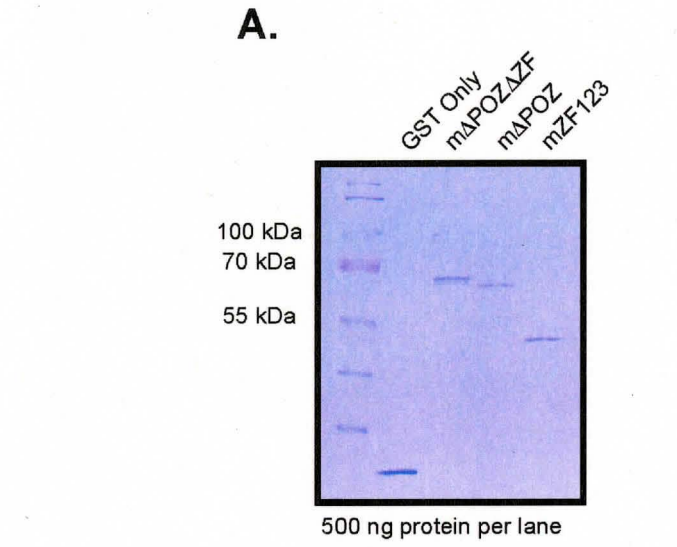
repressors. Lastly our finding that murine Kaiso was still able to elicit a response from the KBS mutant partial *cyclin D1* promoter, like its human counterpart, led us to speculate that in their respective systems murine and human Kaiso may have similar functions.

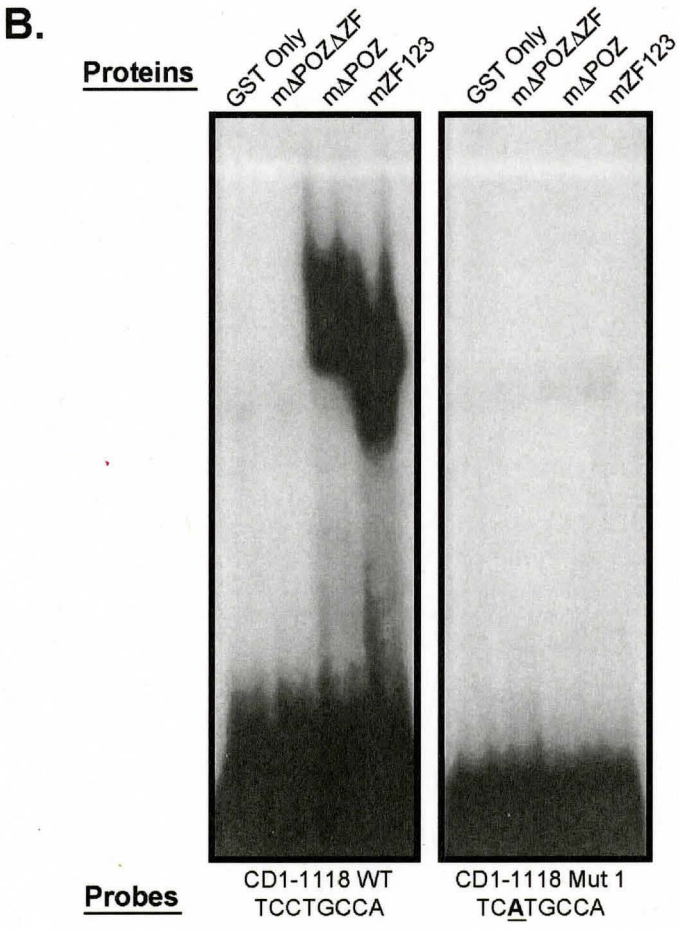
B.4. Murine Kaiso exhibits stronger binding to the cyclin D1 promoter than human Kaiso.

The original EMSA experiments investigating Kaiso DNA-binding specificity in the context of the *cyclin D1* promoter were carried out using murine Kaiso GST-fusion proteins that had been constructed by a previous member of our lab. Murine specific GST-ΔPOZ, GST-ZF123, GST-ΔPOZΔZF, and GST only (Figure B-3,A) were incubated with radiolabelled wild type and mutant *cyclin D1* promoter-derived probes as previously described. Initial experiments demonstrated that the murine Kaiso mutants efficiently bind the CD1-1118 probe (Figure B-3,B, left panel). Like its human counterpart, murine Kaiso is unable to bind a CD1-1118 probe with a single point mutation within the KBS (TCCTGCCA → TC**A**TGCCA) (Figure B-3B, right panel). However, unlike its human counterpart murine Kaiso is able to bind to the CD1+24 probe (Figure B-3,C, left panel). This binding is weaker than what is observed with the CD1-1118 probe and is predominantly observed with the ZF123 protein, but is still significantly noticeable and easily reproduced. Moreover, a C → A mutation within the CD1+24 KBS is insufficient for abolishing this binding (CTGCTA → **A**TGCTA) (Figure B-3, C, centre panel); a G → T mutation must be generated within this KBS before

murine Kaiso binding can be diminished, however, is not completey abrogated (CTGCTA → CT**T**CTA) (Figure B-3,C, right panel). Together these findings suggest that murine Kaiso may bind the KBS more efficiently than human Kaiso. The original CAST that identified the Kaiso sequence-specific DNA binding sequence was performed with murine Kaiso (20). The possibility exists that human Kaiso binds a slightly different sequence preferentially. Coupled with the luciferase assay results these observations allow for the speculation that human and murine Kaiso may differ significantly in function and even perhaps structure.

Figure B-3: Murine Kaiso binds both KBS of the *cyclin D1* promoter *in vitro*. EMSA analyses were carried out using a panel of murine GST-Kaiso fusion proteins mΔPOZΔZF, mΔPOZ, and mZF123, and GST protein only. Prior to EMSA analysis the proteins were expressed and purified from BL21 bacterial cultures (A). The purified proteins were incubated with radiolabelled *cyclin D1* promoter derived probes and the protein-DNA complexes were separated by gel electrophoresis The murine Kaiso proteins bind efficiently to the CD1-1118 probe, and a single base pair mutation introduced into the KBS of this probe (TCATGCCA) completely abolishes this binding (B). Similary, murine Kaiso proteins were able to bind the CD1+24 probe, albeit at a lower affinity than their binding to CD1-1118 (C). A C → A mutation within this KBS (ATGCTA) was not sufficient for abolishing mZF123 binding to this probe, and a G → T mutation (CTTCTA) could inhibit but not completely abolish mZF123 binding (C).


A.
GST Only
mΔPOZΔZF
mΔPOZ
mZF123
100 kDa
70 kDa
55 kDa
500 ng protein per lane


B.
Proteins
GST Only
mΔPOZΔZF
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mZF123
GST Only
mΔPOZΔZF
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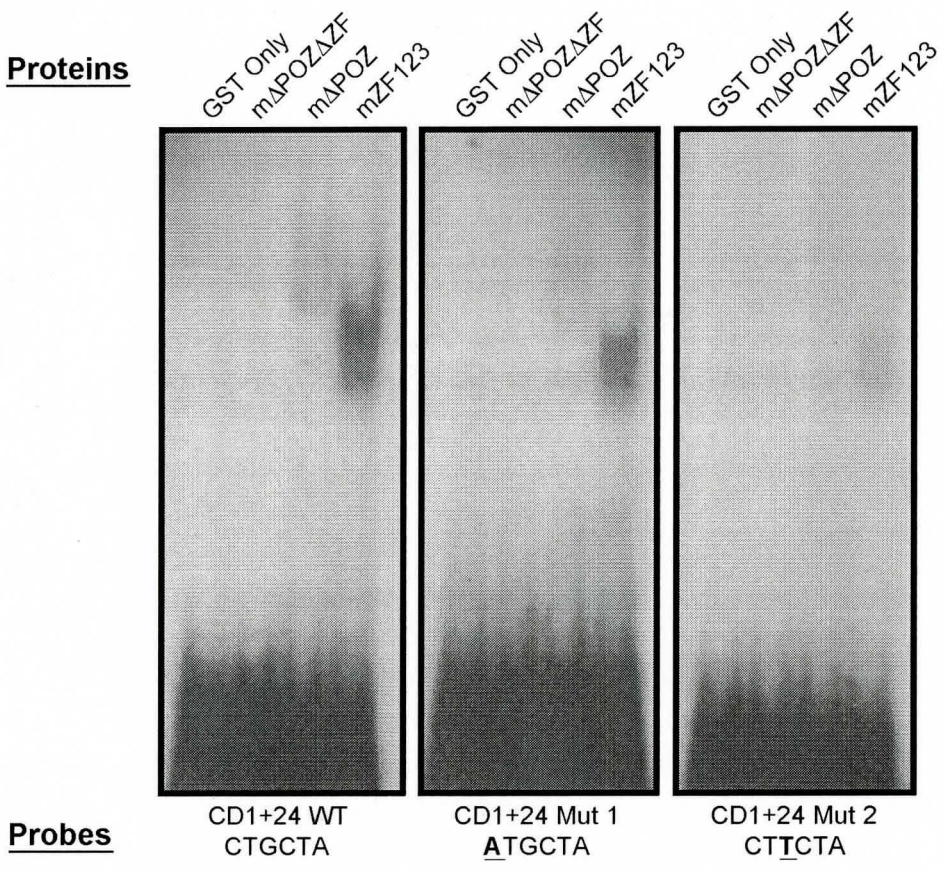
mZF123
Probes
CD1-1118 WT
TCCTGCCA
CD1-1118 Mut 1
TCATGCCA

C.


Proteins
GST Only
mΔPOZΔZF
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GST Only
mΔPOZΔZF
mΔPOZ
mZF123
GST Only
mΔPOZΔZF
mΔPOZ
mZF123
Probes
CD1+24 WT
CTGCTA
CD1+24 Mut 1
ATGCTA
CD1+24 Mut 2
CTTCTA

B.5. Murine Kaiso associates with methylated cyclin D1 promoter probes.

In light of the differing observations of murine and human Kaiso KBS binding, a few small-scale experiments were initiated to investigate murine Kaiso methylated DNA binding. 250 ng of murine Kaiso protein construct was utilized in each experiment to keep consistency with previously utilized human Kaiso protein amounts. Not only were the murine Kaiso protein mutants able to bind the unmethylated *cyclin D1* promoter probes more efficiently than human Kaiso as seen in Figure B-3, they were was also able to bind the methylated wild type CD1+24 probe more efficiently than human Kaiso as well (Figure B-4). Furthermore, murine Kaiso binding to the methylated wild type CD1+24 probe was stronger than its binding to the same probe in the unmethylated state (Figure B-4).

These results suggest that murine Kaiso may have an overall higher affinity for DNA binding than human Kaiso. However, these results also raise the possibility that the protocol used for the purification and preparation of the murine and human Kaiso GST-fusion proteins may be better suited for murine Kaiso and may need further optimization for human Kaiso. Regardless of these implications it is clear that both human and murine Kaiso proteins bind methylated DNA and bind extremely well to methylated DNA fragments containing the KBS. This suggests the exciting possibility that for certain genes, such as *cyclin D1*, Kaiso binding to the KBS as well as to methylated DNA may co-ordinately function to induce transcriptional repression.

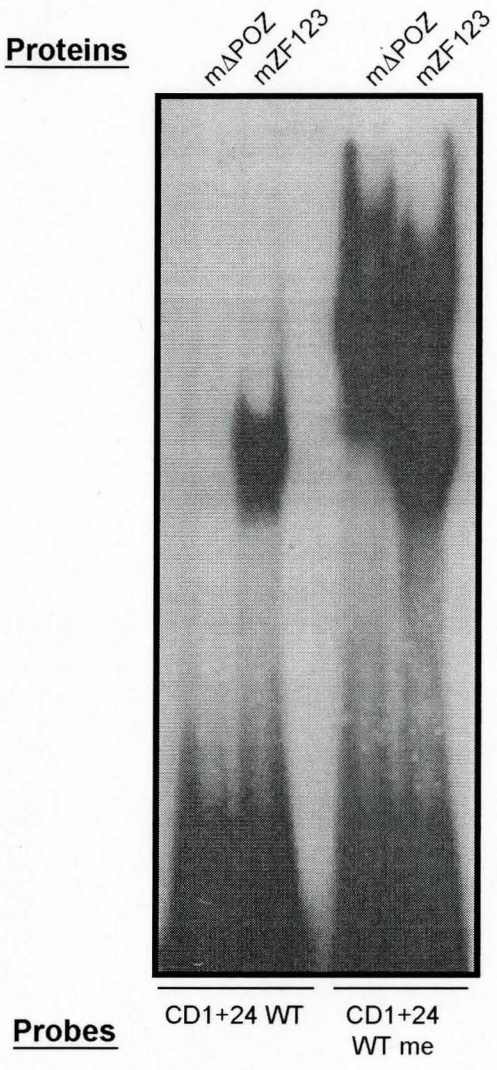

Figure B-4: Murine Kaiso binds methylated *cyclin D1* promoter-derived probes *in vitro*. The CD1+24 KBS is located within a predicted CpG island region of the *cyclin D1* promoter. Since murine Kaiso seemed to exhibit stronger DNA binding ability than human Kaiso we tested its ability to bind methylated DNA as well. The murine mZF123 binds the CD1+24 probe in the unmethylated state, but upon methylation of this probe, mZF123 as well as mΔPOZ binding increases significantly.

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