

KAISO'S REGULATION OF THE CELL CYCLE GENE *CCND1*

CHARACTERIZATION OF THE CELL CYCLE REGULATOR, *CCND1* AS A KAI1 TARGET GENE

BY

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ABSTRACT

Kaiso is a novel member of the BTB/POZ (Broad complex, Tramtrak, Bric à brac/Pox virus and zinc finger) zinc finger family of transcriptional regulators that have many roles in development and tumorigenesis. Kaiso was first identified as a binding partner for p120^{ctn}, an Armadillo catenin with roles in cell adhesion and stabilization of cadherins at the cell membrane. Kaiso is both an activator and repressor of gene transcription and interacts with two distinct types of DNA sequence; a consensus Kaiso binding site (KBS) TCCTGCNA and methylated CpG dinucleotide pairs (i.e. CpGCpG). Thus far p120's nuclear role is to inhibit Kaiso-mediated regulation of its target genes. Some of the Kaiso target genes identified to date include, *matrilysin*, *rapsyn*, and *MTA2*. The Kaiso homologue in *Xenopus laevis* (frog) has also been shown to regulate the cell cycle regulator *CCND1*. Sequence analysis of the human *CCND1* promoter revealed several potential Kaiso binding elements including both KBS and methylatable CpGs.

My research demonstrated that Kaiso binds to the *CCND1* promoter *in vitro* and *in vivo* to both KBS-specific and CpG-specific regions. Furthermore, I determined that Kaiso may act as either a repressor or activator of the human *CCND1* gene depending on the cellular environment. Altogether these data support my hypothesis that Kaiso is a regulator of the *CCND1* gene. Future studies looking at the significance of KBS versus CpG-binding on Kaiso's mechanism of regulation are required to determine the significance of this regulation. Furthermore, studies examining the cell cycle-dependent changes in Kaiso levels may reveal how alterations in Kaiso expression affect Kaiso target genes including *CCND1*.

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CONTRIBUTIONS BY OTHERS

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ABBREVIATIONS

Ab	<u>A</u> nti <u>b</u> ody
APC	<u>A</u> denomatous <u>p</u> olyposis <u>c</u> oli
Ar	<u>A</u> cidic <u>r</u> egion
Arm	<u>A</u> rmadillo
BSA	<u>B</u> ovine <u>s</u> erum <u>a</u> lbumin
b.p.	<u>b</u> ase <u>p</u> air
BTB-POZ	<u>B</u> road-Complex, <u>T</u> ramtrack, and <u>B</u> ric à brac/ <u>p</u> oxviruses and <u>z</u> inc finger
carb	<u>c</u> ar <u>b</u> enicillin
CBD	<u>C</u> atenin- <u>b</u> inding <u>d</u> omain
CDK	<u>C</u> yclin <u>d</u> ependent <u>k</u> inase
ChIP	<u>C</u> hromatin <u>I</u> mmunop <u>r</u> ecipitation
c.p.m.	<u>c</u> ounts <u>p</u> er <u>m</u> illion
DAB	3,3' <u>d</u> i <u>a</u> mino <u>b</u> enzidine
DMEM	<u>D</u> ulbecco's <u>M</u> odified <u>E</u> agle <u>M</u> edium
DNMT	<u>D</u> NA <u>m</u> ethyl <u>t</u> ransferase
Dsh	<u>D</u> is <u>s</u> hevelled
ECL	<u>E</u> nhanced <u>c</u> hemiluminescence
EGF	<u>E</u> pithelial <u>g</u> rowth <u>f</u> actor
EMSA	<u>E</u> lectrophoretic <u>m</u> obility <u>s</u> hift <u>a</u> ssay
FBS	<u>F</u> etal <u>b</u> ovine <u>s</u> erum
GBP	<u>G</u> SK-3 β <u>b</u> inding <u>p</u> rotein
GSK-3	<u>G</u> lycogen <u>s</u> ynthase <u>k</u> inase- <u>3</u>
GST	<u>G</u> lutathione- <u>S</u> - <u>t</u> ransferase
HDAC	<u>H</u> istone <u>d</u> e <u>a</u> cetylase
HS	<u>H</u> orse <u>s</u> erum
IHC	<u>I</u> mmunohistochemistry
IPTG	<u>I</u> sopropyl-beta-D- <u>t</u> hiogalactopyranoside
JMD	<u>J</u> uxta <u>m</u> embrane <u>d</u> omain

KBS	<u>K</u> aiso <u>b</u> inding <u>s</u> ite
LARII	<u>L</u> uciferase <u>A</u> ssay <u>R</u> eagent II
LRP5/6	<u>L</u> ow-density-lipoprotein <u>r</u> eceptor related <u>p</u> roteins <u>5</u> or <u>6</u>
mAb	<u>m</u> onoclonal Ab
MCL	<u>M</u> antle <u>c</u> ell <u>l</u> ymphoma
MBD	<u>M</u> ethyl <u>b</u> inding <u>d</u> omain
MBP	<u>M</u> ethyl <u>b</u> inding <u>p</u> rotein
MBT	<u>M</u> id- <u>b</u> lastula <u>t</u> ransition
MM	<u>M</u> ultiple <u>m</u> yeloma
MMP	<u>M</u> atrix <u>m</u> etallo <u>p</u> roteinases
NLS	<u>N</u> uclear <u>l</u> ocalization <u>s</u> ignal
PBS	<u>P</u> hosphate <u>b</u> uffered <u>s</u> aline
PBST	<u>P</u> BS- <u>T</u> riton X 100
PNK	<u>P</u> olynucleotide <u>k</u> inase
pAb	<u>p</u> olyclonal <u>A</u> b
qRT-PCR	<u>Q</u> uantitative <u>R</u> eal- <u>T</u> ime <u>P</u> olymerase <u>C</u> hain <u>R</u> eaction
Rb	<u>R</u> etinob <u>b</u> lastoma
RLU	<u>R</u> elative <u>l</u> ight <u>u</u> nits
r.p.m.	<u>r</u> evolutions <u>p</u> er <u>m</u> inute
SAM	<u>S</u> - <u>a</u> denosyl- <u>m</u> ethionine
S Phase	<u>S</u> ynthesis <u>P</u> hase
TBS	<u>T</u> ris <u>b</u> uffered <u>s</u> aline
TCF/LEF	<u>T</u> - <u>c</u> ell <u>f</u> actor/lymphoid <u>e</u> nhancer <u>f</u> actor
WB	<u>W</u> estern <u>b</u> lot

INTRODUCTION

1.1. Overview

Tumorigenesis is a process that is concomitant with a number of characteristic changes at the molecular, cellular and tissue level. Cells undergo changes in gene expression that allow them to become more proliferative, undifferentiated, and motile. Tumour cells typically harbour mutations in genes that regulate processes such as the cell cycle; for example, *CCND1* (gain of function), or *RB* (loss of function). These mutations allow cells to divide uncontrollably to create a primary mass or tumour. Phenotypic changes to tumour cells allow them to break away from the primary tumour and metastasize to distant parts of the body. Downregulation of cell adhesion factors such as epithelial-cadherin (E-cadherin) and upregulation of matrix metalloproteinases (MMPs) facilitate tumour cell metastasis. In fact, E-cadherin is often downregulated in 50% of metastatic tumours and this correlates with poor prognosis (Bremnes *et al.*, 2002). Specifically, loss of cell adhesion, especially through loss of E-cadherin, encourages cell metastasis. Since approximately 90% of malignant tumours are carcinomas of epithelial origin, the importance of the E-cadherin cell-cell adhesion complex in tumour progression cannot be overstated (Behrens *et al.*, 1989; Birchmeier and Behrens, 1994).

1.2. The E-cadherin/catenin complex

In epithelial cells, E-cadherin form adherens junctions which are necessary for the development and maintenance of cell-to-cell adhesion and tissue architecture, reviewed in

(Yap *et al.*, 1997). E-cadherin is a transmembrane glycoprotein that forms calcium-dependent homodimers with other E-cadherin molecules on the same cell, reviewed in (Takeichi, 1988). This allows cis-clustering of these homodimers which then form homophilic interactions with other cadherin molecules on adjacent cells and this trans-association allows the cells to adhere to each other. The E-cadherin molecule is anchored to the actin cytoskeleton through the interaction of its cytoplasmic domain with the catenin cofactors α -, β - and γ -catenin. The E-cadherin juxtamembrane domain (JMD) domain interacts with the catenin p120^{ctn} (hereafter p120) which regulates E-cadherin stability and turnover (Figure 1). This interaction is important for cell-cell adhesion as it maintains adhesive strength and stability (Yap *et al.*, 1998). E-cadherin also interacts with β -catenin through its C-terminal catenin binding domain (CBD) (Aberle *et al.*, 1994), and this interaction links E-cadherin to the actin cytoskeleton via α -catenin. Interestingly, defects in E-cadherin regulation is a common hallmark of many tumours of epithelial origin and is often implicated in tumour metastasis. Furthermore, in cells where E-cadherin levels are normal, defects are noted in one or more of the catenins, reviewed in (Birchmeier and Behrens, 1994).

Defects in catenin expression and function have been implicated in metastatic cancers, suggesting that their function is vital in counteracting tumour initiation and/or progression. Normally, cells form tissues through their ability to create and maintain connections with adjacent cells. This process depends largely on three types of cellular junctions that form at the apical regions of the lateral membrane. This includes tight junctions, desmosomes and adherens junctions (Farquhar and Palade, 1963). The latter of

these allows cells to adhere to each other via adhesion molecules, specifically classical cadherins such as E-cadherin.

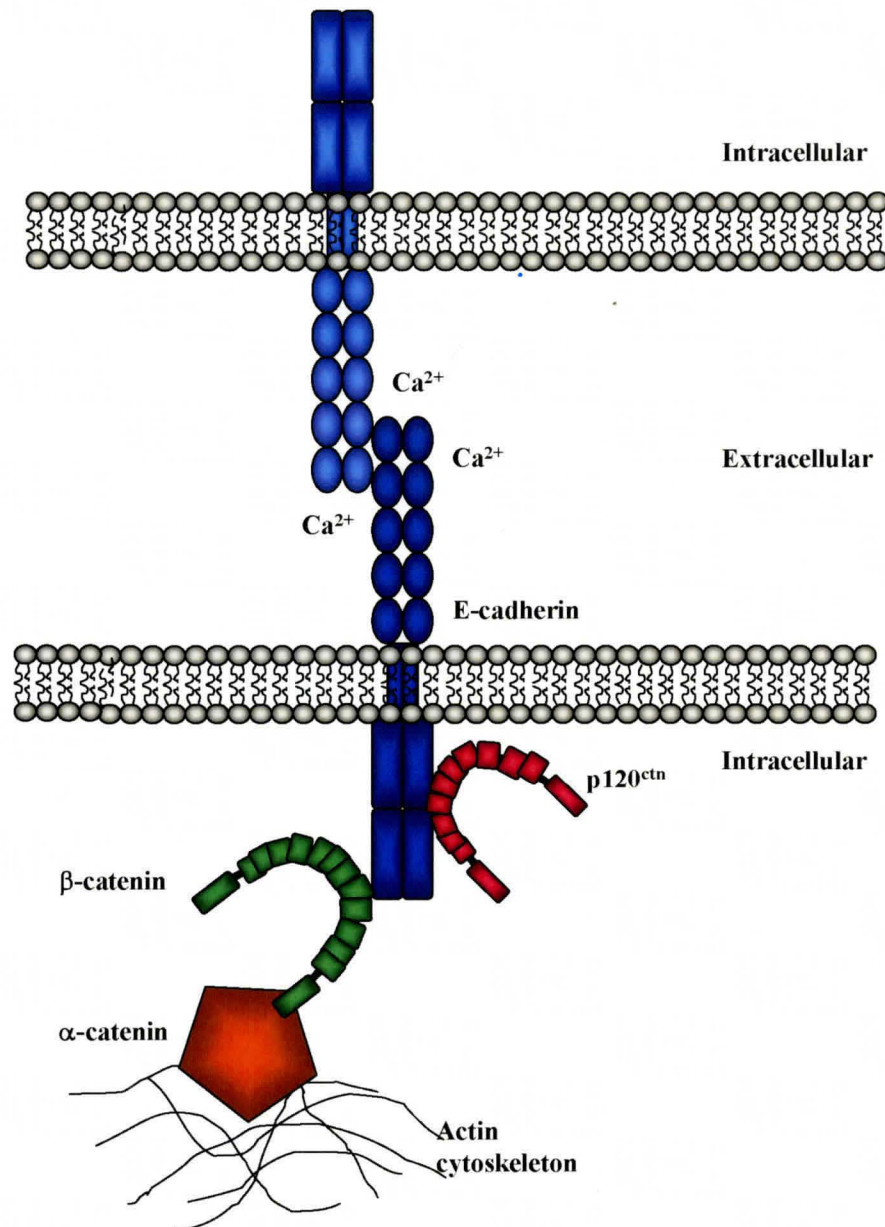


Figure 1: E-cadherin/catenin complex. Schematic of the E-cadherin/catenin complex. The transmembrane protein E-cadherin mediates cell-cell adhesion via Ca^{2+} -dependent homophilic interactions with E-cadherin molecules on adjacent cells. E-cadherin is bound to p120 via its juxtamembrane domain (JMD) whereas β -catenin is bound via a C-terminal catenin binding domain (CBD), linking E-cadherin to the actin cytoskeleton via α -catenin. p120 regulates E-cadherin stability and turnover.

1.3. The multifunctional Armadillo catenins.

The Armadillo catenins (β -, γ -, and p120) are each characterized by a carboxy-terminal Arm domain that consists of 10 or more tandem copies of 42 amino acid repeat first identified in *Drosophila* Armadillo protein (Wieschaus and Riggleman, 1987). The Armadillo catenins were originally thought to function only as cell-adhesion co-factors for the classical cadherin adhesion molecules. Indeed, β -catenin has been shown to interact with E-cadherin and link it to the underlying actin cytoskeleton, thus stabilizing the adhesion complex (Wheelock and Knudsen, 1991). In contrast, the more recently characterized p120 catenin binds and regulates stability and turnover of E-cadherin rather than anchoring it to the actin cytoskeleton (Davis *et al.*, 2003; Davis and Reynolds, 2006; Ireton *et al.*, 2002; Reynolds *et al.*, 1994).

Increasing evidence has shown that these Armadillo catenins are also involved in regulating gene expression changes within the nucleus. It has been established that β -catenin translocates into the nucleus where it activates transcription of downstream target genes such as *CCND1*, *matrilysin* and *Id2* (Behrens *et al.*, 1996; Crawford *et al.*, 1999; Rockman *et al.*, 2001; Tetsu and McCormick, 1999). More recently a similar function has been assigned to p120; p120 translocates to the nucleus and modulates gene expression through interaction with its binding partner, the transcription factor Kaiso (Daniel and Reynolds, 1999; Spring *et al.*, 2005). Altogether, these data demonstrate that Armadillo proteins have multiple diverse functions in the cell; they act as cell adhesion cofactors and they act as signalling molecules in the nucleus, where they alter gene expression. Furthermore, p120 catenin has been shown to have a role in regulating Rho-GTPases in

the cytoplasm, reviews in (Anastasiadis and Reynolds, 2001). Both cell adhesion and signalling functions are altered in tumours and during metastasis, showing that the proper function of catenin proteins is essential for a healthy cell.

1.4. β -catenin and the Wnt Pathway

In addition to its role in the E-cadherin/catenin complex, β -catenin is also involved in cell signalling events as a component of the Wnt signalling pathway. Under normal conditions, in the absence of Wnt ligands, β -catenin is recruited to a multi-protein complex containing Glycogen synthase kinase 3 (GSK-3), Axin and Adenomatous polyposis coli (APC). This results in the phosphorylation of β -catenin by GSK-3 and casein kinase 1 (CK1), ultimately leading to the ubiquitination and subsequent degradation of β -catenin in the cytoplasm (Aberle *et al.*, 1997; Gao *et al.*, 2002; Liu *et al.*, 2002; Orford *et al.*, 1997). The Wnt pathway is activated when Wnt ligands interact with the transmembrane receptor Frizzled, which then complexes with the co-receptor LRP5/6 (low-density-lipoprotein receptor related proteins 5 or 6) (He *et al.*, 2004). This activates the intracellular protein Dishevelled (Dsh), which mediates the release of β -catenin from the APC, Axin, and GSK-3 multiprotein complex. Dsh's activity inhibits phosphorylation of β -catenin by introducing alternate binding partners for various members of the multi-protein complex that normally function to target β -catenin for destruction, reviewed in (Logan and Nusse, 2004). For example, Dsh interacts with GSK-3 binding protein (GBP) which then interacts with GSK-3, thus preventing the phosphorylation of β -catenin (Salic *et al.*, 2000; Yost *et al.*, 1998). This unphosphorylated β -catenin is no longer degraded via

ubiquitin-mediated proteolysis and hence it accumulates in the cytoplasm (Logan and Nusse, 2004). Excess cytoplasmic β -catenin then translocates to the nucleus and interacts with TCF/LEF (T-cell factor/lymphoid enhancer factor) transcription factors to activate transcription of target genes such as, *CCND1*, *matrilysin*, *ID2*, and *c-myc* (Crawford *et al.*, 1999; He *et al.*, 1998; Rockman *et al.*, 2001; Tetsu and McCormick, 1999) (Figure 2).

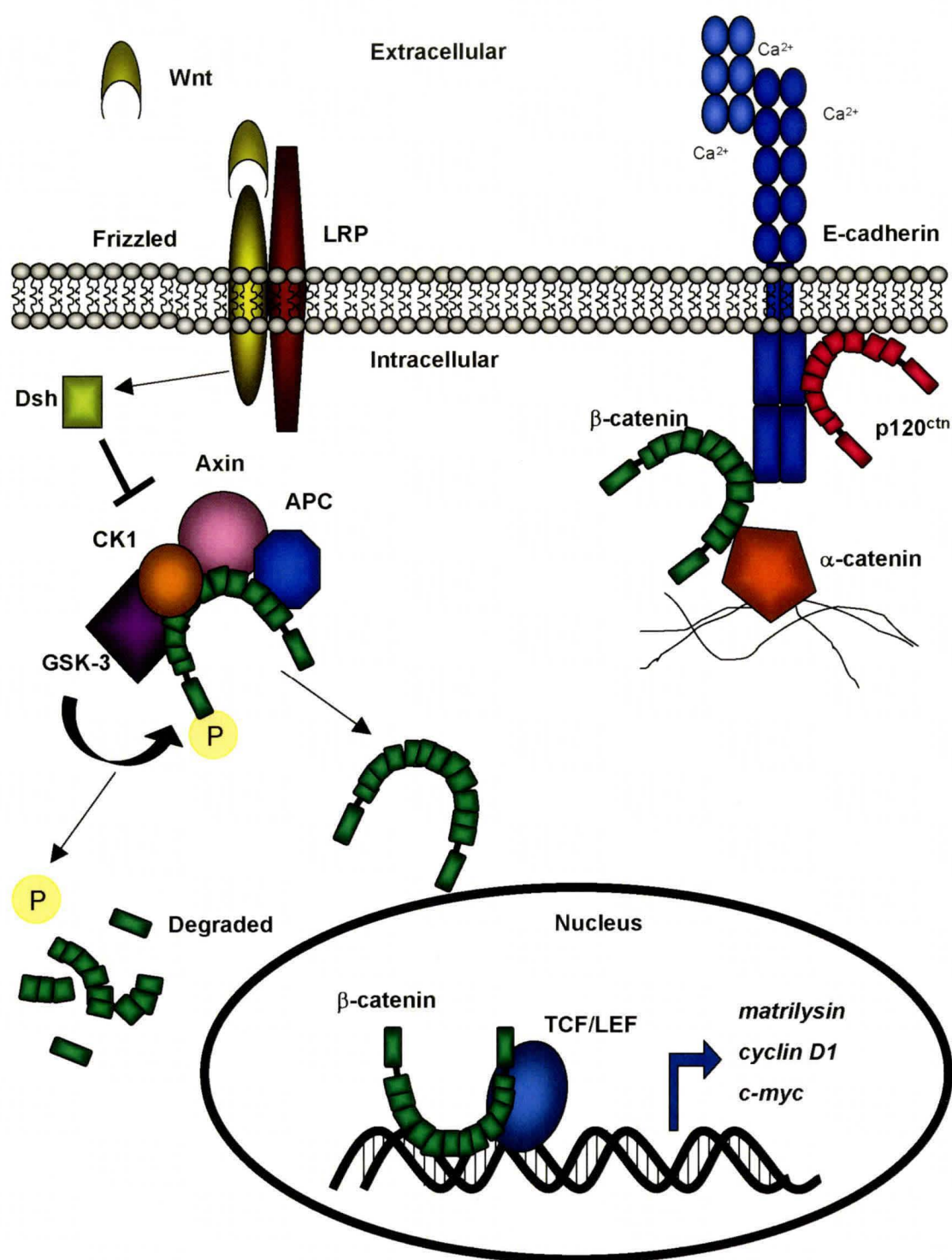


Figure 2: The Canonical Wnt Signalling Pathway. Upon binding of the Wnt ligand to the Frizzled/LRP receptor complex, the Dsh protein is activated. Dsh acts to inhibit the multi-protein complex that normally targets β -catenin for degradation. Cytoplasmic accumulation of β -catenin occurs, allowing it to translocate to the nucleus and activate its downstream target genes via interaction with the TCF-LEF transcription factor.

1.5. p120^{ctn}, a Src substrate and Armadillo catenin.

The catenin p120 (hereafter p120) was originally identified as a substrate for the non-receptor Src tyrosine kinase (Downing and Reynolds, 1991; Kanner *et al.*, 1991) and later found to be phosphorylated by various receptor tyrosine kinases upon growth factor stimulation (Reynolds *et al.*, 1992) (Figure 3). p120 is the prototypical member of the p120 subfamily of Armadillo proteins that includes plakophilin, ARVCF and δ -catenin (Anastasiadis and Reynolds, 2000). These proteins typically contain ten to thirteen Armadillo (Arm) repeats of 42 amino acids each (Peifer *et al.*, 1994). The p120 protein contains 10 Arm repeats and is 991 amino acids in length with a molecular mass of ~120 kDa. Unlike other p120 subfamily members, p120 exists as multiple isoforms due to different start codons and splicing events in the *p120* gene (*CTNND1*) (Keirsebilck *et al.*, 1998). Interestingly, different tissues express different isoforms of p120, with epithelial cells preferentially expressing isoform 3 and fibroblast cells preferentially expressing isoform 1 (Aho *et al.*, 2002).

The presence of an Arm domain and the structural similarity of p120 to β -catenin led to the discovery that p120 was a component of the E-cadherin/catenin complex (Aghib and McCrea, 1995; Reynolds *et al.*, 1994; Shibamoto *et al.*, 1995; Staddon *et al.*, 1995). Like β -catenin, p120 binds E-cadherin through its Arm domain but unlike β -catenin, p120 binds E-cadherin via the JMD (Daniel and Reynolds, 1995). Also unlike β -catenin, p120 does not interact with α -catenin or APC suggesting that it plays a distinct role in cadherin-mediated cell adhesion (Daniel and Reynolds, 1995).

Indeed, p120 has a unique role at the cell membrane relative to the classic catenin family members. One of the core functions of p120 at the plasma membrane is to stabilize E-cadherin, reviewed in (Reynolds and Carnahan, 2004). Initial studies revealed that siRNA knockdown of p120 levels in SW48 colon carcinoma cells resulted in a dramatic decrease in E-cadherin levels that was rescued upon p120 re-expression (Ireton *et al.*, 2002). Furthermore, this effect was shown to be posttranslational since p120 expression had no effect on E-cadherin mRNA levels. In fact, p120 only has an effect on E-cadherin once it reaches the cell membrane. If p120 is absent from the cell membrane E-cadherin is rapidly degraded, reviewed in (Reynolds and Carnahan, 2004). p120 acts to inhibit the binding of alternate inhibitory E-cadherin binding partners at the JMD thereby protecting it from degradation (Baki *et al.*, 2001; Fujita *et al.*, 2002). For example, the relatively uncharacterized protein, Hakai, which is an ubiquitin ligase enzyme, has been shown to bind to E-cadherin at the JMD and target it for ubiquitination (Fujita *et al.*, 2002). Likewise, the protein Presenilin-1 also binds to the JMD and targets E-cadherin for degradation via proteolytic cleavage (Baki *et al.*, 2001). Together this data demonstrates that p120 has a protective role of E-cadherin at the cell membrane, and in its absence E-cadherin is susceptible to proteolytic degradation.

In addition to its role at the plasma membrane, p120 also possesses a cytoplasmic role. Free cytoplasmic p120 not bound to the E-cadherin complex (Papkoff, 1997; Thoreson *et al.*, 2000) plays a role in regulating the activity of Rho-GTPases; p120 inhibits RhoA (Anastasiadis *et al.*, 2000; Noren *et al.*, 2000), and activates Rac and Cdc42 (Grosheva *et al.*, 2001; Noren *et al.*, 2000). Rho-GTPases have important roles in

cytoskeletal organization and cell motility and thus p120's regulation of these proteins demonstrates its role in these processes as well.

In addition to p120's roles in cytoskeletal organization, p120 also has a nuclear role (Kelly *et al.*, 2004b; Rocznik-Ferguson and Reynolds, 2003; Thoreson *et al.*, 2000; van Hengel *et al.*, 1999). A nuclear role for p120 was experimentally demonstrated when it was found that p120 inhibited the transcriptional activity of its binding partner Kaiso (Kelly *et al.*, 2004b) (Figure 3). Specifically, p120 inhibited the DNA-binding ability of Kaiso and the nuclear localization of p120 was necessary for this inhibition (Daniel and Reynolds, 1999; Kelly *et al.*, 2004b).

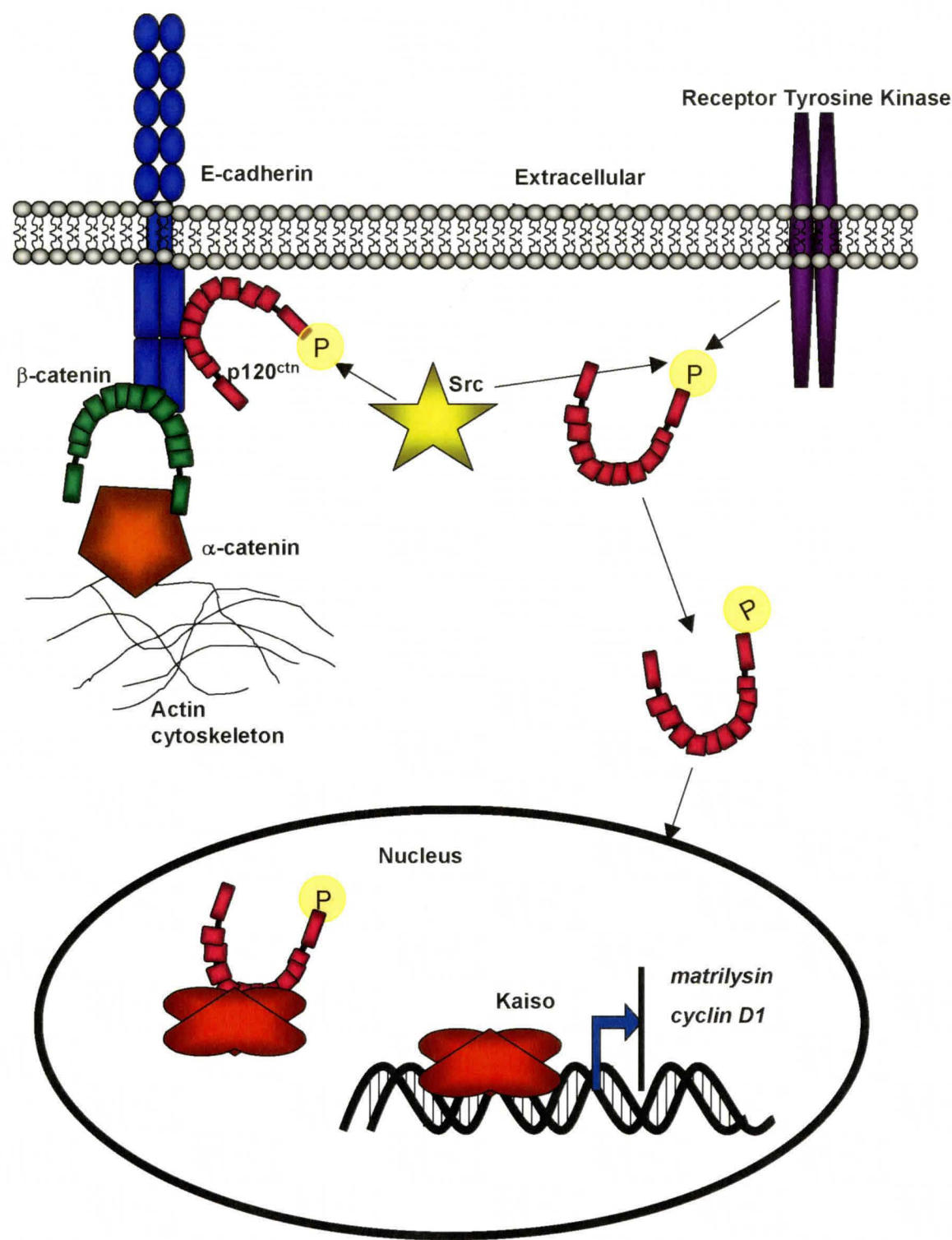


Figure 3: Kaiso/p120 Pathway. Schematic of the Kaiso and p120 interaction pathway. p120 is phosphorylated by Src or in response to receptor tyrosine kinase activation. It is currently unknown what triggers p120 to enter nucleus. The binding of p120 to Kaiso inhibits Kaiso's DNA-binding and hence may inhibit Kaiso's ability to regulate its downstream target genes.

1.6. The p120-binding partner Kaiso

Kaiso is a BTB/POZ (Broad-Complex, Tramtrack, and Bric à brac/poxviruses and zinc finger) domain zinc finger transcription factor (Albagli *et al.*, 1995; Bardwell and Treisman, 1994) that was first identified as a specific p120-binding partner in a yeast two-hybrid screen using the p120 Arm domain as bait (Daniel and Reynolds, 1999). Kaiso is a 672 amino acid protein with a molecular weight of ~100 kDa (Figure 4) (Daniel and Reynolds, 1999). Similar to other POZ-ZF proteins, Kaiso has an amino terminal POZ domain and three carboxy terminal C₂H₂ zinc fingers (Daniel *et al.*, 2002). The p120-Kaiso interaction is mediated by p120 Arm repeats 1 to 7 and a carboxy-terminal region of Kaiso that encompasses its DNA-binding ZF domain (Daniel and Reynolds, 1999). The p120-Kaiso interaction is mutually exclusive of E-cadherin since p120 also binds E-cadherin via its Arm domain (Reynolds *et al.*, 1994).

Interestingly, studies of Kaiso in *Xenopus laevis* embryos have implicated Kaiso in early development. XKaiso is expressed throughout *Xenopus* development from oocytes to the early tadpole stage (Kim *et al.*, 2002). XKaiso expression was localized primarily to areas of the nervous system, namely the brain, eye, ear, branchial arches and the spinal cord (Kim *et al.*, 2002). Like its mammalian counterpart, xKaiso interacts with xp120 (Kim *et al.*, 2002) and this interaction relieves xKaiso-mediated repression of Kaiso target genes (Kim *et al.*, 2004). Functional studies looking at the role of xKaiso in early development revealed that Kaiso knockdown results in gastrulation defects in developing *Xenopus* embryos (Kim *et al.*, 2004). Subsequent studies in *Xenopus* embryos revealed that xKaiso regulates a subset of both canonical (*Siamois*, *c-Fos*, *CCND1*, *c-*

Myc) and non-canonical (*Wnt11*) Wnt target genes (Kim *et al.*, 2004; Park *et al.*, 2005). Furthermore, in early *Xenopus* development xKaiso was shown to be a global regulator of methylation-specific gene silencing of zygotic gene expression prior to the mid-blastula transition (MBT) (Ruzov *et al.*, 2004). Knockdown of xKaiso results in the premature expression of zygotic genes (Ruzov *et al.*, 2004). Previous studies examining the role of classical methyl-binding proteins, containing the methyl binding domain (MBD), showed that their deletion resulted in no global changes of genome-wide gene expression occur (Chen *et al.*, 2001; Guy *et al.*, 2001; Hendrich *et al.*, 2001; Tudor *et al.*, 2002; Zhao *et al.*, 2003). This suggests that Kaiso is unique in this role.

Kaiso has been shown to possess dual-specificity DNA-binding properties; Kaiso binds to both a sequence-specific Kaiso binding site (KBS) TCCTGCnA, where n is any nucleotide, and it binds methyl-CpG dinucleotides (Daniel *et al.*, 2002). Electrophoretic mobility shift assays (EMSAs) using KBS-derived and methyl-CpG oligonucleotides showed that Kaiso binds to the sequence-specific KBS sequence with higher affinity than methyl-CpG islands (Daniel *et al.*, 2002). Although most POZ-ZF proteins act as transcriptional repressors, as exemplified by the oncoprotein Bcl-6 (Dhordain *et al.*, 1997), Kaiso has been shown to have both transcriptional activation and repression ability like ZF5 (Kaplan and Calame, 1997; Rodova *et al.*, 2004; Ruzov *et al.*, 2004; Spring *et al.*, 2005). Kaiso's mechanism of repression involves the recruitment of histone deacetylase (HDAC) co-repressor complex. Complexes containing N-CoR were found to target promoters in a methylation-dependent manner (Yoon *et al.*, 2003) while complexes containing mSin are postulated to target sequence-specific DNA-binding. Kaiso target

genes characterized to date include *matrilysin* (Spring *et al.*, 2005), *metastasin* (Prokhortchouk *et al.*, 2001), *siamois* (Park *et al.*, 2005), *MTA2* (Yoon *et al.*, 2003), *Wnt11* (Kim *et al.*, 2004), *rapsyn* (Rodova *et al.*, 2004) and *CCND1* (Cyclin D1) (Otchere, 2006; Park *et al.*, 2005), many of which have defined roles in tumourigenesis.

Studies looking at the sub-cellular localization of Kaiso have shown that in cultured cells Kaiso is predominately nuclear (Daniel and Reynolds, 1999). Indeed, it was found that Kaiso possesses a highly basic nuclear localization signal (NLS) which when mutated results in cytoplasmic accumulation of Kaiso (Kelly *et al.*, 2004a). However, subsequent studies examining the localization of Kaiso in the tumour microenvironment revealed that in tissues Kaiso is often found in the cytoplasm (Soubry *et al.*, 2005). Furthermore, Kaiso expression is progressively localized in the cytoplasm and completely absent in cells at the center of the tumour (Soubry *et al.*, 2005). This data suggests that Kaiso may function as a tumour suppressor in these tumours due to its absence in the progressing tumour. Interestingly, a subset of Kaiso target genes (i.e. *CCND1* and *matrilysin*) are also regulated by the Wnt/ β -catenin/TCF signalling pathway which has been shown to be aberrant in many cancers (Crawford *et al.*, 1999; He *et al.*, 1998; Rockman *et al.*, 2001; Tetsu and McCormick, 1999). This led us to believe that Kaiso may be acting as a modulator of the canonical Wnt signalling pathway.

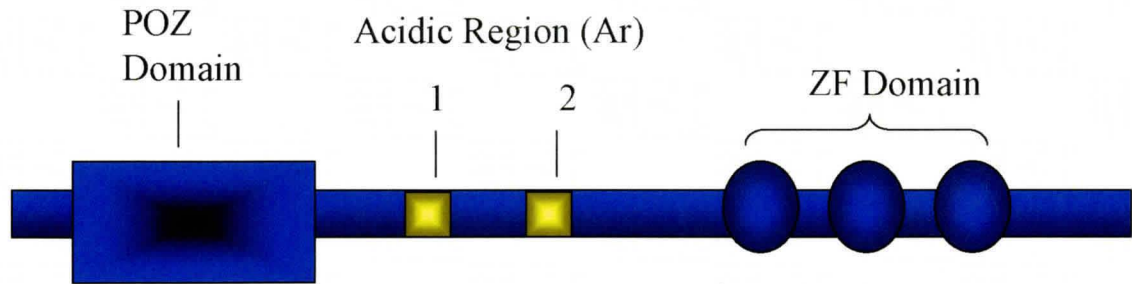


Figure 4: Schematic representation of Kaiso. The amino terminus of Kaiso contains a POZ domain that is involved in protein-protein interactions. In the central region there are two acidic regions that are typically associated with transcriptional activation. At the carboxy-terminus of Kaiso there are three C₂H₂ zinc fingers that mediate DNA binding.

1.7. *Cyclin D1*

Since xKaiso was shown to transcriptionally regulate the *CCND1* gene (Park *et al.*, 2005) in *Xenopus*, we postulated that Kaiso regulates *CCND1* in humans. *CCND1* is a Wnt/ β -catenin target gene whose promoter region contains three sequence-specific KBS's as well as six CpG dinucleotide pairs located within predicted CpG islands (Figure 5). The presence of these KBS in conjunction with these CpG dinucleotides hints at the possibility that *CCND1* may be a putative Kaiso target gene. Cyclin D1 is one of the regulatory subunits of the cyclin dependent kinases (CDKs) that play an important role in cell cycle progression and proliferation. Specifically, Cyclin D1 associates with CDK4/6 to regulate cell cycle progression through the first growth phase (G1) into the DNA synthesis phase (S phase), reviewed in (Stacey, 2003). The CyclinD1/CDK4/6 holoenzyme is responsible for the phosphorylation and inactivation of the Retinoblastoma (Rb) protein. Hyperphosphorylated Rb does not associate with the transcription factor E2F, and the newly freed E2F then activates transcription of target genes, such as cyclin E, which are required for cell cycle progression, reviewed in (Coqueret, 2002).

The Cyclin D1 protein is expressed during G1 phase but is rapidly degraded upon entry into S phase as it is no longer required for continued cell cycle progression (Stacey, 2003). In continuously dividing cells, Cyclin D1 levels are induced at the beginning of G2 phase by Ras to prepare for the next cell cycle (Guo *et al.*, 2002; Hitomi and Stacey, 1999). The *CCND1* gene has been shown to be regulated by a large number of different factors (Coqueret, 2002) and its expression is aberrant in many cancers where its misexpression is thought to contribute to tumorigenesis (Alle *et al.*, 1998; Buckley *et al.*,

1993). For example, in B-cell malignancy the *CCND1* promoter is hypomethylated leading to Cyclin D1 activation (Liu *et al.*, 2004). Furthermore, the rat *CCND1* promoter is regulated by CpG methylation (Kitazawa *et al.*, 1999) leading to the possibility that the human *CCND1* promoter is also regulated by methylation, possibly through its association with the methyl-binding protein Kaiso.

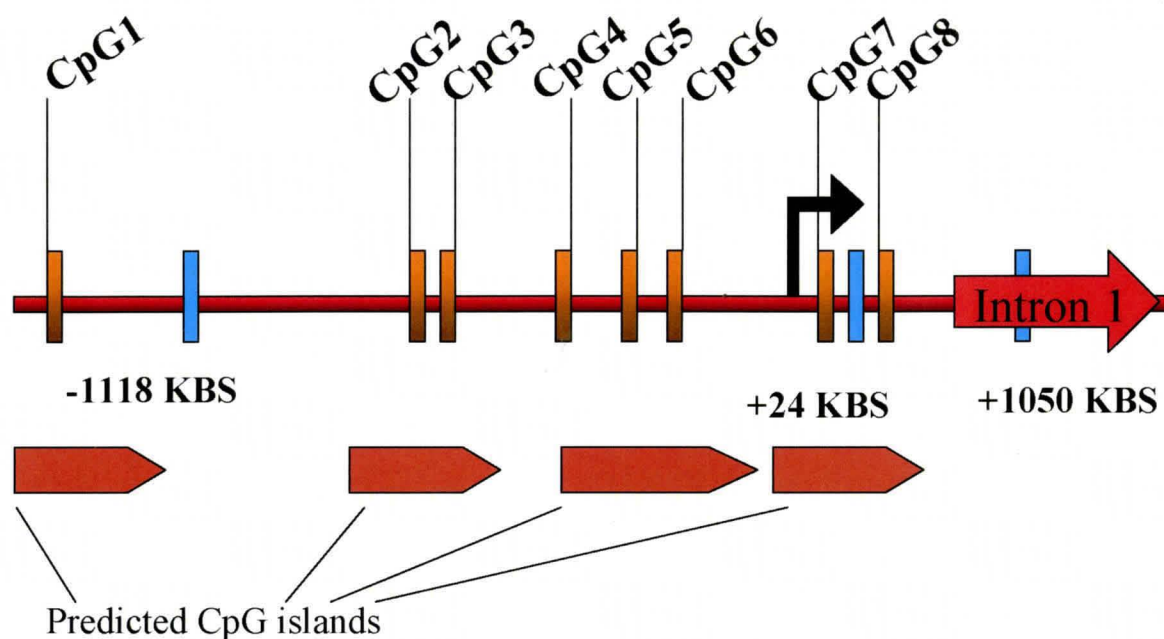


Figure 5: Schematic of *CCND1* promoter highlighting the KBS and CpG sites. The *CCND1* promoter contains three KBS sites (in blue) located at -1118, +24, and +1050 relative to the transcriptional start site. Within the *CCND1* promoter there are also a number of CpG dinucleotide pairs (6 in total) that are located within predicted CpG islands. The eight methylation-specific probes used in EMSA experiments (CpG1 to CpG8) are highlighted in orange.

1.8. Methylation

Epigenetic changes to gene expression are caused not by alterations in the primary nucleotide sequence but rather by other mechanisms such as DNA methylation or histone acetylation and deacetylation. In humans and mammals, methylation of cytosine residues that immediately precede guanosine residues (CpG) occurs frequently and is associated with transcriptional silencing of that particular promoter, reviewed in (Herman and Baylin, 2003). Methylation of DNA occurs through the covalent addition of a methyl group to the fifth carbon residue of the cytosine ring (Figure 6). This reaction is catalyzed by DNA methyltransferase enzymes using S-adenosyl-methionine (SAM) as a substrate (Herman and Baylin, 2003). Gene silencing due to methylation occurs through the association of Methyl-CpG binding proteins (MBP) to methylated DNA and the recruitment of co-repressor complexes (i.e. HDACs), reviewed in (Klose and Bird, 2006). To date, six MBPs have been identified, 5 of which contain a conserved methyl-binding domain (MBD1, MBD2, MBD3, MBD4, MeCP2) and Kaiso. Kaiso is unique among this group as it does not contain the methyl-binding domain (Daniel *et al.*, 2002; Prokhortchouk *et al.*, 2001). Since Kaiso has been shown to exhibit a unique function in repressing methylation-dependent global gene expression in *Xenopus* embryos before the MBT, it could also function independently of the other MBPs in humans (Ruzov *et al.*, 2004).

Abnormal DNA methylation and thus gene silencing has been found to be a hallmark of many human cancers. For example, hypermethylation of the *Rb* tumour suppressor gene is detected in many tumours (Ohtani-Fujita *et al.*, 1994) and correlates

with its downregulation. Consequently, the E2F transcription factor disassociated from Rb and becomes activated and this results in uncontrolled progression through the cell cycle in the absence of an upstream mitogenic signalling event. Conversely, oncogenes such as *CCND1* may be hypomethylated during tumourigenic processes. Activation of Cyclin D1 in B-cell malignancy is associated with loss of methylation and also hyperacetylation of the *CCND1* gene up to 120 kb upstream of the transcriptional start site (Liu *et al.*, 2004).

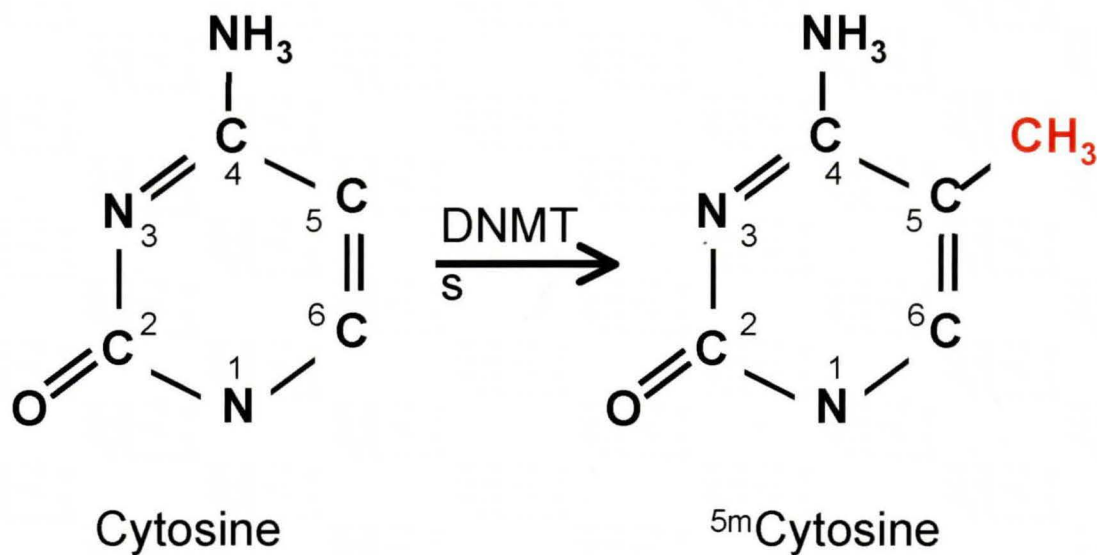


Figure 6: Schematic representing cytosine methylation. DNA methyltransferase enzymes (DNMTs) are responsible for methylation on the 5th carbon of cytosine nucleotides in cytosine-guanine sequences.

RATIONALE:

The regulation of the *CCND1* gene by xKaiso was first uncovered in amphibian (frog) development and raised the possibility that the *CCND1* gene may be a target gene of Kaiso in humans (Ruzov *et al.*, 2004). The presence of a number of Kaiso binding sites in the human *CCND1* promoter (both sequence-specific and methylation-specific) further supported this theory. Changes in the methylation status of the *CCND1* gene correlate with changes in its gene expression (Liu *et al.*, 2004) and since Kaiso is a methyl-CpG DNA-binding protein this raises the possibility that Kaiso may be involved in methylation dependent *CCND1* expression changes (Daniel *et al.*, 2002; Prokhortchouk *et al.*, 2001). Indeed, preliminary studies have shown that Kaiso has a negative regulatory effect on *CCND1* (Otchere, 2006). We sought to assess the association of Kaiso with methylated regions of the *CCND1* promoter and to investigate the effects of Kaiso on *CCND1* expression and protein levels.

My research has shown that Kaiso associates with regions of the *CCND1* promoter both *in vitro* and *in vivo* (both sequence-specific and methylation-dependent regions). Furthermore, in MCF7 cell lines Kaiso misexpression has been positively correlated with changes in Cyclin D1 protein levels. Alternatively, in HeLa cells Kaiso misexpression in minimal reporter assays has shown that Kaiso may repress the *CCND1* promoter in this cell line (and that *in vitro* methylation results in near complete repression from the minimal reporter construct). Altogether, my data implicate *CCND1* as a Kaiso target gene and that this is likely to depend on methylation.

MATERIALS AND METHODS

2.1. Cells and Tissue Culture

The human cervical carcinoma cell line (HeLa) and human breast epithelial cell lines [MCF-12A, MCF-10A (non-tumour) and MCF-7 (tumour)] used in these studies were purchased from ATCC (Manassas, VA). MCF-10A and MCF-12A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California) supplemented with 5% Horse Serum (Gibco/BRL, Grand Island, NY), 0.1 mg/mL penicillin/streptomycin and 0.25 µg/mL Fungizone (Invitrogen), 10 µg/mL Insulin (Sigma-Aldrich, St. Louis, MO), 5 µg/mL Hydrocortizone (Sigma-Aldrich), 20 ng/mL epithelial growth factor (EGF) (Invitrogen), and 100 ng/mL Cholera Toxin (Biomol). All other cells were cultured in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, Utah), 0.1 mg/mL penicillin/streptomycin and 0.25 µg/mL Fungizone, and grown in a humidified atmosphere at 37°C with 5% CO₂.

2.2. Serum Starvation and Stimulation

Prior to DNA, RNA or protein isolation, cultured cells were subjected to either serum stimulation or starvation conditions for 24 hours. Cells were washed once with 1X PBS and cells lysed according to the appropriate protocol. Media used for serum stimulation contained all supplements including the Fetal Bovine Serum (FBS) or Horse Serum (HS). Media used for serum starvation contained all supplements except the serum

(FBS or HS). Cells were then incubated as usual at 37°C in a 5% CO₂ incubator for 24 hours.

2.3. Protein Overexpression and Purification

Escherichia coli (*E. coli*) BL21 bacteria transformed with various pGEX-5X-1 glutathione-S-transferase (GST)-Kaiso fusion constructs were incubated overnight at 37°C in 12 mL of LB media supplemented with carbenicillin (carb) (Carbenicillin Direct, UK). The following morning 10 mL of the overnight culture were inoculated into 90 mL of fresh LB-carb media and incubated at 30°C for 90 minutes. Protein expression was induced by adding 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to the bacterial culture which was incubated for an additional 3 hours at 30°C. Bacteria were pelleted by centrifugation at 6,000 r.p.m. for 15 minutes at 4°C and stored at -20°C overnight.

The next morning the bacterial cell pellets were thawed on ice for 10 minutes before resuspension in ice-cold PBS/0.1% NP-40. Cells were lysed on ice by sonication for 3 minutes at 40% output and duty cycle 4. Lysates were cleared by centrifugation at 10,000 r.p.m. for 20 minutes at 4°C. 800 µL of GST beads equilibrated in PBS/0.1% NP40 were added to the lysates and incubated end-over-end at 4°C for one hour. The GST bead-protein complexes were captured and washed with 1X binding buffer (1 mM DTT, 25 mM HEPES [pH 7.5], 100 mM KCl, 1 mM EDTA, 10 mM MgCl₂, 0.1% NP-40, and 5% glycerol). The GST-Kaiso fusion proteins were eluted from the beads using 10 mM glutathione and the proteins were quantified by Coomassie stain on an SDS-PAGE

gel and compared to Bovine serum albumin (BSA) standards before use in EMSA experiments.

2.4. Stable Cell Lines

MCF-7 cells were stably transfected with the wild-type Kaiso cDNA cloned into the pcDNA3 overexpression plasmid using LipofectamineTM2000 (Invitrogen) according to the manufacturer's protocol. 24 hours post-transfection cells were split 1:10 and treated with G418 (Invitrogen) at 1200 $\mu\text{g/mL}$. Media was replaced every three days for approximately three weeks until individual colonies were formed. Individual colonies were picked and placed in 24-well plates, grown until confluency, moving them up to 6-well dishes until finally reaching the 100 mm dish size. Cells were then tested for Kaiso expression levels using Western blot analysis of whole cell lysates.

2.5. Electrophoretic Mobility Shift Assays

Specific oligonucleotides were created to span the three KBS sites as well as the 6 CpG dinucleotide pairs that are located in the *CCND1* promoter. A panel of GST-Kaiso fusion proteins (full-length Kaiso, Kaiso Δ POZ, Kaiso Δ POZ Δ ZF, Kaiso Δ ZF, Kaiso Δ AR1 (acidic region), Kaiso Δ AR2, and KaisoZF mutant) were used to test Kaiso binding to these DNA sites. The oligonucleotides were radiolabelled with 1 μL $\gamma\text{P}^{32}\text{ATP}$ in a reaction containing 1 μL polynucleotide kinase (PNK) (New England Biolabs, Beverly, ME) and 1 μL Buffer 2 (New England Biolabs) in a 10 μL final volume reaction. This reaction was incubated at 37°C for 45 minutes, stopped with 25 mM EDTA and then purified through

a Chromaspin TE-10 column (Clontech, Palo Alto, CA). Labeled probes (20,000 - 50,000 c.p.m) were incubated with 150-250 ng of GST-Kaiso fusion protein for 30 minutes on ice followed by 25 minutes at room temperature in a reaction containing (3 μ g Poly dI-dC, 5 μ g BSA, 4.8% glycerol, 10 mM MgCl_2 , 0.1% NP-40, and 5 μ L binding buffer). Samples were then run on a 4% non-denaturing polyacrylamide gel at 200V for 2½ hours. Gels were dried, exposed on Kodak XAR film at -80°C for 5 - 48 hours and visualized by autoradiography.

Table 1: *CCND1* promoter-derived KBS probes used for EMSA.

(A) *CCND1* promoter-derived KBS probes: KBS are double-underlined and *italicized*, mutant nucleotides are depicted in ***bold***. CpG dinucleotides are underlined. The KBS located at position +24 is on the complementary strand (5' to 3'). ID numbers in brackets represent re-ordered oligonucleotides.

Name	ID	Sequence	Ref
-1118	ML5102 (ML072484)	Forward: 5'-TTATGCCGGCT <u><i>CCTGCCAG</i></u> CCCCCTCACGC-3'	(Otchere, 2006)
	ML5103 (ML072485)	Reverse: 5'-GCGTGAGGGGGCT <u><i>TGGCAGG</i></u> AGCCGGCATAA-3'	
-1118 mut	ML14543	Forward: 5'-TTATGCCGGCT <u><i>CATTAC</i></u> AGCCCCCTCACGC-3'	(Otchere, 2006)
	ML14544	Reverse: 5'-GCGTGAGGGGGCT <u><i>GTAAATG</i></u> AGCCGGCATAA-3'	
+24	ML7934 (ML072486)	Forward: 5'-CTGTCGGCGCAG <u><i>TAGCAGCG</i></u> AGCAGCAGAC-3'	(Otchere, 2006)
	ML7935 (ML072487)	Reverse: 5'- GTCTGCTGCTCG <u><i>CTGCTA</i></u> CTGCTGCGCCGACAG-3'	
+1050	ML18260	Forward: 5'-GGAGAGCACGGCAG <u><i>CTGCAA</i></u> GGTCCGGTGG-3'	This study
	ML18261	Reverse: 5'-CCACCGGACCT <u><i>TGCAG</i></u> CTGCCGTGCTCTCC-3'	

Table 2: CCND1 promoter-derived methylation probes used for EMSA.

(B) CCND1 promoter-derived CpG probes used for EMSA: CpG dinucleotides are underlined and **bolded**. Core KBS sites are *italicized* and double-underlined.

Name	ID	Sequence
CpG1	ML17974	Forward: 5'- <u>GCGGGGGAGGGGG</u> <u>CGCG</u> GGAGGAATTCACC-3'
	ML17975	Reverse: 5'-GGTGAATTCCTCC <u>CGCG</u> CCCCCTCCCCCGC-3'
CpG2	ML17976	Forward: 5'-CGTTCTTGAAATGCGCCATTCTGCCGGC-3'
	ML17977	Reverse: 5'-GCCGGCAGAATGGGCGCATTTCGAAGAACG-3'
CpG3	ML17978	Forward: 5'-TATGGGGTGTCGC <u>CGCG</u> CCCCAGTCACCCC-3'
	ML17979	Reverse: 5'-GGGGTGACTGGGG <u>CGCG</u> GGCGACACCCCAT-3'
CpG4	ML17980	Forward: 5'-GCCGCAGGGCAGG <u>CGCG</u> GGCGCCTCAGGGAT-3'
	ML17981	Reverse: 5'-ATCCCTGAGGCGC <u>CGCG</u> CCTGCCCTGCCGC-3'
CpG5	ML17982	Forward: 5'-CCCGGCGTTTGGCGCC <u>CGCG</u> CCCCCTCCCC-3'
	ML17983	Reverse: 5'-GGGGAGGGGG <u>CGCG</u> GGCGCCAAACGCCGGG-3'
CpG6	ML17984	Forward: 5'-GCCCCCTCCCCCTGCGCCCGCCCCCGCCCC-3'
	ML17985	Reverse: 5'-GGGGCGGGGGCGGGCGCAGGGGGAGGGGGC-3'
CpG7	ML17986	Forward: 5'-CAGAGGGCTGTCGGCGCAGT <u>AGCAGCGAGC</u> -3'
	ML17987	Reverse: 5'-GCTCGCTGCT <u>ACTGCGCCGACAGCCCTCTG</u> -3'
CpG8	ML17988	Forward: 5'-GAGGGGCAGAAGAG <u>CGCG</u> AGGGAG <u>CGCG</u> GG-3'
	ML17989	Reverse: 5'-CC <u>CGCG</u> CTCCCT <u>CGCG</u> CTTCTGCCCTC-3'

2.6. Luciferase Assays

24 hours prior to transfection, cells were seeded into 6-well dishes and grown to 60-70% confluency at the time of transfection. Cells were transfected using ExGen500 reagent (MBI Fermentas, Burlington, ON) according to the manufacturer's protocol. 24 hours post-transfection the cells were washed twice with 2 mL ice-cold PBS per well. Each well was next treated with 350 μ L of 1X Passive Lysis Buffer (Promega, Madison, Wisconsin) and the 6-well plates were incubated at room temperature for 20 minutes on a shaker. Cell lysates were harvested by pipetting up and down a few times to resuspend and lyse the cells. 50 μ L of lysate from each well were assayed for luciferase activity by adding 100 μ L of Luciferase Assay Reagent II (LARII) (Promega, Madison, Wisconsin) and analysis with a Thermo Luminoskan Ascent Luminescent Plate Reader (Labsystems, Ramsey, MN). Each reaction was performed in triplicate and numbers were normalized by co-transfection with the internal control vector pRL-CMV which was assayed using 100 μ L of Stop and Glo (Promega) or by performing a Bradford assay and normalizing to protein levels.

Table 3: Reporters and effectors used in promoter reporter luciferase assays.**(A) Reporters:**

Construct Name	Characteristics	Ref.
pGL3 Basic backbone	Reporter vector used to quantitatively analyse factors that potentially regulate mammalian gene expression using a modified coding region for firefly (<i>Photinus pyralis</i>) luciferase.	Promega
pGL3 Basic 1748CD1	Backbone reporter vector containing ~1.7 kb fragment of the human <i>CCND1</i> promoter (nucleotides -1748 to +134 relative to the transcriptional start site). Possess two KBS.	(Tetsu and McCormick, 1999)

(B) Effectors:

Effector Name	Characteristics	Ref.
pcDNA3-hKaiso	Human Kaiso overexpression plasmid	(Kelly, 2007)
pRC/RSV-p120 1A	Murine p120 overexpression plasmid	(Daniel and Reynolds, 1999)
pCS-ΔN89 β-catenin	Constitutively active human β-catenin overexpression plasmid	(Imbert <i>et al.</i> , 2001; Munemitsu <i>et al.</i> , 1996)
pSilencer - hKaiso	Human Kaiso siRNA silencing plasmid	(Graham, 2005)

2.7. Immunoblot Analysis

Cells were grown to 90% confluency on 100 mm dishes and washed twice with 1X ice-cold PBS. Cells were then incubated 10 minutes on ice with Nonidet P-40 lysis buffer (0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM PMSF, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, 1 mM sodium orthovanadate, and 25 mM EDTA). The lysates were harvested using a cell scraper and transferred to a 1.5 mL microfuge tube. Lysates were cleared by centrifugation at 4°C for 5 minutes at 14,000 r.p.m. and the supernatants were transferred to new tubes. Protein concentration was determined by performing a Bradford Assay. Equal amounts of protein were 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), boiled for 2 minutes, and run on a 7% SDS-poly-acrylamide gel for 4 hours at 25 mA. The resolved proteins were then transferred to a nitrocellulose membrane (Perkin Elmer) using the Hoeffer semi-dry transfer apparatus (Amersham/Pharmacia, San Francisco, California) at 125 mA for 2 hours. The membrane was incubated for 5 minutes at room temperature in 3% milk in Tris-buffered saline (TBS) [pH 7.4]. The blocked membrane was incubated overnight at 4°C with primary antibodies (Ab) in 3% milk-TBS. The following antibodies were used at the specified concentrations for Western blotting: anti-Kaiso polyclonal (1:12,000), anti- β -tubulin monoclonal antibody (1:40,000) and anti- β -catenin polyclonal antibody (1:1000, 0.5 μ g/ μ L). The following day the membranes were washed 5 times with TBS for 5 minutes to remove any unbound primary antibody and then incubated for 2 hours at room temperature with horseradish peroxidase-conjugated secondary antibody (diluted 1:40,000 in 3% milk-TBS). The membranes were

finally washed 5 times with TBS for 5 minutes and processed using the enhanced chemiluminescence (ECL) system (Amersham/Pharmacia) or Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer, Waltham, MA) according to the manufacturer's protocol.

2.8. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed using the ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA). The cells were grown to 70-80% confluency, fixed with 1% formaldehyde in DMEM media for 10 minutes followed by a wash with 5 mL of ice-cold 1X PBS. Formaldehyde-crosslinking was terminated by adding 5 mL of Glycine Stop-Fix Solution and incubating for 5 minutes at room temperature. Cells were then washed with 5 mL of ice-cold 1X PBS and harvested by the addition of 1 mL of ice-cold 1X PBS with 0.5 mM PMSF to each plate, then transferred to 15 mL conical tubes. Cells were pelleted by centrifugation for 10 minutes at 2,000 rpm at 4°C and the supernatant removed. The cells were next resuspended in 1 mL of ice-cold Lysis Buffer (with protease inhibitor cocktail and PMSF) and incubated on ice for 30 minutes. Cells were then transferred to an ice-cold dounce homogenizer and gently lysed on ice with 10 strokes. Nuclei were pelleted by centrifuging at 5,000 rpm for 10 minutes at 4°C. The nuclei pellets were resuspended in 250 µL of Digestion Buffer (supplemented with protease inhibitor cocktail and PMSF) and pre-warmed at 37°C for 5 minutes. 12.5 µL of Enzymatic Shearing cocktail (200 U/mL) was added and the mixture incubated at 37°C for 5 minutes. The reaction was stopped by adding 5 µL ice-cold 0.5 M EDTA and

chilling on ice for 10 minutes. Sheared chromatin (4-6 μg) was immunoprecipitated overnight at 4°C with 25 μL of Protein G magnetic beads, 10 μL ChIP Buffer 1, 1 μL of protease inhibitor cocktail, 4 μg of Ab and dH_2O brought up to 100 μL . The following antibody amounts were used; 4 μg Kaiso 6F mAb (monoclonal Ab), 4 μL Kaiso pAb (polyclonal Ab), 4 μg 12CA5 negative control mAb, 10 μL preimmune IgG negative control, 10 μL RNA Polymerase II positive control mAb. The next day the beads were pelleted on a magnetic stand and the supernatant removed. Beads were washed twice with 800 μL ChIP Buffer 1 and three times with ChIP Buffer 2, with 10 minutes agitation for each wash. DNA was eluted from the beads by incubation for 15 minutes with 50 μL of Elution buffer AM2. To reverse the cross-links, 50 μL of Reverse Cross Link Buffer was added to the beads and incubated at 94°C for 15 minutes. Tubes were returned to room temperature and 2 μL of Proteinase K was added to the reaction and incubated for 1 hour at 37°C. The reaction was stopped by adding 2 μL of Proteinase K Stop Solution. Recovered DNA was then subjected to PCR using promoter-specific primers to identify Kaiso-associated chromatin targets.

Table 4: *CCND1* primers for Chromatin Immunoprecipitation (ChIP).

Name	ID	Sequence	Ref
-1118-for	ML4850	5'-TTTACATCTGCTTAAGTTTGCG-3'	(Otchere, 2006)
-1118-rev	ML4851	5'-TTAGAATTTGCCCTGGGACT-3'	(Otchere, 2006)
+24-for	ML6191	5'-CACACGGACTACAGGGGAGTT-3'	(Otchere, 2003)
+24-rev	ML6192	5'-CTCGGCTCTCGCTTCTGCTG-3'	(Otchere, 2003)
CpG5-for	ML072480	5'-TTTGCATTTCTATGAAAACCGG-3'	This study
CpG5-rev	ML072481	5'-GCAACTTCAACAAAACCTCCC-3'	This study
CpG8-for	ML072482	5'-ACACGGACTACAGGGGAGTTTTG-3'	This study
CpG8-rev	ML072483	5'-ATTTCGAACCCCTGGGGAGG-3'	This study

2.9. Immunohistochemistry

Paraffin-embedded tissue slides were incubated in a 60°C oven for 1 hour to de-wax. Next they were treated with xylene for 5 times 4 minutes each and rehydrated in a series of ethanol washes. The slides were incubated with 100%, 95%, 75% ethanol and tap water twice for 3 minutes each. An antigen retrieval step was performed at 37°C for 5 minutes in the following unmasking solution; 1 mg/mL Proteinase K, 0.05M Tris pH 8, and 5mM EDTA pH 8. This was followed by two PBS washes lasting 2 minutes each. Endogenous peroxidase activity was quenched by incubation with a 3% H₂O₂ solution in PBS for 15 minutes followed by three 3 minutes PBS washes. Slides were then blocked by incubation with a 5% milk solution in PBS-Triton X 100 (PBST) for 2 hours. Primary Ab diluted in 1% milk in PBS was incubated on the slides overnight at 4°C in a humidified container. The following Ab concentrations were used: Kaiso 6F mAb (10-12 µg/mL), and Cyclin D1 pAb (1:50). The following day the slides were washed three times 10 minutes each with 1X PBS before adding the secondary antibody. The appropriate secondary Abs were diluted at 1:250 in 1% milk in PBS and the slides incubated with 2° Ab for 1 hour in a humidified container at room temperature. Excess Ab was washed off the slides with PBS three 10 minute washes. Slides were next incubated with ABC reagent (Vector labs) for 30 minutes at room temperature followed by three 10 minute washes with PBS. Slides were incubated with DAB (3,3'-diaminobenzidine) substrate (Vector labs) for 2 - 10 minutes to develop a brown colour and put in dH₂O for 5 minutes. Slides were then counterstained with hematoxylin (Poly Scientific) and dehydrated in a series of ethanol washes (75%, 80%, 95%, and 100%) for 1 minute each.

Slides were finally cleared in xylene for 20 minutes, mounted in Permount (Fisher Scientific) and dried overnight before imaging.

Table 5: Antibody concentrations for WB, IHC and ChIP. WB; western blot, IHC; immunohistochemistry, ChIP; chromatin immunoprecipitation.

Antibody Name	Monoclonal or Polyclonal	Clone Name(s)	Concentration/dilution used	Manufacturer or Reference
Anti-Kaiso	Polyclonal	N/A	WB: 1:12,000 IHC: N/A ChIP: N/A	(Daniel <i>et al.</i> , 2001)
Anti-Kaiso	Monoclonal	6F	WB: N/A IHC: 2-4 µg/mL ChIP: 10-12µg/mL	(Daniel <i>et al.</i> , 2001)
Anti-p120 ^{ctn}	Monoclonal	15D2	WB: 2 µg/mL IHC: N/A ChIP: N/A	(Wu <i>et al.</i> , 1998)
Anti-β-catenin	Polyclonal	N/A	WB: 1:1000 IHC: N/A ChIP: N/A	Sigma-Aldrich
Anti-Cyclin D1	Monoclonal	DCS-6	WB: 1:500 IHC: 1:10-1:50 ChIP: N/A	Santa Cruz
Anti-Cyclin D1	Polyclonal	N/A	WB: N/A IHC: 1:10-1:50 ChIP: N/A	US Biological
Anti-β-tubulin	Monoclonal	SAP.4G5	WB: 1:20,000,000 IHC: N/A ChIP: N/A	Sigma-Aldrich

RESULTS

The first evidence that Kaiso may be a regulator of the *CCND1* gene came from studies in *Xenopus laevis* embryos where it was shown that xKaiso negatively regulated the *CCND1* gene (Kim *et al.*, 2002). Coincident with this publication, initial studies in our lab revealed that Kaiso associated *in vitro* with the *CCND1* promoter via two regions containing potential KBSs (-1118 and +24). Kaiso bound to the -1118 KBS region in a methylation-independent manner but bound to the +24 region in a methylation-dependent manner.

3.1. Kaiso specifically binds to the -1118 KBS site of CCND1 in vitro.

As a first step towards determining if *CCND1* is repressed or activated by Kaiso, we determined how Kaiso binds to the *CCND1* promoter. To this end EMSA experiments were performed to confirm and extend the initial results obtained by Ms. Otchere (Otchere, 2006). A 30 b.p. oligonucleotide sequence encompassing the -1118 KBS site in the *CCND1* promoter and an oligonucleotide in which the KBS had been mutated (TCCTGCCA → TCATTACA) (-1118 mut) were used in EMSA experiments to confirm the specificity of Kaiso binding to the -1118 KBS site. These experiments were performed using purified GST-Kaiso fusion deletion proteins. A protein lacking the Kaiso POZ domain was used since full-length POZ proteins are known to associate weakly with DNA *in vitro* due to inhibition by the POZ domain (Bardwell and Treisman, 1994). The following fusion proteins were used in the EMSA experiments: GST-Kaiso Δ POZ which

lacks the amino-terminal POZ domain; GST-Kaiso Δ AR1 and GST-Kaiso Δ AR2 which lack the amino-terminal region up to and including acidic region (AR) 1 and 2 respectively; GST-Kaiso-ZF contains the carboxy-terminal region of Kaiso encompassing the three zinc fingers; GST-Kaiso Δ POZ Δ ZF which lacks the amino-terminal POZ domain and the carboxy-terminal zinc finger regions (Figure 7). Finally, GST-tag alone was used as a negative control since it does not contain any intrinsic DNA-binding ability.

In repeated independent experiments I confirmed that Kaiso does bind to the -1118 KBS region of the *CCND1* promoter (Figure 8A). Surprisingly, although the GST-Kaiso Δ POZ and GST-Kaiso-ZF fusion proteins both lacked the inhibitory DNA binding POZ domain, GST-Kaiso-ZF bound oligonucleotides with higher efficiency than Kaiso Δ POZ. This raises the possibility that other DNA-binding inhibitory regions may exist outside the POZ domain. Binding was not seen, as expected, in the negative control, GST-alone or GST-Kaiso Δ POZ Δ ZF lanes. To confirm the specificity of Kaiso-KBS binding, GST-Kaiso deletion mutants were incubated with a 30 b.p. oligonucleotide harbouring three point mutations in the core KBS sequence (-1118 mut). The introduction of these point mutations in the KBS completely abolished binding of Kaiso to the oligonucleotide (Figure 8B).

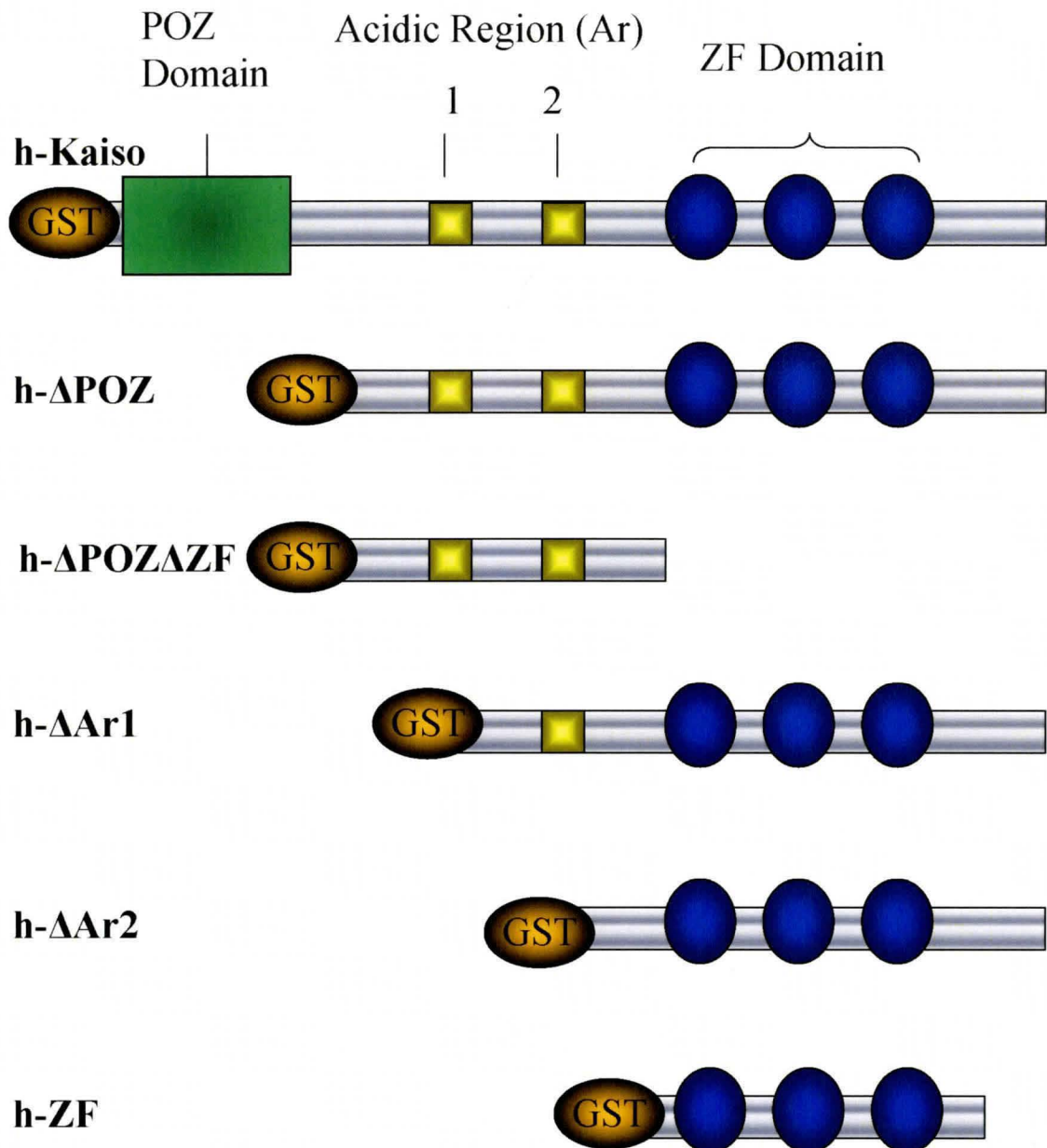


Figure 7: Schematic of the human GST-Kaiso fusion proteins used in EMSA. A panel of human Kaiso cDNAs were subcloned into the pGEX-5X-1 protein expression vector. The Kaiso POZ domain is shown in green, the acidic regions are shown in yellow and the zinc fingers in blue. Amino-terminal GST tag is shown in gold.

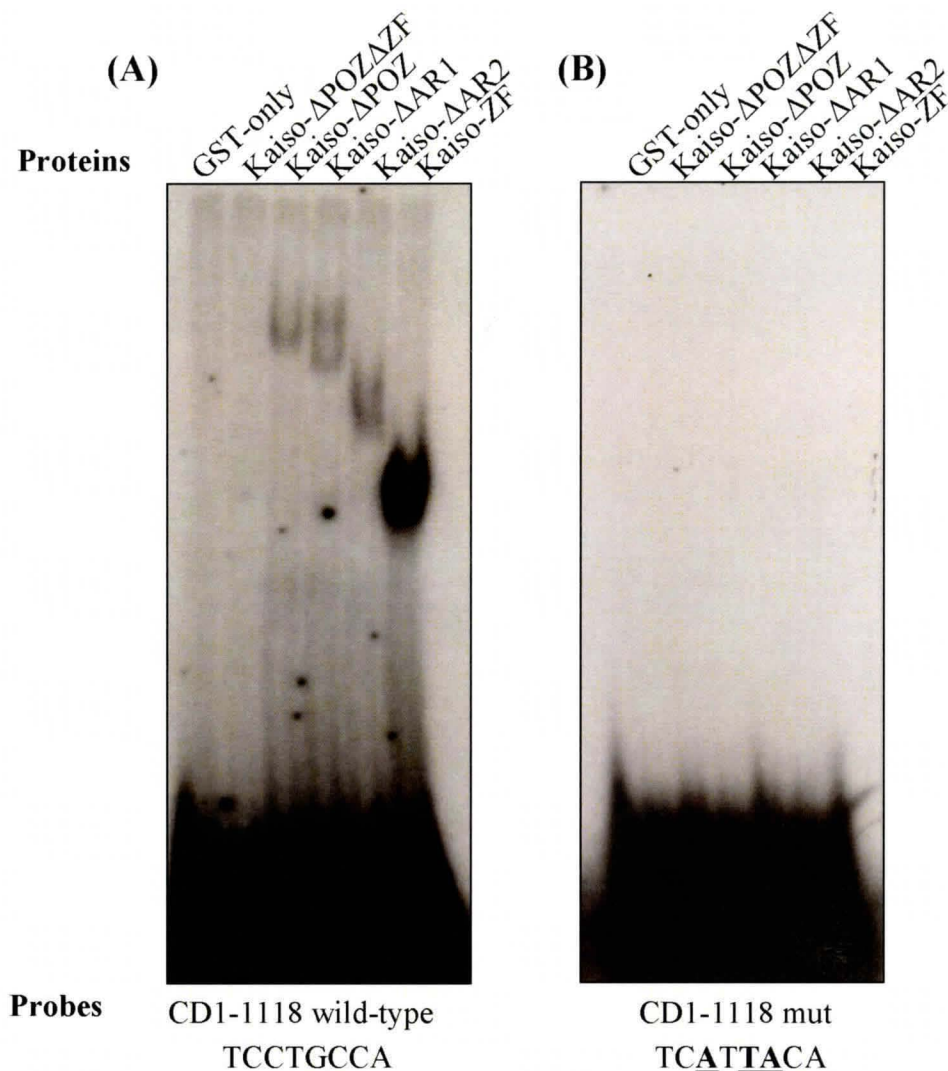


Figure 8: Kaizo binds to the -1118 KBS site of the *CCND1* promoter *in vitro*. Wild type and KBS-mutated oligonucleotides spanning the -1118 KBS site were synthesized. These oligonucleotides were then radiolabelled and incubated in a binding reaction with 250 ng of various GST-human Kaizo proteins. Negative controls include GST-tag alone, and the GST-Kaiso Δ POZ Δ ZF protein lacking both the POZ and ZF domains. Other fusion proteins that are N-terminally deleted but still contain the GST-Kaiso-ZF DNA-binding domain and should bind DNA (h Δ POZ, h Δ AR1, h Δ AR2 and hZF). (A) Only GST-Kaiso fusion proteins possessing the ZFs bound to the -1118 KBS oligonucleotide. (B) Binding to the oligonucleotide was abolished when point mutations were introduced in the KBS.

3.2. Kaiso specifically binds to the +24 KBS of CCND1 promoter in a methylation-dependent manner.

EMSA experiments were performed to confirm Kaiso binding to the +24 KBS region of the *CCND1* promoter and to optimize the conditions to reduce non-specific binding. In these experiments I used a panel of GST-Kaiso deletion mutants in a binding reaction with a 30 b.p. oligonucleotide that span the +24 KBS region. Oligonucleotides were also *in vitro* methylated with *Sss I* methylase to determine if methylation affected Kaiso DNA binding. As seen previously, Kaiso only bound to the +24 KBS region of the *CCND1* promoter when methylated (Figure 9A and B). This data suggested that Kaiso associated with +24 KBS region of the *CCND1* promoter in a methylation-dependent but KBS-independent manner. To determine the specificity of Kaiso DNA-binding in this region, EMSAs using excess unlabelled probes were performed. Methylated +24 KBS oligos were incubated with the GST-Kaiso ZF deletion mutants as well as with the GST-Kaiso full-length protein which contains the POZ domain that partially inhibits DNA binding *in vitro* (Daniel *et al.*, 2002). The unmethylated unlabelled probe was unable to compete with both the GST-Kaiso full-length and deletion mutant GST-Kaiso ZF fusion proteins for DNA binding (Figure 10). However, methylated excess unlabelled probe out-competed both the GST-Kaiso full-length and GST-Kaiso ZF fusion proteins. This result indicated that Kaiso binds specifically to the +24 KBS region of the *CCND1* promoter and this binding is methylation-dependent. It should be noted that the +24 KBS oligonucleotide, along with harboring the core KBS site, also contains CpG dinucleotides; this would explain the methylation-dependent binding to this probe. The finding that

Kaiso binds to regions of the *CCND1* promoter in a methylation-dependent but KBS-independent manner led to an analysis of the *CCND1* promoter for CpG dinucleotide pairs (i.e. CGCG) that may be recognized and bound by Kaiso.

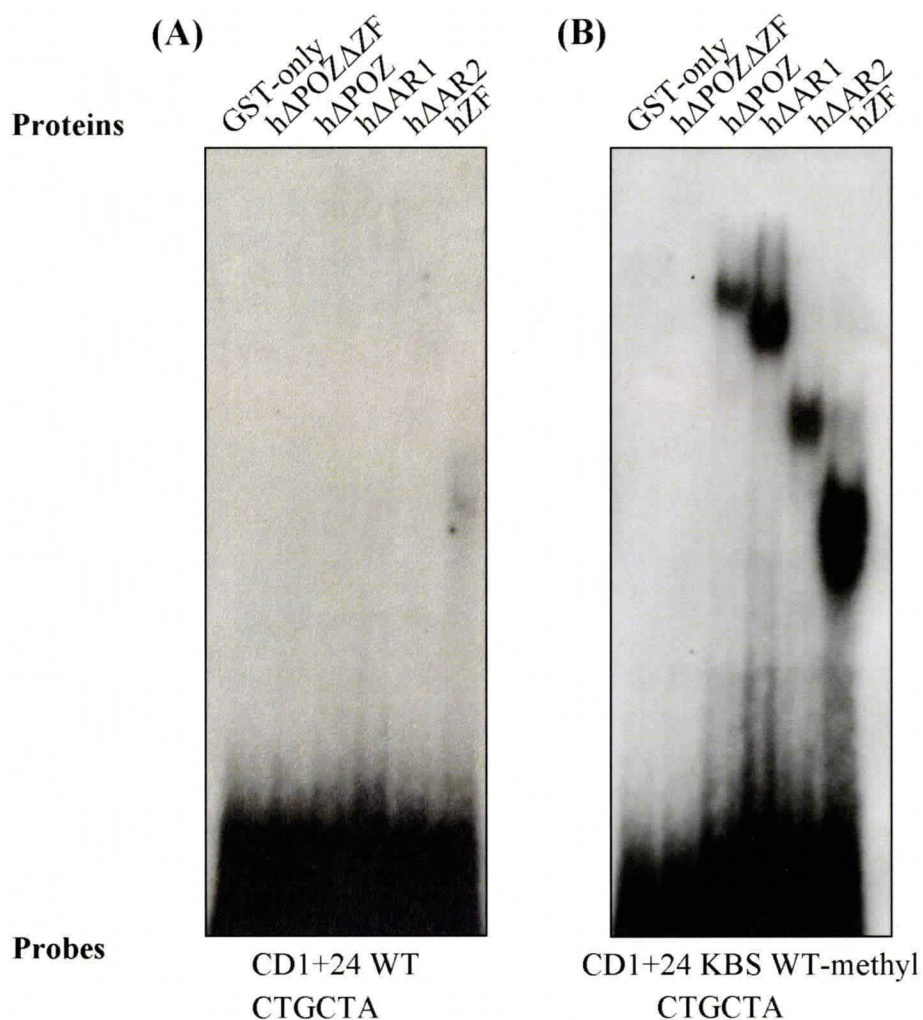


Figure 9: Kaiso binds to the +24 KBS site in a methylation-dependent manner. Oligonucleotides spanning the +24 KBS sites were created, radiolabelled and incubated in a binding reaction with 250 ng of various GST-hKaiso fusion proteins. (A) The GST-Kaiso fusion proteins did not bind the +24 KBS region when unmethylated. (B) The GST-Kaiso fusion proteins bound to the *in vitro* methylated +24 KBS oligonucleotide.

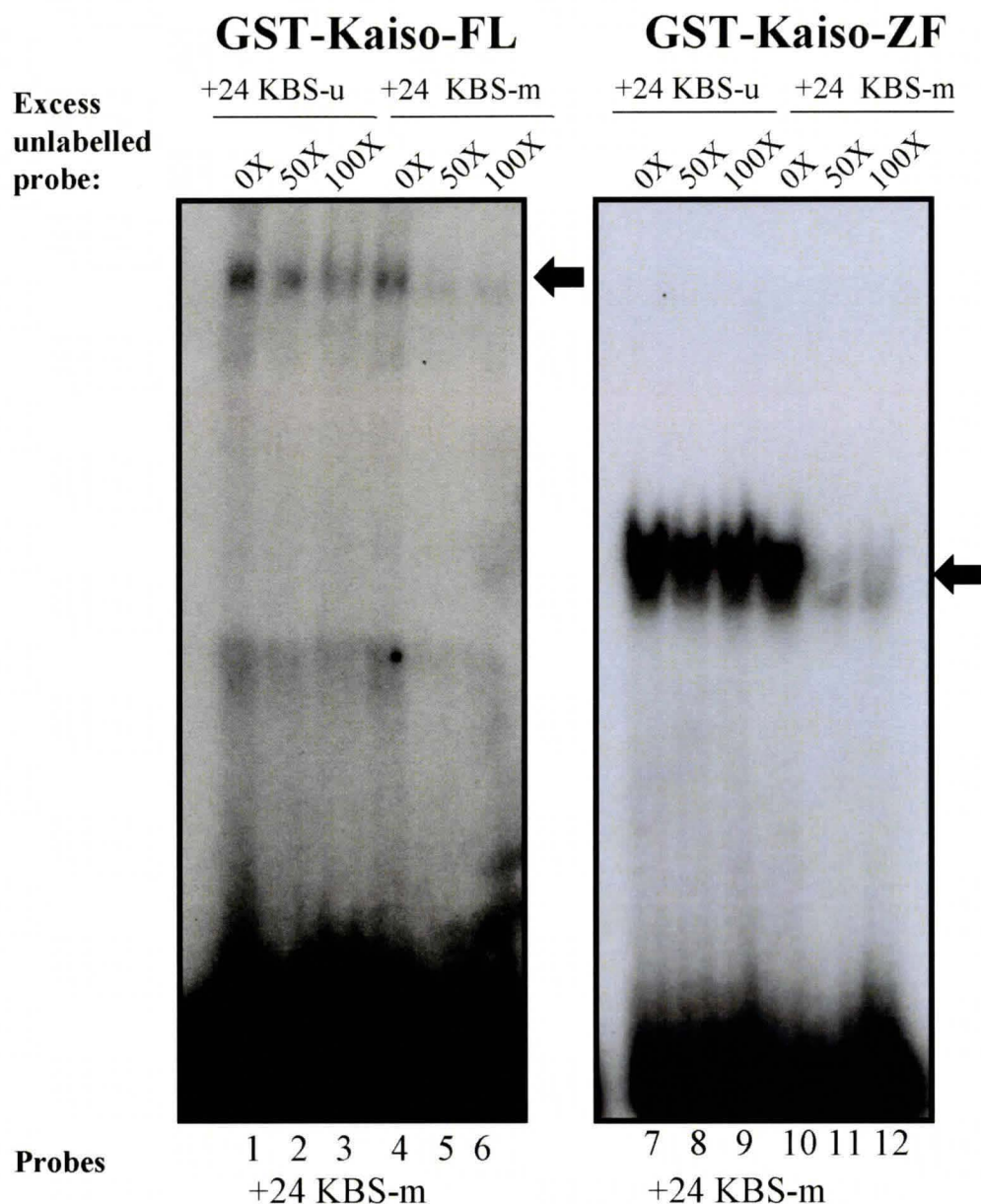


Figure 10: Kaiso specifically binds to the +24 KBS oligonucleotide when methylated. *CCND1*-promoter-derived +24 KBS oligonucleotides were methylated (m) *in vitro*, radiolabelled and incubated with GST-Kaiso proteins. Unlabelled probe was added to the reactions at 50X and 100X molar excess of labelled probe. Methylated unlabelled probe competed for binding to the GST-Kaiso-ZF and GST-Kaiso full-length (FL) protein. As a negative control unmethylated (u) +24 KBS probe was tested and did not decrease binding (lanes 2,3,8,9).

3.3. *Kaiso binds to the CCND1 promoter in a methylation-dependent manner in vitro.*

Previous experiments performed in our lab showed that Kaiso repressed the expression of a minimal *CCND1* promoter reporter construct that did not contain the -1118 KBS (Othchere, 2006). This suggested that Kaiso may be repressing the *CCND1* promoter in a KBS-independent manner and raised the possibility that repression was occurring in a methylation-dependent manner.

Analysis of the 961CD1 construct (used in luciferase assays and lacking the -1118 KBS) showed that it harboured six CpG dinucleotide pairs (i.e. CpGCpG) to which Kaiso could potentially bind. These sites are located within a number of predicted CpG islands in the promoter and also surround the three KBS (Figure 6). To explore the possibility that Kaiso may be interacting with CpG dinucleotides within the *CCND1* promoter, eight oligonucleotides were created that spanned these sites (one oligonucleotide, CpG8 contains two CpG sites). The oligonucleotides were methylated *in vitro* with *Sss I* methylase and then incubated with the GST-Kaiso fusion proteins to determine whether they would bind Kaiso. EMSA studies revealed that Kaiso did not bind any of the eight non-methylated oligonucleotides (Figure 11). However, when the oligonucleotides were *in vitro* methylated they were all bound by the GST-Kaiso fusion proteins possessing the ZF domain. The negative controls GST-only, and GST-Kaiso Δ POZ Δ ZF did not bind any of the methylated or unmethylated oligonucleotides (Figure 11). Interestingly, although Kaiso bound all eight of the CpG oligonucleotides, some oligonucleotides were bound with higher efficiency (i.e. CpG5 and CpG8). The binding sites bound by Kaiso stronger possibly represent physiological relevant CpG sites that are bound by Kaiso *in vivo*.

To confirm that Kaiso was binding the CpG oligonucleotides with different affinities, another EMSA experiment was performed to analyze all of the eight CpG oligonucleotides in one experiment. To qualitatively determine which CpG sites were binding to Kaiso with the highest affinity, all eight oligonucleotides were incubated with GST-Kaiso-ZF fusion proteins and run on the same gel for comparison. The GST-Kaiso-ZF fusion protein bound to oligonucleotides CpG5 and CpG8 with the highest affinity and CpG3 and CpG6 with the lowest (Figure 12). The CpG5 and CpG8 sites may thus represent more physiologically relevant Kaiso binding sites. To test this possibility an EMSA was performed using the two highest affinity (CpG5 and CpG8) and two lowest affinity (CpG3 and CpG6) binding site oligonucleotides, in conjunction with GST-Kaiso full-length and GST-Kaiso-ZF mutant fusion proteins that should not bind DNA. It was found that full-length Kaiso bound to CpG5 and CpG8 while the Kaiso-ZF mutant did not (as would be expected) (Figure 13). In contrast to these findings, CpG3 and CpG6 were not bound by the full-length Kaiso (data not shown). These findings support our hypothesis that the two highest affinity sites, which do bind to the GST-Kaiso full length protein *in vitro*, may be physiologically relevant Kaiso binding sites.

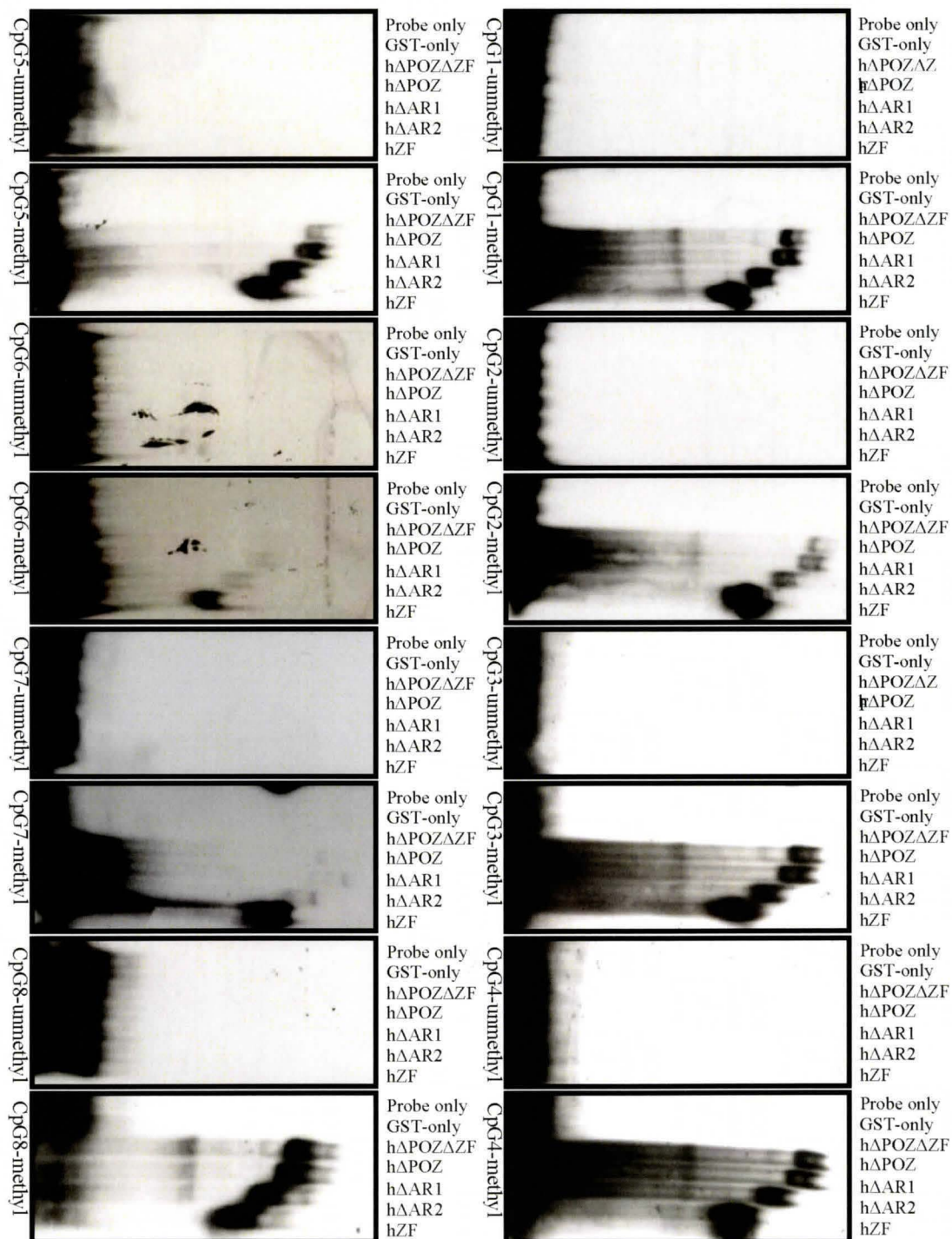


Figure 11: Kaiso binds to all *CCND1*-derived oligonucleotides possessing methylated CpGs. *CCND1*-promoter derived oligos (CpG1 to CpG8) were methylated *in vitro* with *Sss I* methylase, radiolabelled & incubated with GST-Kaiso fusion proteins as indicated. In all oligonucleotides tested, Kaiso bound to methylated probes but not to unmethylated probes .

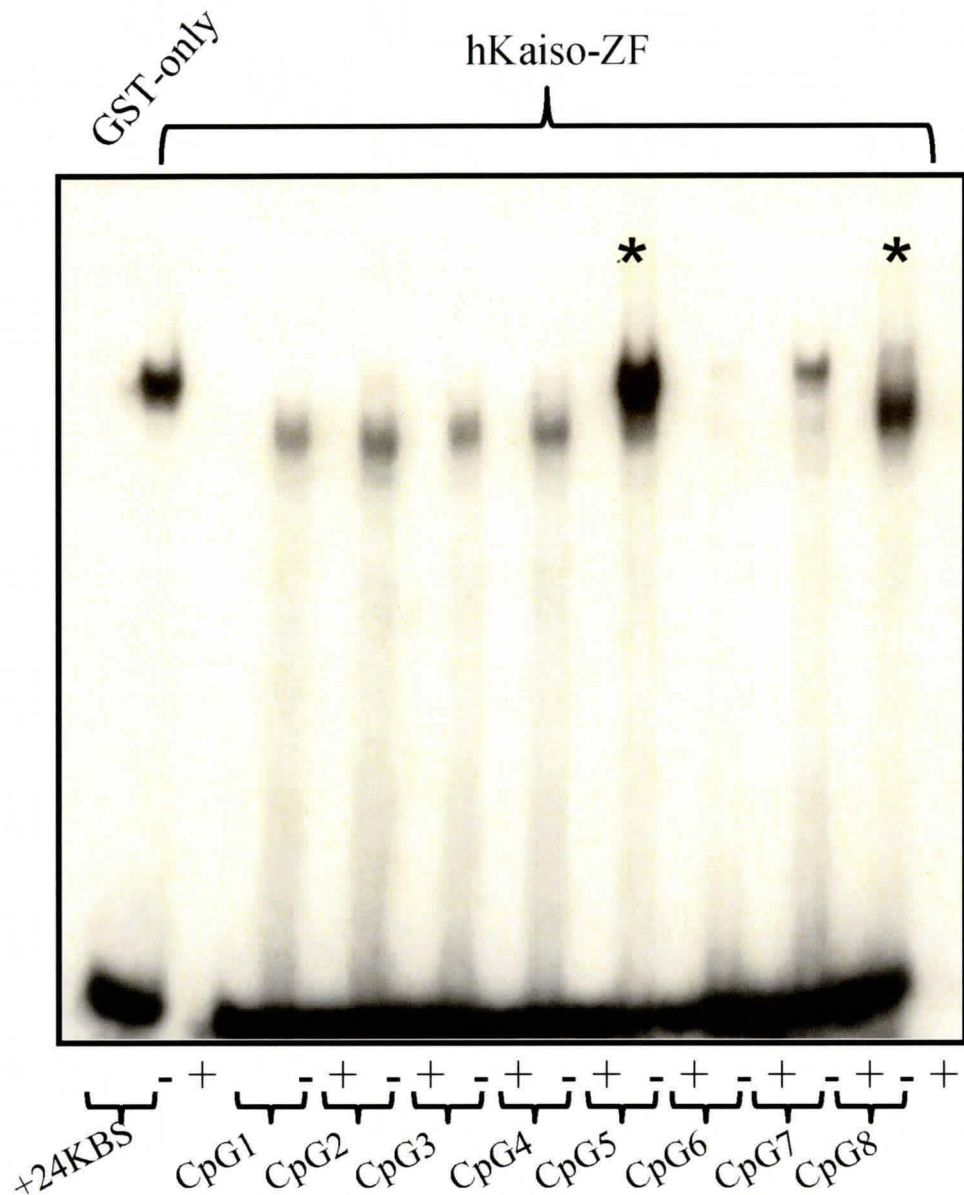


Figure 12: Kaiso binds the methylated CpG5 and CpG8 sites with the highest affinity. *CyclinD1*-promoter-derived oligos (CpG1 to CpG8) were methylated *in vitro*, radiolabelled & incubated with GST-hKaiso ZF fusion protein. Kaiso bound to methylated probes but not to unmethylated probes. +; methylated, -; unmethylated.

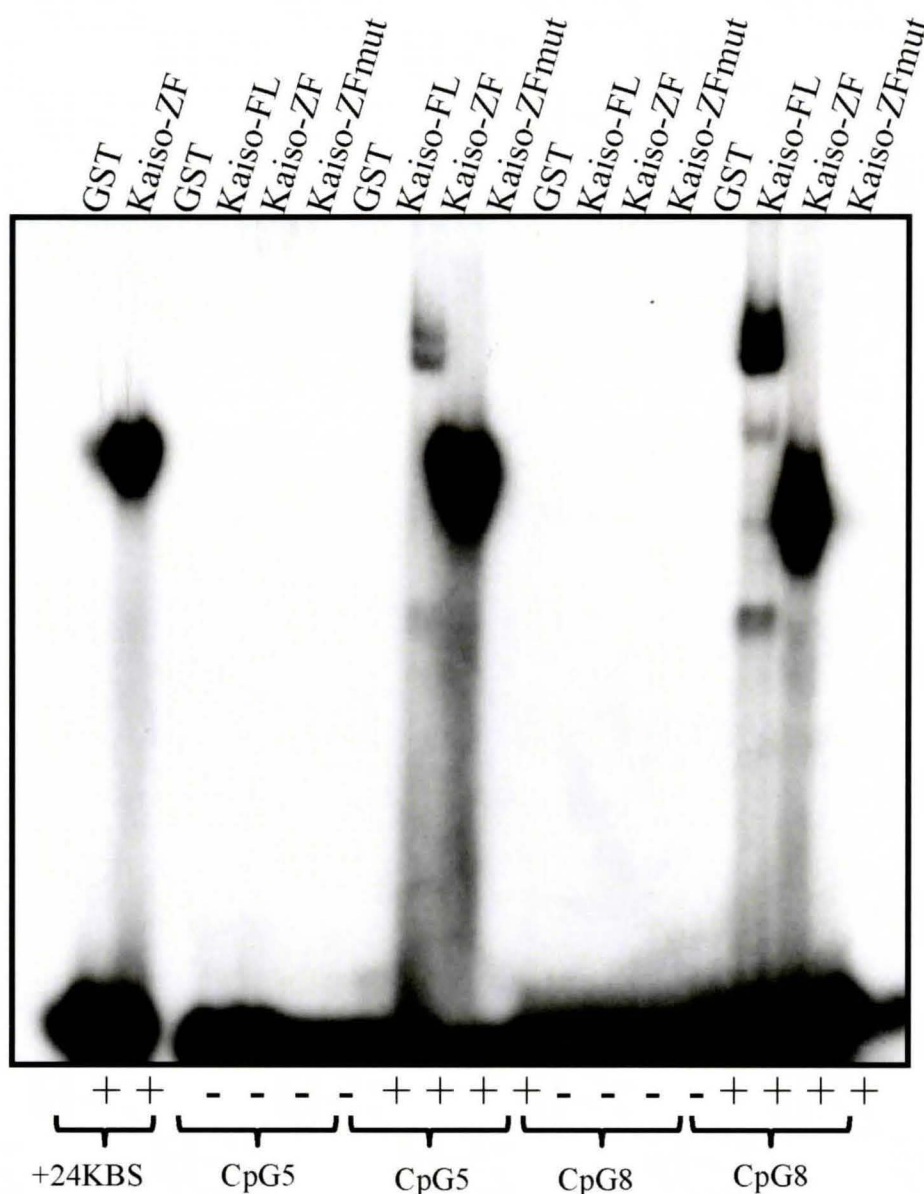


Figure 13: Full-length Kaiso binds CpG5 and CpG8. Oligonucleotides CpG5 and CpG8 were methylated *in vitro*, radiolabelled & incubated with GST-Kaiso full-length (FL), GST-Kaiso-ZF and GST-Kaiso-ZF mutant fusion proteins. Full-length Kaiso bound to CpG5 and CpG8 but the Kaiso ZF mutant protein did not. Full-length Kaiso binds less efficiently than the Kaiso ZF fusion protein because the POZ domain is believed to sterically hinder DNA binding of POZ-ZF proteins *in vitro*. (+); methylated, (-); unmethylated.

3.4. Kaiso specifically binds to the CpG5 and CpG8 methylated regions of the CCND1 promoter.

To determine the specificity of Kaiso binding to the CpG5 and CpG8 regions, EMSAs using excess unlabelled probes were performed. The GST-Kaiso-ZF protein was incubated with both radiolabelled and unlabelled oligonucleotides. As seen in Figure 14, excess amounts of unlabelled methylated probe competed with the labeled probes. This confirms the specificity of Kaiso binding to the CpG5 and CpG8 probes in a methylation-dependent manner.

3.5. Kaiso does not bind to the +1050 KBS in vitro.

In addition to the -1118 and +24 KBS, a third KBS is located in the *CCND1* promoter (+1050 within the first intron). As a first step towards characterizing this +1050 KBS and to determine if it may be a physiologically relevant KBS, oligonucleotides spanning the +1050 KBS were created and tested for Kaiso binding in EMSA experiments. The +1050 KBS oligonucleotides were incubated with the various GST-Kaiso deletion mutants and it was found that GST-Kaiso Δ POZ, GST-Kaiso Δ AR1, GST-Kaiso Δ AR2 and GST-Kaiso-ZF fusion proteins did not bind the +1050 oligonucleotide in either a methylation-dependent or independent-manner (Figure 15). The negative control, GST-Kaiso Δ POZ Δ ZF fusion protein likewise did not bind to the +1050 oligonucleotide. These results suggest that the putative KBS at the +1050 site may not be physiologically relevant for Kaiso-mediated regulation of the *CCND1* promoter.

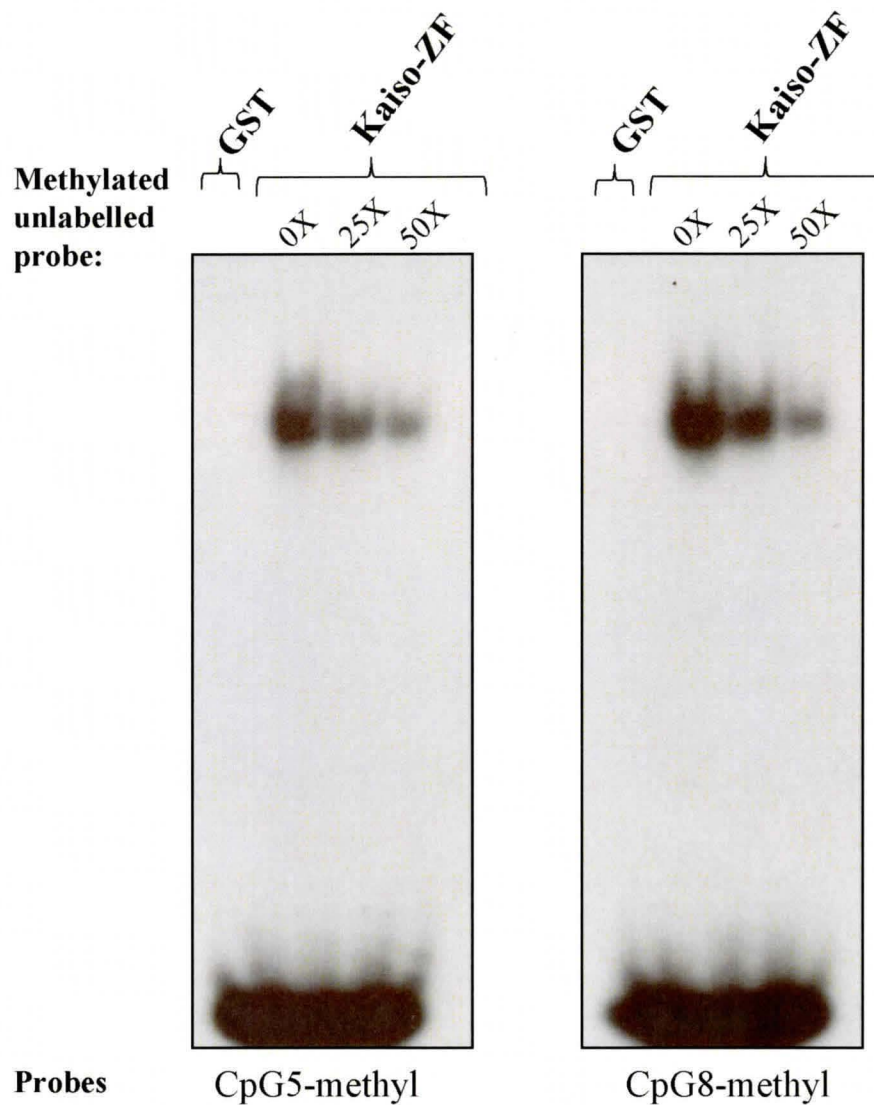


Figure 14: Kaiso specifically binds to the CpG5 and CpG8 regions when methylated. *CCND1*-promoter-derived CpG5 and CpG8 oligonucleotides were methylated *in vitro*, radiolabelled and incubated with GST-Kaiso proteins. Unlabelled probe was added to the reactions at 25X and 50X excess labelled probe. Methylated unlabelled probe was able to outcompete the binding of the GST-Kaiso-ZF and full-length protein. As a negative control GST protein alone was tested.

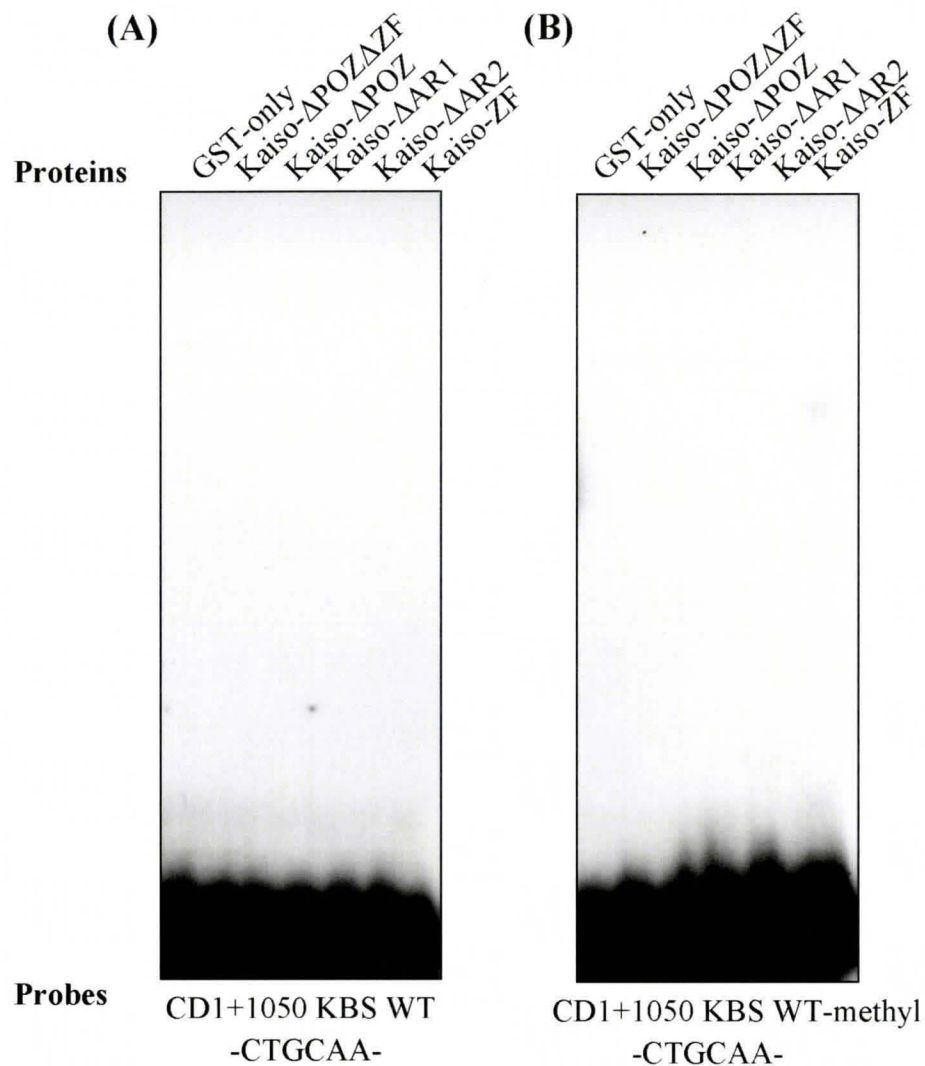


Figure 15: Kaiso does not bind to the +1050 KBS. Oligonucleotides spanning the +1050 KBS site were radiolabelled and incubated in a binding reaction with 250 ng of various GST-Kaiso fusion proteins. The Kaiso-GST fusion proteins did not bind the +1050 KBS region when unmethylated (A) or methylated (B).

3.6. Kaiso binds to the *CCND1* promoter *in vivo*.

To determine whether Kaiso binds to the *CCND1* promoter *in vivo*, chromatin immunoprecipitation (ChIP) analysis was performed. It had previously been shown that Kaiso binds to the -1118 KBS region of the *CCND1* promoter in MCF7 (breast tumor) cells (Otchere, 2006). To determine whether Kaiso binds to the highest affinity CpG dinucleotide regions (as determined *in vitro*) *in vivo*, primers were created that spanned the CpG5 and CpG8 regions of the *CCND1* promoter. These primers should amplify a fragment of approximately 200 b.p. centered upon either of these regions. Indeed primers that surround the +24 KBS region (which also contains CpG dinucleotides) amplified a fragment from Kaiso immunoprecipitated chromatin isolated from MCF7 cells (Figure 16).

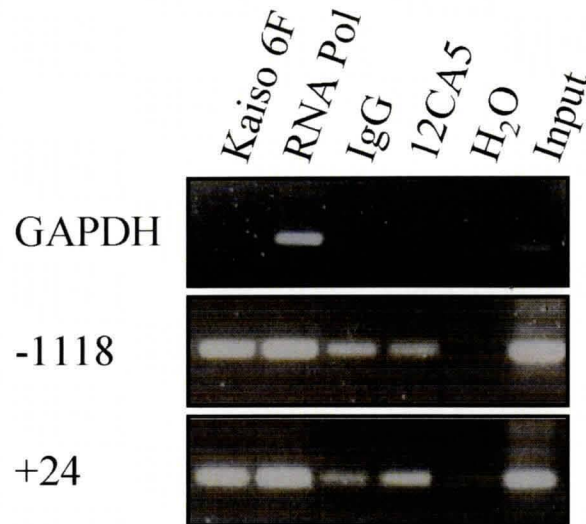


Figure 16: Chromatin Immunoprecipitation of Kaiso on *CCND1* promoter fragments. MCF7 breast tumor cells were used to isolate Kaiso-chromatin immunocomplexes using a Kaiso-specific mAb (6F). Primers surrounding the -1118 KBS site on the *CCND1* promoter were used to successfully amplify chromatin associated with Kaiso. Primers targeting the +24 KBS site of the *CCND1* promoter were also able to successfully amplify chromatin associated with Kaiso. Positive control (anti-RNA Pol II) derived immunocomplexes amplified the positive control primers (GAPDH), and to a lesser extent the negative control (IgG and 12CA5).

3.7. Stable MCF-7 Kaiso overexpression cell lines.

Since Kaiso associates with the *CCND1* promoter *in vivo* in breast cancer cell lines [(Figure 16) and (Otchere, 2006)] changes in protein levels were examined. To further explore the effects of Kaiso-mediated regulation on the *CCND1* promoter *in vivo*, it was first necessary to create human breast tumour cells lines that stably overexpress Kaiso. MCF-7 cells were transfected with the pcDNA3-Kaiso construct and selected for stable integration in the presence of G418 drug for three weeks (See Section 2.4 in Materials and Methods). A number of putative clones were subsequently picked, expanded and tested for Kaiso overexpression (Figure 17). Clone #27 appeared to be a good candidate as a high Kaiso overexpressor, and clones #28 and #65 appeared to be candidates as moderate Kaiso overexpressors. These cells lines, along with previously made MCF-7 Kaiso depletion cell lines (Almardini, 2006), were subsequently used to test the effects of Kaiso misexpression on Cyclin D1 protein levels *in vivo*.

3.8. Stable Kaiso misexpression indicates a positive correlation between Cyclin D1 and Kaiso in MCF7 cells.

To determine if there is a correlation between Kaiso and Cyclin D1 levels in the Kaiso depletion and overexpression cell lines cell synchronization and re-stimulation with serum studies were performed. Due to the fact that unsynchronized cells are heterogeneous with respect to cell cycle stage and thus Cyclin D1 levels, cell synchronization was necessary. Cells were grown to approximately 50% confluency prior to serum starvation for 24 hours. After 24 hours of serum starvation, a 24 hour time

course of serum stimulation and harvesting at 0, 4, 8, 12, 18 and 24 hours was performed. These synchronized cells were tested for Cyclin D1 and Kaiso protein levels. The results obtained indicated that as seen previously in the Kaiso-depleted cell lines, knockdown of Kaiso correlated with a decrease in Cyclin D1 expression (Figure 18A). Furthermore, this trend was seen in all cell cycle stages. Interestingly, Kaiso levels seemed to decrease as the cell cycle progressed, being highest during serum starvation and decreasing as the cells were serum stimulated and nearly absent at 24 hours. In the MCF7 overexpression cell line (#27) it was also seen that Kaiso overexpression resulted in an increase in Cyclin D1 expression (Figure 18B). Furthermore, Cyclin D1 levels which peak at 4 hours post synchronization remained high for a longer period of time (up to 24 hours) in the Kaiso overexpression cell line. In summary, both the Kaiso overexpression and depletion MCF7 cell lines demonstrated that Kaiso and Cyclin D1 exhibit a positive correlation with respect to protein expression levels, indicating that, in this cell line, Kaiso acts as a positive regulator of Cyclin D1 expression.

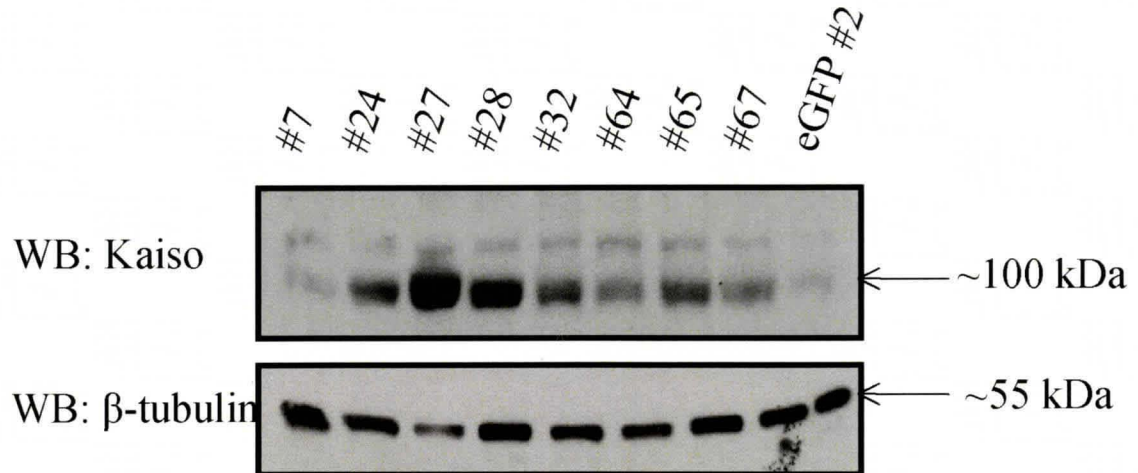


Figure 17: Generation of a stable MCF7 Kaiso overexpression cell line. The Kaiso overexpression plasmid, pcDNA3-hKaiso, which contains a neomycin resistance gene, was transfected into MCF7 cells. Cells were selected in G418 drug for 3 weeks and clones were isolated and tested for Kaiso overexpression. A number of positive clones were identified and expanded, with clone #27 and #28 having the highest expression of Kaiso. These cell lines were subsequently used in a number of experiments to assess the effects of Kaiso misexpression on Cyclin D1 expression. β -tubulin serves as a protein loading control. Endogenous levels of Kaiso were determined using a cell line which was transfected with a GFP-Kaiso construct (whose molecular weight would be higher than the endogenous Kaiso levels).

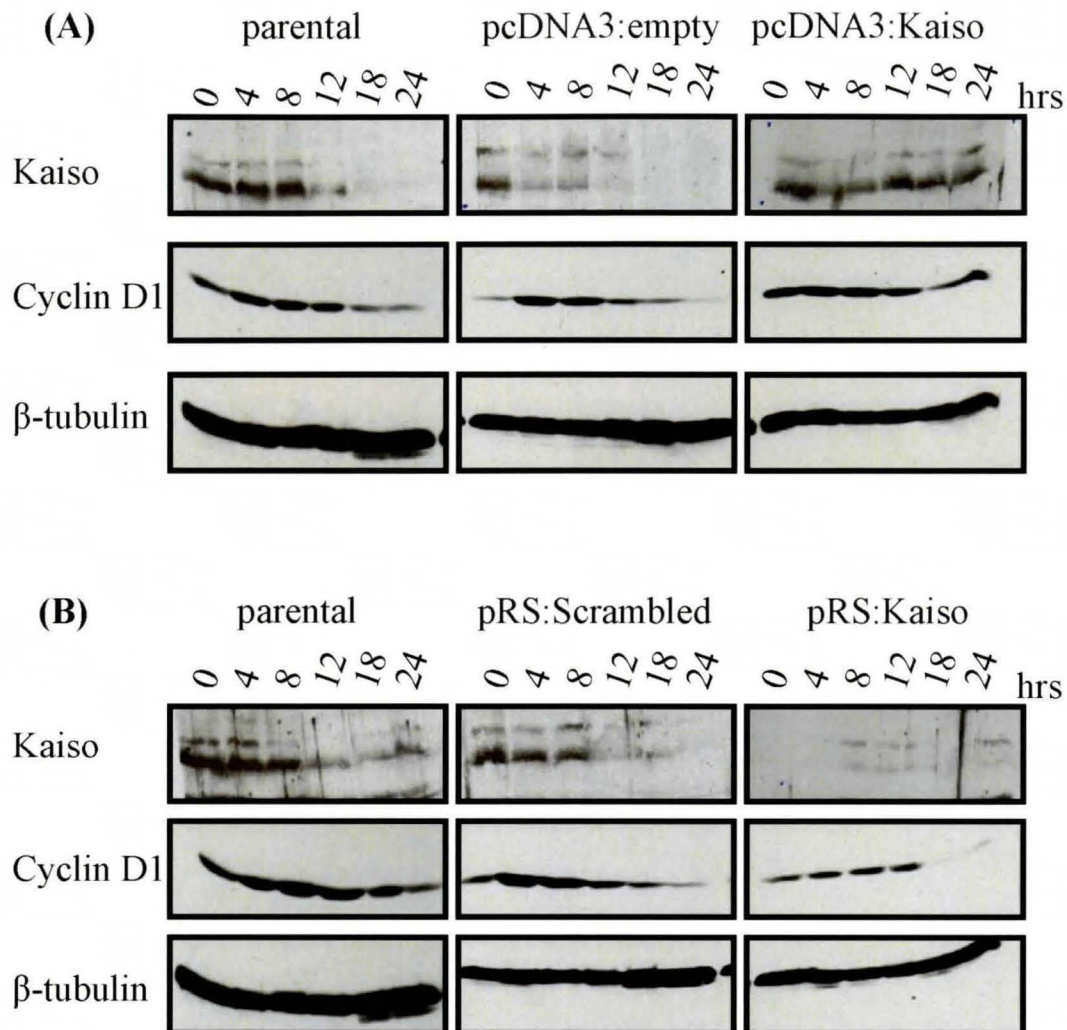


Figure 18: Effects of cell synchronization and Kaiso misexpression on Cyclin D1. MCF7 parental and misexpression cells were serum-starved for 24 hours to synchronize the cells and then serum was reintroduced. Protein was harvested from the cells at 0, 4, 8, 12, 18 and 24 hours after serum readdition and cell lysates were subjected to Western blot analysis. pcDNA3-Kaiso clone #27 (See Figure 17) was used as the Kaiso overexpression cell line. A) Kaiso overexpression cell line #27 showed increased Cyclin D1 expression later into the cell cycle. B) Kaiso depletion cell line (pRS-Kaiso) showed decreased Cyclin D1 levels.

3.9. Kaiso is highly expressed in various human tissues.

If *CCND1* is a *bona fide* Kaiso target gene, one would expect that their proteins levels may correlate in tumour tissue. To compare Kaiso and Cyclin D1 levels in tumour tissue, Kaiso expression levels were first examined in various human tissues using immunohistochemistry. A human tissue array slide with various different tumour and non-tumour tissue types was subjected to immunohistochemistry using Kaiso-specific antibodies. It was found that Kaiso is highly expressed and localized in the cytoplasm and nucleus of human skin tissue (Figure 19A). It was also found that Kaiso was expressed predominately in the cytoplasm of normal liver tissue (Figure 19B).

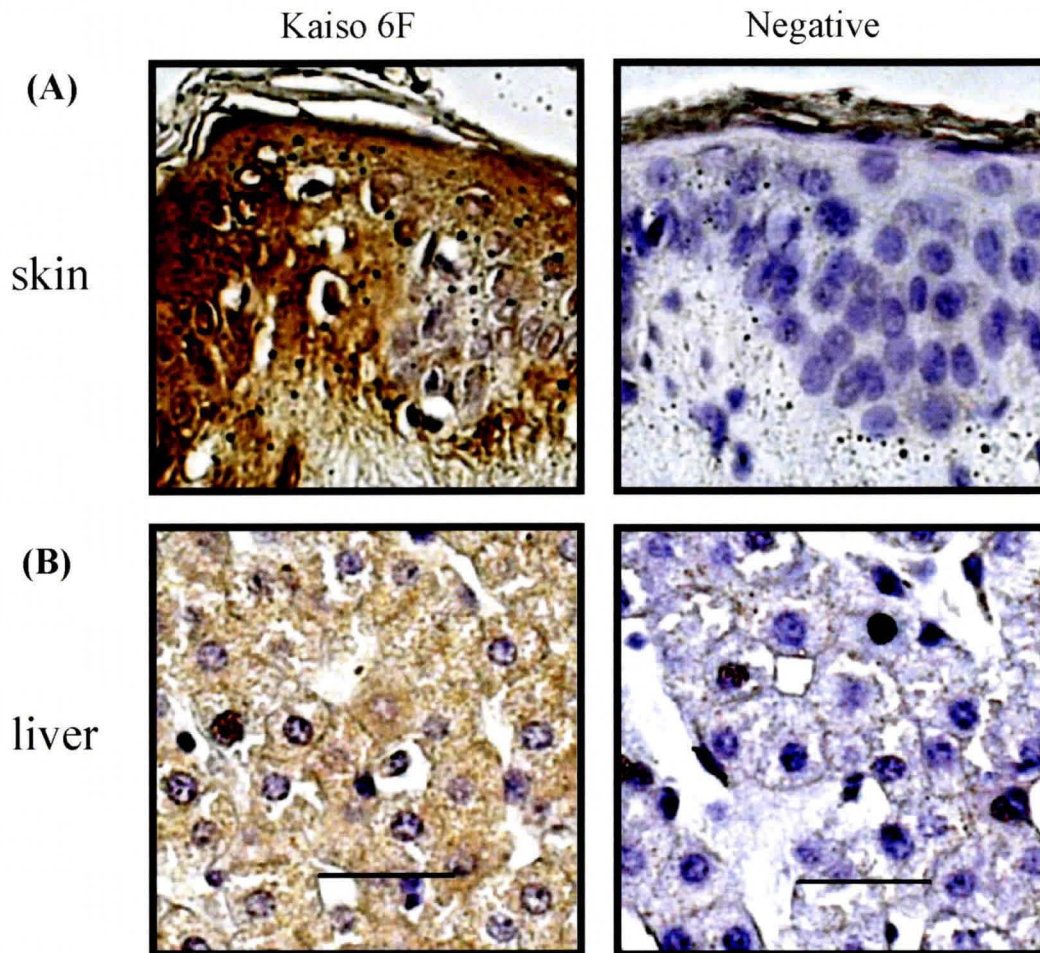


Figure 19: Kaiso expression and localization in various tumor and non-tumor tissue samples. Immunohistochemistry analysis of various tissue types using the Kaiso 6F mAb at 2 $\mu\text{g/mL}$. Negative control images were incubated without primary Ab, but incubated with secondary Ab. A) Normal skin tissue from a 36 year old female. B) Normal liver tissue from a 42 year old female. Scale bar represents 20 μm .

3.10. Kaiso represses the CCND1 gene in a dose-dependent manner.

To determine the functional consequence of Kaiso overexpression on *CCND1* expression, minimal promoter-reporter luciferase assays were performed. A minimal *CCND1*-promoter reporter construct, -1748CD1 in the pGL3Basic backbone vector was used along with the pcDNA3-Kaiso overexpression effector plasmid. Reporter and effector plasmids were transfected into HeLa cervical carcinoma cells (which are highly transfectable and well established in our lab for luciferase assays) and assayed 24 hours later for luciferase levels. 500 to 2500 ng of pcDNA3-Kaiso plasmid were transfected into the cells in 500 ng increments. The -1748CD1 promoter-reporter was increasingly repressed as increasing amounts of Kaiso DNA were co-transfected (Figure 20). Relative lights units (RLU) were normalized using the internal control plasmid pRL-CMV by measuring Renilla luciferase levels.

3.11. In vitro methylated CCND1 promoter reporter plasmids are highly repressed with and without Kaiso.

In order to further explore Kaiso's methylation-specific repression of *CCND1*, minimal promoter reporter assays were performed on *in vitro* methylated 1748CD1-pGL3Basic constructs. The 1748CD1 reporter construct was first methylated with *Sss I* methylase and transiently transfected into HeLa cells. It was observed that expression from the 1748CD1 reporter was almost completely repressed in both the absence and presence of ectopic Kaiso (Figure 21). This led us to speculate that the repression of the

methyated 1748CD1 construct in the absence of ectopic Kaiso, may be due to the presence of endogenous Kaiso.

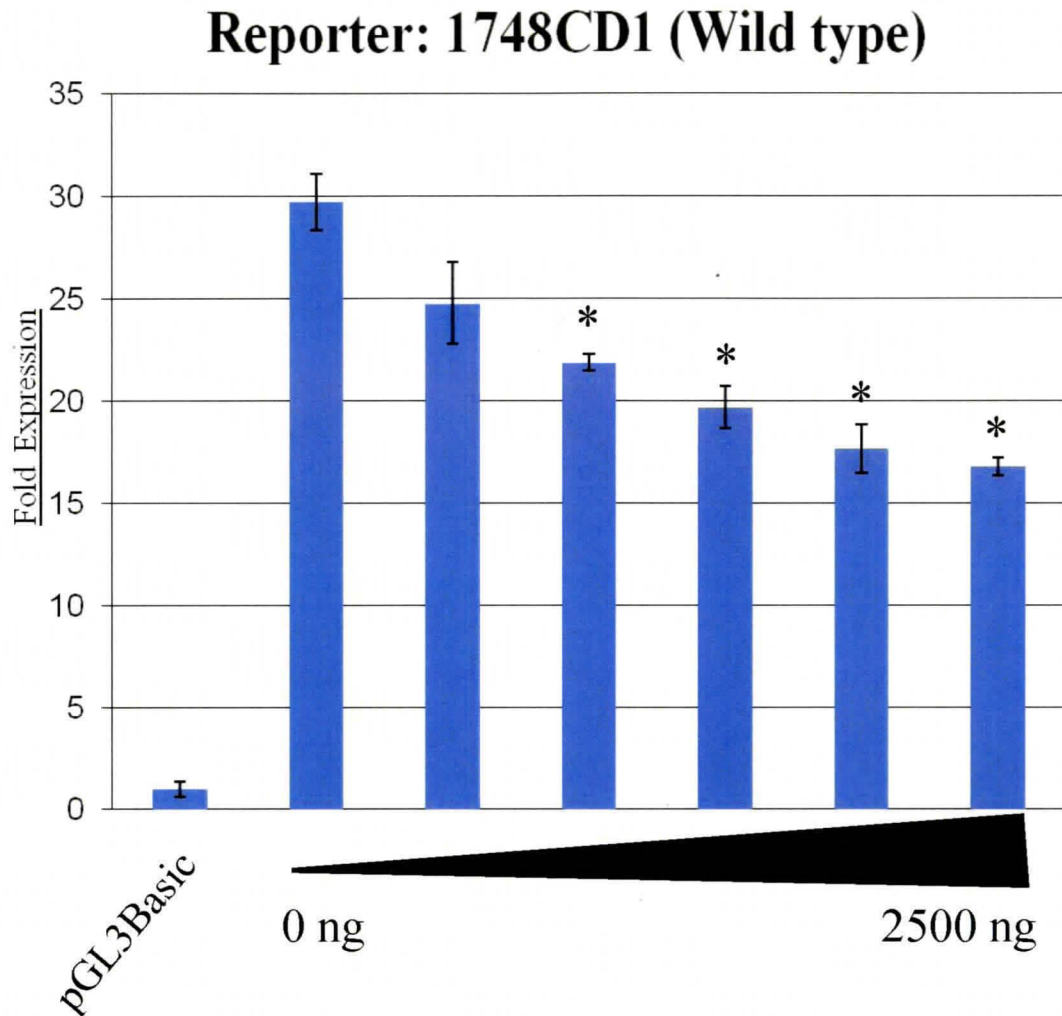


Figure 20: Kaiso represses the *CCND1* promoter in a dose-dependent manner. Promoter-reporter assays were performed using the -1748CD1 reporter vector co-transfected with the pcDNA3-Kaiso overexpression plasmid. The *CCND1* reporter construct was repressed ~ 2-fold when 2500 ng of Kaiso effector was transfected. Relative light units were normalized using the Renilla internal control, pRL-CMV. A one-way ANOVA was performed on each of the data sets compared to the 0 ng Kaiso transfection along with a Tukey's multiple comparison test. The data sets highlighted with a (*) represent those that were deemed statistically significant ($P > 0.05$).

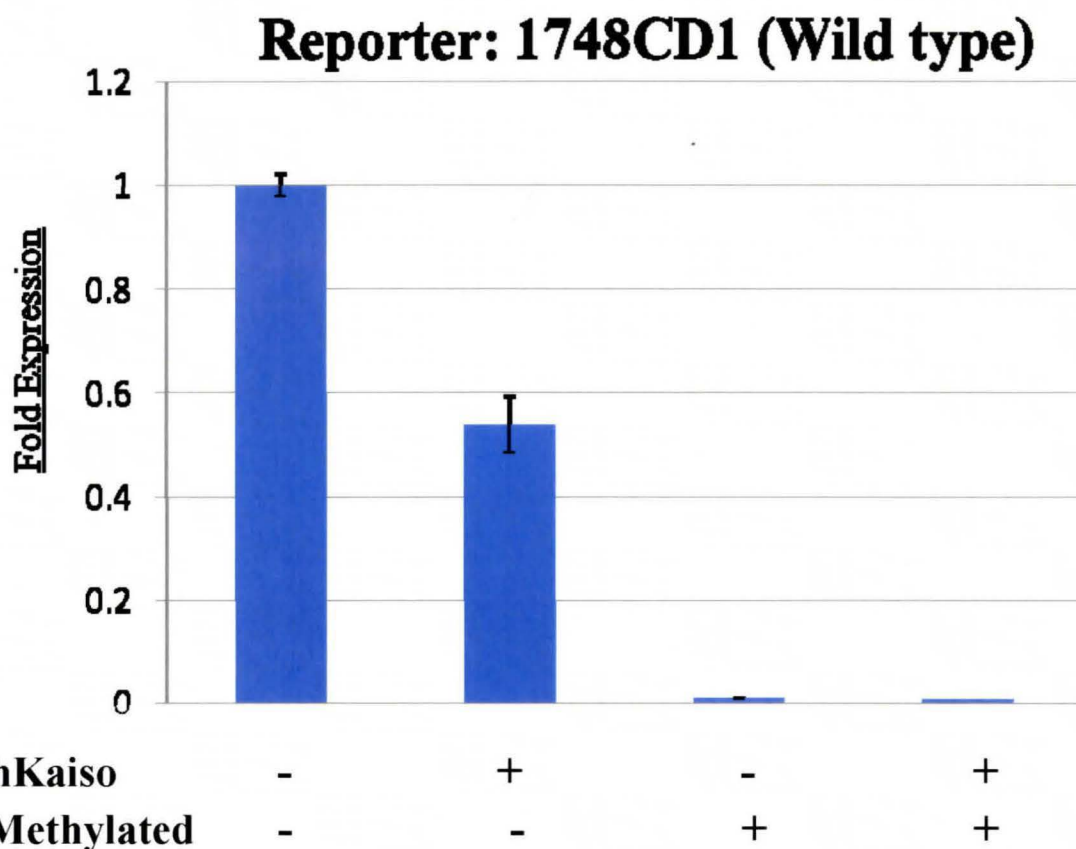


Figure 21: *In vitro* methylated 1748CD1 is completely repressed in the presence or absence of exogenous Kaiso. To investigate whether complete methylation of the 1748CD1 *CCND1* promoter reporter construct will affect Kaiso's ability to repress the *CCND1* promoter, we co-transfected it with Kaiso after methylation by *Sss I* methylase. We observed a complete repression of the *CCND1* promoter when methylated in the presence and absence of exogenous Kaiso. Expression levels are normalized to protein levels as determined by Bradford assay.

3.12. Kaiso-mediated repression modulates β -catenin activation of the *CCND1* promoter.

Previous work in our lab showed that Kaiso co-expression with β -catenin decreased β -catenin-mediated activation of the minimal *CCND1* reporter construct in luciferase assays. In order to confirm these results, and determine the cumulative effect of co-expression of β -catenin, p120 and Kaiso on the *CCND1* promoter, minimal promoter reporter assays were performed. As seen previously, Kaiso repressed, while β -catenin and p120 activate, the 1748CD1 reporter construct (Figure 22). It was also shown that co-expression of Kaiso and β -catenin resulted in a decreased activation of the *CCND1* promoter compared to when β -catenin was transfected alone (Figure 22). Furthermore, Kaiso's ability to decrease β -catenin activation of the *CCND1* promoter was eliminated when p120 was co-transfected. This confirms that Kaiso is able to modulate β -catenin-mediated activation of *CCND1* and suggests cross-talk between the canonical Wnt-signaling pathways and the Kaiso/p120 pathway. Again it also suggests that p120 is able to modulate Kaiso's repression of the *CCND1* promoter as well as Kaiso's ability to modulate β -catenin.

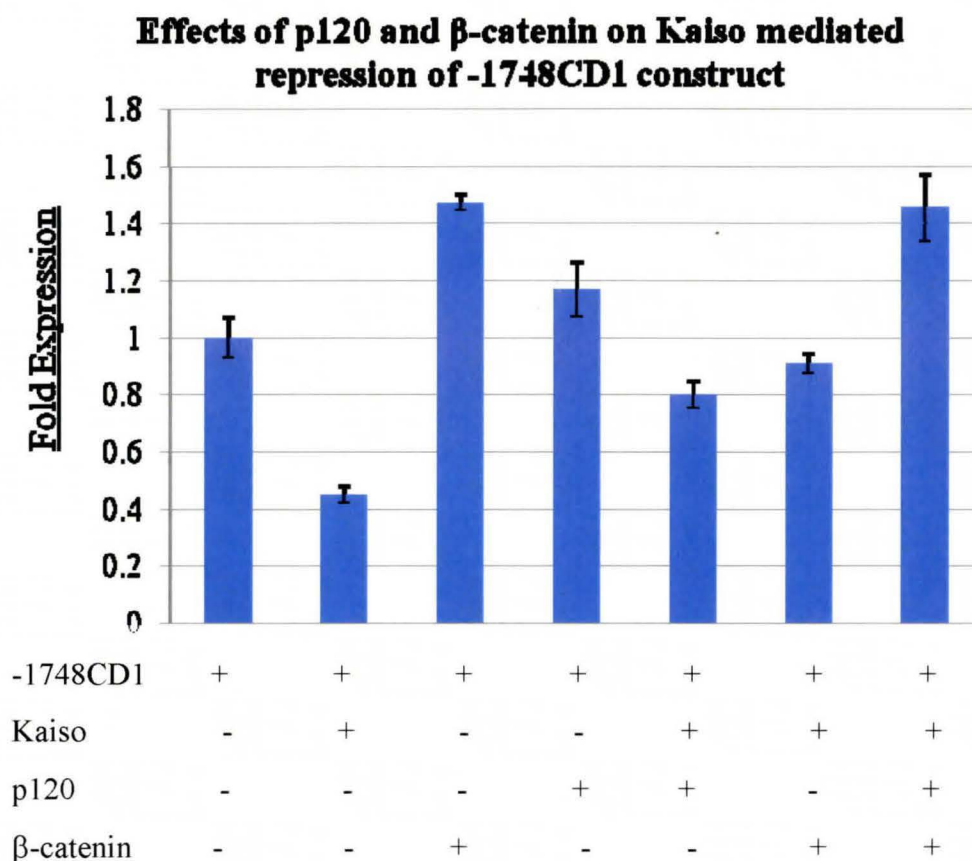


Figure 22: p120 relieves Kaiso's modulation of β -catenin-mediated activation of *CCND1*. To determine the effects of p120, β -catenin and Kaiso on the *CCND1* promoter, the proteins were transfected individually or in combination for luciferase assay. Co-expression of all three proteins resulted in expression levels similar to those found with β -catenin alone, indicating that Kaiso's modulation of β -catenin is relieved by the presence of p120. Expression levels are normalized to protein levels as determined by Bradford assay.

DISCUSSION

4.1. Kaiso associates with the CCND1-promoter in vitro.

Kaiso is a member of the BTB/POZ family of transcription factors, with roles in different aspects of development and tumorigenesis, reviewed in (Kelly and Daniel, 2006). In developing *Xenopus laevis* (frog) embryos xKaiso regulates downstream targets of canonical (*Siamois*, *c-Fos*, *CCND1*, *cMyc*) and non-canonical (*Wnt11*) Wnt signalling (Kim *et al.*, 2004; Park *et al.*, 2005). Along with roles in development, Kaiso has also been implicated in tumorigenesis. A cancer profiling array showed that Kaiso was misexpressed in 42% of the breast tumour samples, being underexpressed in 30% and overexpressed in 12% respectively (Daniel, 2003). This suggests that Kaiso may possess both oncogenic and tumour suppressor roles. This may be promoter-specific and could be attributed to Kaiso's dual specificity DNA binding ability as Kaiso binds sequence-specific and methylated CpG dinucleotides (Daniel *et al.*, 2002; Prokhortchouk *et al.*, 2001). The Wnt target gene, *matrilysin*, is repressed by Kaiso in human colon and cervical carcinoma cell lines (HT-29 and HeLa) (Spring *et al.*, 2005). Since Kaiso was also shown to regulate *CCND1* expression in *Xenopus* (Park *et al.*, 2005) we examined the human *CCND1* promoter region for potential KBS and methyl CpG sites. Three potential KBSs were identified in the *CCND1* promoter located at -1118, +24 and +1050 b.p. upstream of the transcriptional start site. In addition to these, a number of CpG dinucleotide pairs were identified in CpG islands within this promoter. To determine if Kaiso would associate with these distinct regions of the *CCND1* promoter, EMSA

experiments were performed. Oligonucleotides specific to each of the KBSs were created and Kaiso's ability to bind these regions was tested. We found that although Kaiso could bind to the -1118 KBS-specific probe in a sequence-specific manner (Figure 8A) it did not bind to either the +24 (Figure 9A) or +1050 (Figure 15A) KBS specific probes in a sequence-specific manner. The specificity of the interaction of Kaiso with the -1118 KBS probe was tested by introducing point mutations in the KBS (see Table 1A). This mutation abolished Kaiso's binding to the probe (Figure 8B). The fact that Kaiso can associate with the *CCND1* promoter in a sequence-specific manner supports the notion that Kaiso may be a regulator of the *CCND1* gene. Kaiso binding to the -1118 KBS may potentially elicit either a repression or activation response. Studies in *Xenopus* as well previous work in our lab have implicated Kaiso as a negative regulator of *CCND1* (Otchere, 2006; Park *et al.*, 2005). However, Kaiso DNA-binding via the KBS sequence can elicit both repression and activation of target gene transcription (Rodova *et al.*, 2004; Spring *et al.*, 2005). Specifically, Kaiso is known most commonly to repress target genes, such as *matrilysin* (Spring *et al.*, 2005). Alternatively, Kaiso activates at least one of its target genes, *rapsyn* (Rodova *et al.*, 2004), a synapse-specific protein required for acetylcholine receptor clustering, along with δ -catenin. Sequence inspection of the *CCND1* promoter region also identified a number of CpG dinucleotide pairs to which Kaiso may bind to regulate gene expression. Unlike Kaiso KBS-specific DNA binding, binding via methylated CpGs will result in repression of a target gene.

4.2. Kaiso binds the CCND1-promoter in a methylation-dependent manner in vitro.

Visual inspection of the +24 and +1050 KBS probes used in these experiments revealed the presence of a CpG dinucleotide pair sequence within these probes (See Table 1A). To test whether Kaiso may associate with regions of the *CCND1* promoter in a methylation-specific manner, the +24 and +1050 KBS probes were *in vitro* methylated with *Sss I* methylase and used in EMSA experiments. Interestingly, Kaiso DNA-binding was observed with the methylated +24 KBS probe (Figure 9B) but not to the methylated +1050 KBS probe (Figure 15B). These results suggest that Kaiso is binding to the +24 KBS probe via methylated CpG dinucleotides and not via the core KBS located within it. The specificity of Kaiso binding to the +24 probe was tested by performing EMSA using excess unlabelled probes using both methylated and unmethylated excess unlabelled probe. Both 50X and 100X excess of methylated unlabelled probe greatly reduced DNA binding whereas the same amount of unmethylated probe did not affect Kaiso DNA binding (Figure 10). This data further supports the idea that Kaiso may be regulating the *CCND1* promoter via dual mechanisms; methyl CpG and KBS. Within the +24 KBS probe, there are two CpG dinucleotides, separated by only one nucleotide. It is thus not surprising that methylation-specific binding is observed with the +24 KBS probe as this sequence is very similar to the preferred Kaiso methylation-specific binding sequence which consists of two sequential CpG dinucleotide pairs (i.e. CpGCpG). In contrast, the +1050 KBS probe has two CpG dinucleotide pairs separated by 14 intervening nucleotides, thus making it unlikely that Kaiso would bind them. Further analysis of the

CCND1 promoter revealed numerous CpG dinucleotides within CpG islands in the promoter.

To determine if Kaiso binds additional regions in the *CCND1* promoter in a methylation-dependent manner, EMSA experiments were performed using probes that span each of the CpG dinucleotide pairs in the *CCND1* promoter. Interestingly, we found that Kaiso could associate with all such regions of the promoter (Figure 11). However, it was evident that Kaiso was binding these regions with varying affinities, as indicated by the amount of time required to detect the signal after exposure of the autoradiogram to film. To assess Kaiso's relative binding affinity for these probes (as indicated by signal strength), the GST-Kaiso ZF protein was incubated with each probes and the binding reactions run on the same gel (Figure 12). The highest binding was seen for probes CpG5 and CpG8, which were also shown to bind specifically to Kaiso via cold-competition assays (Figure 14). These sites likely represent physiologically relevant Kaiso binding sites *in vivo* as they also bind to the full-length Kaiso protein in our EMSA experiments (Figure 13), which is usually inhibited *in vitro* by the POZ domain (Bardwell and Treisman, 1994).

Interestingly, the *CCND1* promoter is regulated by CpG methylation in B-cell malignancies (Liu *et al.*, 2004). Histone hyperacetylation and DNA hypomethylation have been correlated with increased *CCND1* expression in various mantle cell lymphoma (MCL) and multiple myeloma (MM) cell lines (Liu *et al.*, 2004). Methylation changes of the *CCND1* promoter have also been shown in Long-Evans rat leukemic cell lines (Kitazawa *et al.*, 1999). In normal rat endometrial stromal cells, methylated cytosines are

observed. This methylation was lost in the functioning layer, where *CCND1* expression increased (Kitazawa *et al.*, 1999). These data show that the methylation status of the *CCND1* promoter correlates with *CCND1* expression in both human and rat cancer models. This also shows that methylation of *CCND1* is aberrant in at least one type of tumorigenesis (Liu *et al.*, 2004). Furthermore, as shown in the rat model, loss of *CCND1* methylation may be part of a normal program of regulating *CCND1* expression in different tissue types. Collectively these data raise the exciting possibility that Kaiso may be involved in methylation-dependent regulation of the *CCND1* gene.

4.3. Kaiso associates with the CCND1-promoter in vivo.

In tumorigenesis, cells undergo uncontrolled proliferation and often harbour defects in cell-cycle regulators, such as Cyclin D1 (Malumbres and Barbacid, 2001). Cyclin D1 plays a role in both normal breast development and in breast tumorigenesis (Brennan and Brown, 2004). Since Kaiso is misexpressed in breast tumours, this misexpression may be associated with changes in Cyclin D1 levels (Daniel, 2003). Kaiso's association with its target gene promoters *in vivo* is postulated to be cell type specific since its association with the *rapsyn* promoter is exclusive to neuromuscular cell types (Rodova *et al.*, 2004). Given the role of Cyclin D1 in breast tumorigenesis and the fact that Kaiso had been shown to be misregulated in a number of breast tumour tissues we wanted to determine if Kaiso associates with the *CCND1* promoter in breast tumour cell lines. Excitingly, we showed that Kaiso associates with the *CCND1* promoter in MCF7 breast tumour cells (Figure 16). It associates with the -1118, KBS specific region,

just as previously described (Otchere, 2006), as well as with a methyl-CpG site which surrounds the transcriptional start site (named +24). This provides the first evidence that Kaiso could associate with both KBS, methylation-independent, and CpG, methylation-dependent sequences in the same promoter. Just as Kaiso can selectively activate or repress different sets of genes depending on the cell type, so too may Kaiso activate or repress the *CCND1* gene depending on the cellular environment. Now that it was established that Kaiso binds to the *CCND1* promoter the functional relevance of this association needed to be addressed.

4.4. Kaiso misexpression in the MCF7 breast cancer cell line positively correlates with Cyclin D1 levels.

To determine the effect of Kaiso misexpression on Cyclin D1 protein levels, a stable Kaiso overexpression cell line was generated in MCF7 cells (See Section 2.4 of Materials and Methods). This cell line along with a Kaiso depletion cell line previously created in the lab (Almardini, 2006) was used to assess changes in Cyclin D1 levels. MCF7 cells are derived from a pleural effusion of a metastatic breast tumour and possess characteristics of differentiated epithelial (Soule *et al.*, 1973). MCF7 cells double every 29 hours (ATCC, 2008); therefore the G1 phase occurs from 10-12 hours. Interestingly, unlike many breast tumour cell lines, MCF7 cells do not contain an amplification of the *CCND1* gene (Zwijssen *et al.*, 1996). This makes them ideal for studying changes in Cyclin D1 levels. We found that after synchronization by serum starvation, Cyclin D1 followed a typical pattern of relative expression showing increased expression after serum

stimulation for about 12 hours, the approximate length of time for G1 phase (Lewin, 2004), followed by a dramatic decrease in Cyclin D1 levels (when cells are entering S-phase) (Figure 18). Interestingly, when Kaiso was depleted, the absolute levels of Cyclin D1 dramatically decreased while still following the typical pattern associated with the cycling cell. When Kaiso was overexpressed, the results were less striking but increased levels of Cyclin D1 were observed for a longer period of time when compared to parental cells (Figure 18). Altogether these data suggest that in MCF7 cells, Kaiso may be positively regulating Cyclin D1 levels. This was an unexpected finding since previous work showed that Kaiso is a negative regulator of the *CCND1* gene in *Xenopus* (Park *et al.*, 2005). Since these studies were carried out in different organisms, the possibility exists that alternate co-factors may be involved in Kaiso's regulation of the *CCND1* promoter. This may explain why there is repression and activation in these two systems. This is the first study to examine the correlation between Kaiso and Cyclin D1 in human breast tumour cells. Since we know that Kaiso associates with the *CCND1* promoter via two mechanisms, sequence-specific and methylation-dependent (Daniel *et al.*, 2002), it is possible that Kaiso may also dually regulate the *CCND1* gene through those two mechanisms. Since DNA-methylation is associated with gene silencing, it is unlikely that Kaiso would be activating *CCND1* through binding to those regions. Interestingly, in a study looking at the association between Cyclin D1 levels and methylation status in B-cell malignancy, the methylation status of the *CCND1* promoter was also determined for MCF7 cells. It was found that the *CCND1* promoter is hypomethylated in MCF7 cells (Liu *et al.*, 2004) suggesting that Kaiso may be regulating *CCND1* expression via the

KBS in this cell line. Further studies aimed at looking at the proliferation rate of Kaiso misexpression MCF7 cells lines would help to shed light on cell growth changes that are associated with changes in Kaiso levels.

Another remarkable observation that was made during these experiments is that Kaiso levels were at their highest following serum starvation and continually decreased throughout the time course until Kaiso was almost completely absent. This is the first study looking at the changes in Kaiso levels with respect to the cell cycle. It appears that Kaiso levels in MCF7 cells are highest in quiescent state and decrease as the cell cycle progresses. Similar observations have been made by other researchers in breast cell lines; Kaiso protein levels continually decrease until 100% confluency is reached (Witt, 2008). Altogether these data further supports the hypothesis that *CCND1* is a Kaiso target gene.

4.5. Kaiso represses CCND1 expression in HeLa cells.

To functionally determine the transcriptional effects of Kaiso on a minimal *CCND1* promoter, promoter-reporter luciferase assays were conducted. Experiments were conducted using HeLa cervical carcinoma cells, a cell line that was well established for this assay in our lab. Overexpression of Kaiso in these cells repressed the luciferase reporter gene in a dose-dependent manner (Figure 20). This data suggests that Kaiso is acting as a transcriptional repressor of the *CCND1* gene in this cell line. This data is in support of previously conducted experiments in our lab (Otchere, 2006), as well as data in *Xenopus* which first described *CCND1* as a potential Kaiso target gene (Park *et al.*, 2005). Interestingly, this data contradicts what was observed in MCF7 cells, as described

previously. Collectively these data supports the idea that Kaiso may both activate and repress the *CCND1* promoter depending on the environmental context. Differences in Kaiso regulation of the *CCND1* promoter may be a part of normal development but may also be aberrant in some tumourigenic settings. Changes in methylation are a well characterized phenomenon in cancer, in particular hypermethylation of tumour suppressor genes such as RB and p16 (Lo *et al.*, 1996; Ohtani-Fujita *et al.*, 1997; Ohtani-Fujita *et al.*, 1993). Along with specific sites of hypermethylation occurs global loss of methylation, or hypomethylation, at other sites (Wilson *et al.*, 2007). As the *CCND1* promoter has been previously shown to be hypomethylated during tumorigenesis (Liu *et al.*, 2004), this may explain why we do not see Kaiso-specific repression in this cell line. As the methylation status of the *CCND1* promoter in HeLa cells is unknown it is not possible to determine if maintenance of methyl-CpG sites in *CCND1* allow Kaiso to repress this gene in this scenario. However, my data indicates that Kaiso regulates the *CCND1* gene in a dual-specificity manner through its different DNA-binding abilities; it may repress *CCND1* through binding methyl-CpG site and activate *CCND1* through binding the -1118 KBS site. Whereby, unmethylated conditions Kaiso may repress *CCND1* and in the absence of methylation Kaiso may gain access to KBS sites where it can cause activation. As past experiments have shown that Kaiso represses gene expression through binding sequence-specific KBS in other genes (Daniel *et al.*, 2002; Spring *et al.*, 2005) this theory is perhaps a little over simplified. Future experiments looking at the methylation status of an array of different cell lines along with changes in Cyclin D1 levels after Kaiso misexpression may help to support this hypothesis.

4.6. CCND1 promoter methylation silences gene expression.

To examine the effects of methylation of Kaiso-mediated regulation of *CCND1* in HeLa cells, luciferase reporter constructs were methylated *in vitro*. We found that when methylated, expression from the *CCND1*-derived reporter construct was completely repressed (Figure 21) both in the presence and absence of Kaiso. Recent studies have shown that there are two Kaiso-like proteins, ZBTB4 and ZBTB38 which may be functionally redundant with Kaiso (Filion *et al.*, 2006). Like Kaiso, both proteins can bind to methylated CpG dinucleotides and surprisingly ZBTB4 also binds to the KBS specifically (Filion *et al.*, 2006). The presence of these proteins may functionally replace Kaiso when Kaiso is absent. This may explain why we still detect repression of the *CCND1* promoter when Kaiso is depleted. The hypothesis that ZBTB4 may be functionally redundant to Kaiso was supported by recent work which showed that a Kaiso knock-out mouse was phenotypically normal and there were no changes in target gene expression (Prokhortchouk *et al.*, 2006). Functional overlap between Kaiso and ZBTB4 or ZBTB38 may also explain differences in Kaiso function in different cells lines (i.e. HeLa vs. MCF7 cells). Future studies looking at the role of Kaiso and ZBTB4 in gene expression need to be conducted to determine if there is functional overlap.

4.7. Kaiso modulates β -catenin-mediated activation of CCND1.

A number of Kaiso target genes are also downstream targets of the canonical Wnt signalling pathway (Park *et al.*, 2005; Spring *et al.*, 2005). Due to the overlap in target genes regulated by both proteins it was postulated that Kaiso may be acting to modulate

β -catenin mediated activation of target genes. To functionally assess whether Kaiso can modulate Wnt signalling, luciferase reporter assays were conducted using a *CCND1*-derived minimal promoter. As seen previously, β -catenin activates the *CCND1* promoter (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999) and Kaiso represses the *CCND1* promoter in HeLa cells (Figure 22). When Kaiso was co-transfected with β -catenin, Kaiso abrogates β -catenin-mediated activation of the *CCND1* minimal promoter (Figure 22). Furthermore, when cells were transfected with p120, activation of the *CCND1* minimal promoter was observed. This could be due to p120's modulation of endogenous Kaiso protein. When all three effectors, Kaiso, β -catenin and p120, were co-transfected, *CCND1* expression levels were close to those observed when β -catenin was transfected alone. This may be due to p120 sequestering Kaiso away from the promoter, thereby inhibiting Kaiso ability to modulate β -catenin activation of *CCND1*. These data present the already established trend that Kaiso is a modulator of the canonical Wnt signalling by selectively repressing β -catenin target genes.

4.8. Kaiso is differentially expressed in various human tissues.

Most of the data presented thus far was derived from *in vitro* assays in tissue culture cells. As such, experiments in whole tissues need to be conducted to support these data. As a first step towards performing correlative analysis of Kaiso and Cyclin D1 levels in a large panel of human tumour tissues, immunohistochemistry was performed on a tissue array containing various tissue types. In these preliminary experiments Kaiso-specific staining of breast tissue was not observed in the conditions tested (data not

shown). It is likely that different conditions will likely increase the sensitivity of these assays to allow detection of the Cyclin D1 antigen in breast tissue. Indeed, recent experiments from our lab showed strong Kaiso binding in breast tissue (Beatty, 2008). Excitingly, Kaiso staining was observed in a number of other tissues including, skin and liver (Figure 19). Strong Kaiso staining was observed in normal skin tissue (Figure 19A) where it may be hypothesised to be repressing *CCND1* levels. One of the first steps toward skin tumorigenesis is the overexpression of Cyclin D1 (Burnworth *et al.*, 2006) perhaps loss of Kaiso regulation of *CCND1* may help contribute to this phenomenon, either through hypomethylation or through loss of Kaiso function. Disruption of the β -catenin function has also been shown to occur in melanoma lesions (Demirkan *et al.*, 2007). It was shown that melanomas derived from chronically sun damaged skin are more likely to have activated Wnt/ β -catenin pathway. The possibility exists that in some cases Wnt/ β -catenin pathway activation may be due to loss of Kaiso expression/function and thus loss of regulation of Kaiso target genes.

Expression of Kaiso in normal liver tissue suggests that it may play a regulatory role in liver cells. A recent study in BEL-7404 hepatoma cells showed that p120 overexpression results in down regulation of survivin and Cyclin D1 expression (Nong *et al.*, 2006). In this study they also showed that p120 overexpression enhanced the β -catenin-E-cadherin binding. The possibility exists that the increase in p120 expression could be acting to modulate Kaiso-mediated regulation of *CCND1*. This would be in support of the MCF7 Kaiso misexpression data where in a tumourigenic state Kaiso may be activating the *CCND1* promoter and in the case of BEL-7404 hepatoma cells; p120

overexpression may act to decrease Cyclin D1 via its interaction with Kaiso. Interestingly, the Kaiso binding partner, Znf131, is highly expressed in the liver (Donaldson, 2007), suggesting that the Znf131/Kaiso complex may have a role in liver. Future studies examining the effect of Kaiso misexpression on Cyclin D1 levels in hepatoma cells will help outline a role for Kaiso or the Kaiso-Znf131 complex in liver tumorigenesis.

4.9. Significance and Conclusions.

In this thesis I have characterized the cell cycle regulatory gene, *CCND1*, as a Kaiso target gene. We have shown that Kaiso may act as either an activator or repressor of the *CCND1* expression depending on the environmental conditions (i.e. certain co-repressors or co-activators only being expressed in specific cell lines). Our results indicate that this may depend on cell type and that Kaiso's dual specificity DNA-binding ability (both KBS sequence-specific and methylation-dependent CpGs) may determine how it regulates the *CCND1* gene. Our results show that misexpression of Kaiso directly correlates with changes in Cyclin D1 protein levels in MCF7 breast tumour cell lines. Furthermore, our results show that Kaiso and p120 modulate canonical Wnt signalling through β -catenin. Altogether, these data support our hypothesis that ***CCND1* is a physiologically relevant Kaiso target gene.**

These studies have addressed a number of questions regarding Kaiso's regulation of *CCND1*. However, there are still a number of unanswered questions which still need to be addressed. Given the opposing effects of Kaiso misexpression in either HeLa or MCF7

cells there are still a number of studies that need to be performed to address this. In particular, it is necessary to determine the functional overlap between Kaiso and its other family members, ZBTB4 and ZBTB38. Perhaps the differential expression of one of these Kaiso-like proteins in different cells lines may explain why we are seeing opposing effects in HeLa and MCF7 cell lines when Kaiso is depleted. Future studies examining the methylation status of the *CCND1* promoter, via bisulphite sequencing, are needed to correlate changes in *CCND1* expression with changes in methylation. Furthermore, changes in *CCND1* transcript levels following Kaiso misexpression are relatively uncharacterized (See Appendix A for preliminary data). Therefore future studies examining changes in *CCND1* levels following Kaiso misexpression still need to be addressed, particularly how this is related to the cell cycle. The novel discovery that Kaiso protein levels are noticeably decreased in MCF7 synchronized cells as the cell cycle progresses is also a potential area of future in study. This would address a largely uncharacterized area of research looking at how Kaiso itself is regulated. Altogether, my studies have suggested Kaiso as having a role in the cell cycle, mainly through its regulation of *CCND1*, but also due to its own expression changes in the cell cycle.

Appendix A: Supplementary Data

The data presented here are a summary of results obtained during the final weeks of this thesis project that are still in their preliminary stage. These results may act as a starting point for future experiments looking at Kaiso function.

Materials and Methods

A.1. Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

Cells were serum starved as described in Section 2.2 and RNA was isolated using the Norgen Total RNA Isolation kit (Norgen). The isolated RNA samples were diluted to 0.5 µg/mL and 2 µL of each of the samples were treated with DNaseI (Invitrogen). DNaseI treated RNA was synthesized into cDNA using the SuperScript™ One-Step (Invitrogen) according to manufacturer's protocol. qRT-PCR was carried out using 12.5 µL of SybrGreen (Biorad), 1 µL of forward and reverse gene-specific primers (10 µM each) (Table 5), 5 µL of cDNA and 5.5 µL of dH₂O (Ultra Pure Distilled Water; Invitrogen). Specific genes were amplified from the cDNA using the Applied Biosystems 7900HT real-time PCR system (Applied Biosystems) using the following program: an initial denaturation period of 10 minutes, followed by 40 cycles of a 30 second denaturing step at 94°C, a 45 second annealing step at 55°C and a 30 second elongation step at 72°C. A final step was added to create a dissociation curve in order to ensure that non-specific amplification did not occur.

Table A-1: Primers used in qRT-PCR Experiments.

Name	ID	Sequence	Ref.
hKaiso-for	ML10859	5'-TGCCTATTATAACAGAGTCTTT-3' (binds 491-512 of cDNA)	(Kelly, 2004)
hKaiso-rev1	ML10860	5'-AGTAGGTGTGATATTTGTTAAAG-3' (binds 716-738 of cDNA)	(Kelly, 2004)
β-actin-for	ML16943	5'-CTCTTCCAGCCTTCCTTCCT-3'	(Kelly, 2004)
β-actin-rev	ML16944	5'-AGCACTGTGTTGGCGTACAC-3'	(Kelly, 2004)
CyclinD1-for	ML083222	5'-CCGTCCATGCGGAAGATC-3'	(Medeiros <i>et al.</i> , 2002)
CyclinD1-rev	ML083223	5'-GAAGACCTCCTCCTCGCACT-3'	(Medeiros <i>et al.</i> , 2002)

Results and Discussion

A.2. CCND1 transcript levels are higher in Kaiso overexpression cell line.

Initial experiments using the stable MCF7 misexpression cell lines revealed that changes in Kaiso protein levels positively correlated with Cyclin D1 protein levels (See Section 3.8). To determine if these changes were due to changes in *CCND1* transcript levels, quantitative real-time PCR (qRT-PCR) was performed. Total RNA was extracted from the stable MCF7 misexpression cell lines at 0, 4, 8, 12, and 24 hours following serum stimulation. *Kaiso* transcript levels were overexpressed, as would be expected, in the MCF7 overexpression cell line (#27) (Figure A-1). Interestingly, in this cell line we observed a decrease in *kaiso* transcript levels over time following serum stimulation. This is in agreement with our observations of Kaiso protein levels which decrease as time progresses following serum addition (Figure 18). The Kaiso knockdown cell line (pRS-Kaiso) showed modestly lower levels of *kaiso* transcript levels than the control (pRS-Scrambled) cell line. Unlike our observations with Kaiso protein levels there does not seem to be a decrease in *kaiso* transcript levels in the control cell lines, (Empty and pRS-Scrambled) following serum addition. As this experiment has only been performed once, these experiments will need to be repeated in order to verify this finding.

Interestingly, *CCND1* transcript levels are much higher in the MCF7 Kaiso overexpression cell line (Figure A-2). Unexpectedly, *CCND1* levels are highest immediately following 24 hours of serum starvation. Perhaps this aberrant *CCND1* expression is due to the overexpression of Kaiso in this cell line. This is in agreement

with our observations of Cyclin D1 protein levels in this cell line. However, *CCND1* expression levels are not seen to be decreased in the Kaiso knockdown cell line. As this cell line only showed a modest decrease in *kaiso* levels, it is not possible to rule out that this may be due to inefficient knockdown of *kaiso* in this experiment. Future studies aimed at reproducing and confirming this data are needed in order to make any conclusive statements about the effect of Kaiso misexpression on *CCND1* transcript levels.

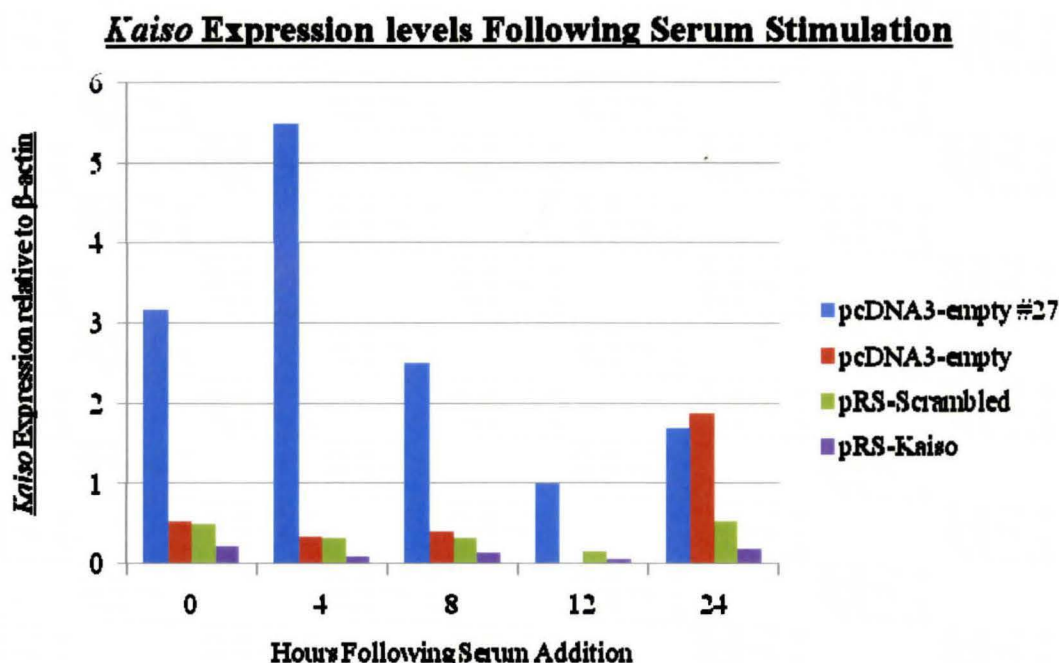


Figure A-1: *Kaiso* transcript levels following serum stimulation in stable MCF7 *Kaiso* misexpression cell lines. Cells were serum starved for 24 hours then treated for serum for 0, 4, 8, 12 and 24 hours to synchronize them. RNA was extracted and used to create cDNA for use in qRT-PCR studies. Values are normalized to β -actin transcript levels. *Kaiso* transcript levels are highest in the *Kaiso* overexpression cell line but exhibit levels similar to the control pcDNA3-empty cell line at 24 hours post serum addition. The *Kaiso* depletion cell line (pRS-*Kaiso*) displayed a modest decrease in transcript levels compared to the control cell lines.

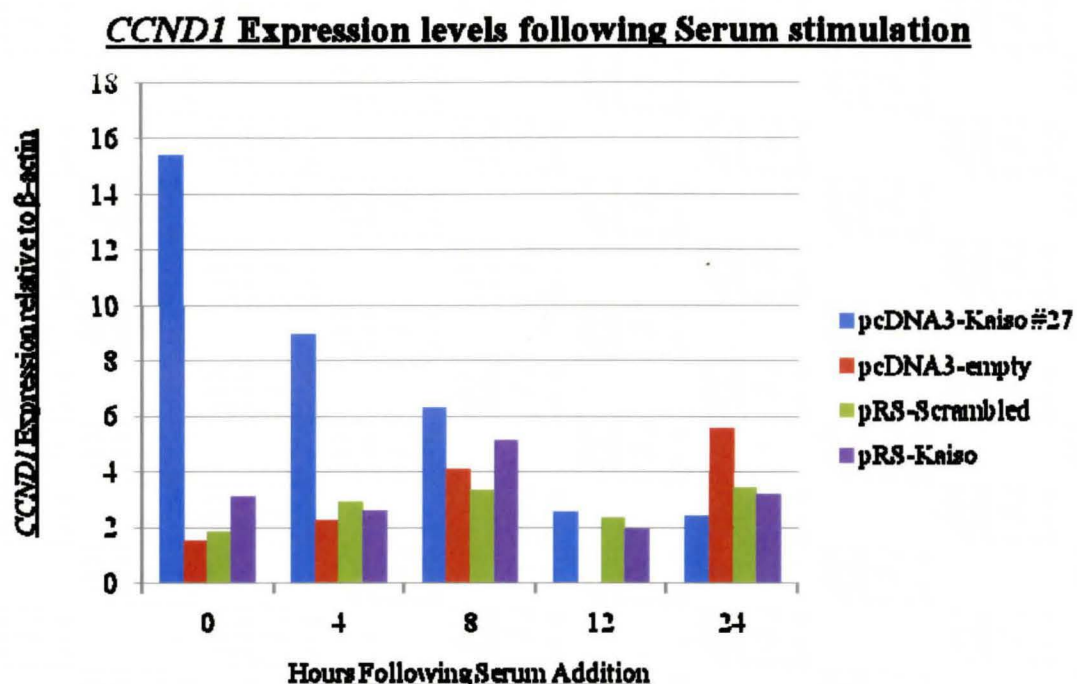


Figure A-2: *CCND1* transcript levels following serum stimulation in stable MCF7 Kaiso misexpression cell lines. Cells were serum starved for 24 hours then treated for serum for 0, 4, 8, 12 and 24 hours to synchronize them. RNA was extracted and used to create cDNA for use in qRT-PCR studies. Values are normalized to β -actin transcript levels. *CCND1* transcript levels are highest in the Kaiso overexpression cell line (pcDNA3-Kaiso #27) but gradually decrease to levels similar to the other cell lines after 12 hours. The Kaiso depletion cell line (pRS-Kaiso) does not exhibit a change in *CCND1* levels.

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