THE CATENIN p120^{ctn} REGULATES KAISO-MEDIATED TRANSCRIPTIONAL REPRESSION

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By

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Abstract

Kaiso is a POZ-ZF transcription factor initially identified as an interaction partner for the cell adhesion co-factor $p120^{ctn}$. Kaiso-DNA binding is inhibited by $p120^{ctn}$, implicating $p120^{ctn}$ in the regulation of Kaiso transcriptional activity. In this study, Kaiso repressed transcription of a luciferase reporter carrying four copies of the sequence-specific Kaiso-binding site (4xKBS) in artificial promoter assays. Mutation of the 4xKBS which is known to disrupt Kaiso-DNA binding also abrogated Kaiso-mediated transcriptional repression. Moreover, $p120^{ctn}$ inhibited Kaiso-mediated transcriptional repression via the 4xKBS, yet neither the $p120^{ctn}$ deletion mutant ΔR3-11 (lacking the Kaiso binding site) or $p120^{ctn}$ NLS mutant (which cannot enter the nucleus) inhibited transcriptional repression. Furthermore, in NIH 3T3 cells (which do not demonstrate a Kaiso-$p120^{ctn}$ interaction), $p120^{ctn}$ failed to inhibit transcriptional repression. Many POZ-ZF transcriptional repressors recruit an HDAC complex via their POZ domain to repress transcription. To investigate the mechanism of Kaiso-mediated transcriptional repression, the POZ domain of Kaiso was deleted, which abrogated transcriptional repression. Kaiso immunoprecipitates contained HDAC activity, and the HDAC co-repressor Sin3A co-immunoprecipitated with Kaiso, implying that Kaiso recruits Sin3A to repress transcription in an HDAC-dependent manner. Lastly, Kaiso repressed transcription via a human *matrilysin* promoter fragment. This suggests that the KBS element is functionally relevant and implicates *matrilysin* as a Kaiso target-gene. Collectively, these data establish Kaiso as a sequence-specific, HDAC-dependent transcriptional repressor that is regulated by the adhesion co-factor $p120^{ctn}$.

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Contributions by Others

The *pSilencer*^{TM} Kaiso and 4xKBS wild-type constructs were cloned by Monica Graham and Akeel Baig, respectively, and both the -296 Hmat promoter in pGL3 and the original 4xKBS sequence in the pGL2 Basic vector were kind gifts from Dr. Howard C. Crawford. The p120^{ctn} NLS mutant was cloned and characterized (Appendix B) by Kevin F. Kelly. Synthetic oligonucleotides that were either cloned into the *pSilencer*^{TM} vector or used for EMSA analyses were provided by Dinsdale Gooden and Alison Cowie of the Central Facility. All other experimental procedures were performed by Christopher Spring.

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Abbreviations

APC	**A**denomatous **p**olyposis **c**oli
Bcl-6	**B**-**c**ell lymphoma-**6**
BTB	**B**road complex, Tramtrack, and **b**ric à brac
CamKII	**Ca**lcium/cal**m**odulin-dependent protein **k**inase **II**
CAST	**C**yclic **a**mplification and **s**election of **t**argets
CBD	**C**atenin **b**inding **d**omain
Cdc42	**C**ell **d**ivision **c**ycle-**42**
CHD4	**Ch**romodomain-helicase-**D**NA-binding protein **4**
ChIP	**Ch**romatin **I**mmuno**p**recipitation
CMV	**C**yto**m**egalovirus
CTCF	**C**CTC-binding **f**actor
Cthrc-1	**C**ollagen **t**riple **h**elix **r**epeat **c**ontaining protein **1**
Dsh	**Dsh**evelled
EC	**E**xtracellular **c**adherin domain
ETO-1	**E**ight **t**wenty-**o**ne t(8;21) protein **1**
ETS	**E**26 **t**ransformation **s**pecific domain protein
EMSA	**E**lectrophoretic **m**obility **s**hift **a**ssay
FAZF	**F**ancomi **a**nemia **z**inc **f**inger
Fz	**F**rizzled receptor
GFP	**G**reen **f**luorescent **p**rotein
GSK	**G**lycogen **s**ynthase **k**inase

GST	**G**lutathione **S**-transferase
HDAC	**H**istone **deac**etylase
HIC-1	**H**ypermethylated **i**n **c**ancer-**1**
Id2	**I**nhibitor of **d**ifferentiation **2**
JMD	**J**uxta**m**embrane **d**omain
JNK	**J**un **N**-terminal **k**inase
KBS	**K**aiso **b**inding **s**ite
LEF	**L**ymphoid **e**nhancer **f**actor
MAR	**M**atrix **a**ssociated **r**egions
MBD3	**M**ethyl CpG **b**inding **d**omain protein **3**
MeCP1	**Me**thyl **C**pG binding **p**rotein **1**
MMLV	**M**oloney **m**urine **l**eukemia **v**irus
MMSV	**M**oloney **m**urine **s**arcoma **v**irus
MMTV	**M**ouse **m**ammary **t**umour **v**irus
mSin3	**m**ammalian **Sin3**
MTA2	**M**etastasis **a**ssociated protein **2**
NaB	**Na**$^+$ **b**utyrate (sodium butyrate)
NCoR	**N**uclear **co**-**r**eceptor
NES	**N**uclear **e**xport **s**ignal
NLS	**N**uclear **l**ocalization **s**ignal
NuRD	**Nu**cleosome **r**emodeling and histone **d**eacetylase complex
Pgk	**P**hospho**g**lycerate **k**inase

POZ	**P**oxvirus **z**inc finger
PLZF	**P**romyelocytic **l**eukemia **z**inc **f**inger
PMA	Phorbol 12-**m**yristate 13-**a**cetate
qRT-PCR	**q**uantitative **r**eal-**t**ime **p**olymerase **c**hain **r**eaction
Rac	**Ra**s-related **C**3 botulinum toxin substrate
Rapsyn	**R**eceptor **a**ssociated **p**rotein of the **syn**apse
Rb	**R**etino**b**lastoma protein
RbAp46/48	**R**etino**b**lastoma-**a**ssociated **p**rotein **46** and **48**
Rho	**R**as **ho**mology
RNAi	**RNA i**nterference
RSV	**R**ous **s**arcoma **v**irus
SAP 18/30	Sin3-**a**ssociated **p**roteins **18** and **30**
siRNA	**s**mall **i**nterfering **RNA**
SMRT	**S**ilencing **m**ediator of the **r**etinoid and **t**hyroid hormone receptor
Src	**Sarc**omagenic Avian sarcoma virus protein
TBL1	**T**ransducin (**b**eta)-**l**ike **1**
TBLR1	**TBL** **r**elated protein **1**
TCF	**T**-**c**ell factor
TSA	**T**richo**s**tatin **A**
Wg	**W**in**g**less protein
Xist	**X**-**i**nactivated chromosome **s**pecific **t**ranscript
ZF	**Z**inc **f**inger
ZF5	**Z**inc **f**inger **5** protein

Introduction

1.1 Cadherins: The Molecular Basis of Cell-Cell Adhesion

1.1.1 Classical Cadherins

The classical cadherins are a family of cell-cell adhesion proteins originally found in the **E**pithelial, **N**euronal, the **P**lacental, and **R**etinal tissues (E-, N-, P-, and R-cadherin) of the body, respectively. Cadherins form large multiprotein complexes (adherens junctions) that mediate cell-cell adhesion in all solid tissues of the body and are a major determinant of cellular organization and morphogenesis (46, 141). Cells expressing a specific cadherin form productive cell-cell contacts with other cells expressing the same cadherin (i.e. E-cadherin expressing cells will form cadherin-based cell-cell contacts with other E-cadherin expressing cells but not with N-cadherin expressing cells) (95, 141). Therefore, the cadherins are distinguished not only by their cell-type specific expression patterns, but also determine which cells will make productive adhesive contacts, thereby regulating cellular organization. However, while each of the classical cadherins differs in their tissue-specific expression profile, they share many molecular and functional properties. The classical cadherins are characterized by; i) an extracellular domain consisting of five **e**xtracellular **c**adherin (EC) domains, ii) a single pass transmembrane domain, and iii) an intracellular domain that complexes with a family of cell-cell adhesion co-factors, the catenins.

Functionally, the extracellular EC domains of the cadherins are responsible for the formation of cadherin homomultimers and as such regulate cadherin adhesive activity. The EC repeats initially mediate the formation of a cadherin-cadherin homodimer on the

surface of one cell, and disruption of this cadherin dimer abrogates cell-cell adhesion (15). Cadherin dimers on the surface of neighboring cells then complex together and physically adjoin neighboring cells **(Figure 1)**.

Morphogenesis is defined as the process by which individual cells organize themselves into tissues, organs, and functional multicellular structures (108). However, transformed tissue demonstrates altered morphogenetic features, concomitant with aberrant cadherin activity (113, 127). It is thus theorized that cadherins regulate "normal" morphogenetic organization of tissues, which is perturbed during tumourigenesis.

Experiments demonstrated that the expression of a specific classical cadherin determines which cells will associate with one another (40, 95, 136). Cadherins are therefore at least partially responsible for the basis of cellular locomotion and patterning. As a result, it was subsequently proposed that a change in cadherin expression within a tumour might promote aberrant morphogenesis by "convincing" the mutant cell population that the appropriate cell adhesion partners are not present. In an epithelial carcinoma, tumour cells initially express E-cadherin but a shift in cadherin expression (i.e. to N-cadherin) would "force" the tumour cells to migrate from the epithelial tissue (expressing E-cadherin) in search of cells which express the same cadherin as the tumour cells (N-cadherin)(57, 92). Consequently, tumour cells that undergo such a shift in cadherin expression would metastasize and form productive cell-cell contacts at a secondary site. In this manner, a shift in cadherin expression could promote the metastatic and invasive phenotype (48, 57, 92, 154).

Cadherin-Based Cell-Cell Adhesion

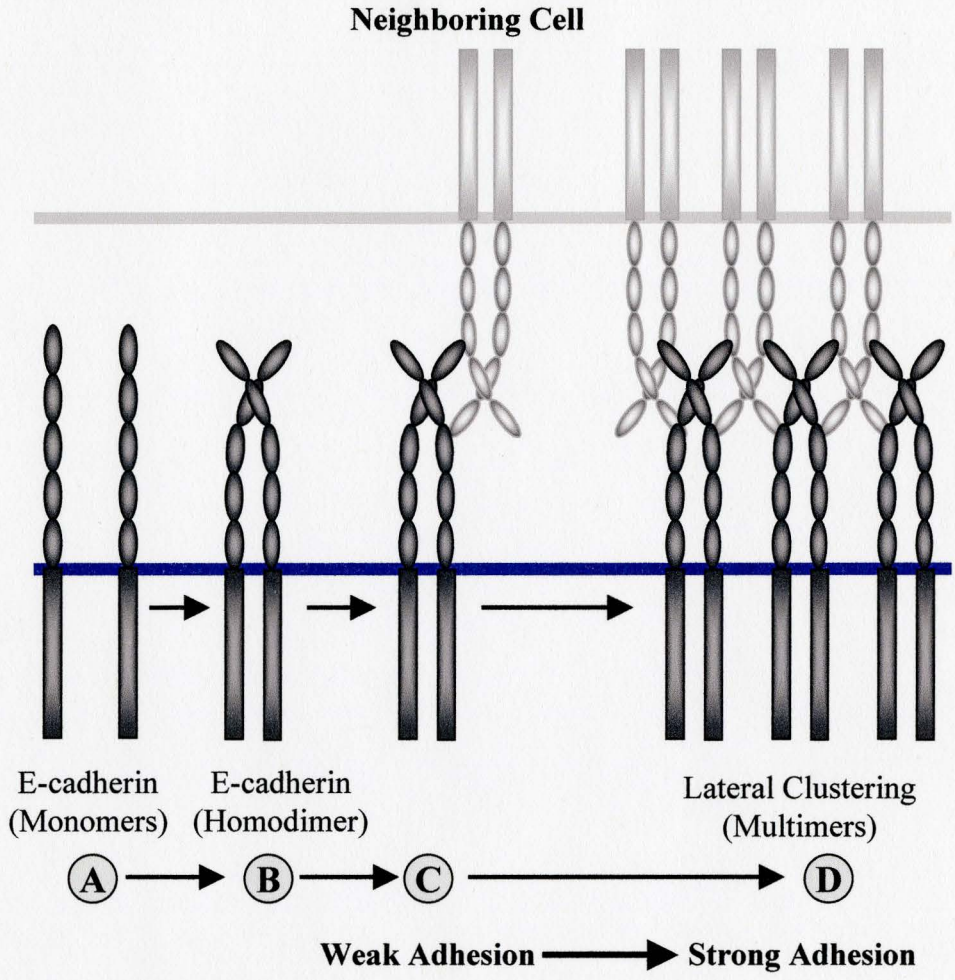


Figure 1: *Cadherin-based cell-cell adhesion.* E-cadherin is composed of 5 extracellular EC domains, a single-pass transmembrane domain, and an intracellular domain. E-cadherin monomers (A) homodimerize (B) via their EC domains and associate with homodimers (C) on the surface of neighboring cells, tethering adjacent cells together. Strong cell-cell adhesion requires the "lateral clustering" (D) of cell adhesion complexes.

1.1.2 E-Cadherin

E-cadherin is the most studied and well understood of the classical cadherins. Studies have focused on the cancer-related roles of E-cadherin in epithelial cells because epithelial carcinomas contribute to the majority of human tumours (9). *Frixen et al.1991* demonstrated that loss of E-cadherin expression not only correlates with epithelial tumour metastasis, but with the de-differentiation of epithelial carcinoma cell lines of the bladder, breast, lung, and pancreas (39). Subsequently, *Becker et al. 1994* demonstrated that E-cadherin is mutated in 50% of diffuse-type human gastric carcinomas (6). These studies clearly establish a correlation between tumourigenesis and E-cadherin mutation. Together these studies and others (7, 78, 134) firmly establish a correlation between aberrant E-cadherin expression and several aspects of tumourigenesis (transformation, metastasis and de-differentiation).

E-cadherin expression not only *correlates* with tumourigenesis, but also plays a *causal* role in promoting cellular transformation and the invasive phenotype. Ectopic expression of E-cadherin in de-differentiated, metastatic carcinomas inhibits invasive tumour growth, thus confirming a causal relationship between E-cadherin function and the inhibition of the invasive phenotype of carcinoma cells (39, 91, 147). In addition, *Graff et al. 1995* demonstrate that the E-cadherin promoter is specifically methylated and transcriptionally inactive in breast carcinomas, but not in normal breast tissue. In these studies, exogenously introduced, unmethylated E-cadherin promoters were normally expressed. Furthermore, demethylation of the endogenous,

transcriptionally silent E-cadherin promoter partially restores endogenous E-cadherin expression (44). These data led to the hypothesis that E-cadherin is a tumour suppressor that is specifically silenced in transformed cells. These studies also established that E-cadherin plays a causal role in inhibiting tumourigenesis by altering cell adhesion and invasiveness.

Despite strong evidence that loss of E-cadherin expression correlates with tumourigenesis, a number of gastric carcinomas maintain expression of E-cadherin (130). Transformed cells that maintain E-cadherin expression instead lack functional cadherin co-factors, the catenins. In the de-differentiated lung carcinoma cell line (PC9) E-cadherin expression is unaltered but the catenin co-factor, α-catenin (that cytosolically bridges E-cadherin to the actin cytoskeleton), is absent (50, 97, 131). Upon restoration of α-catenin expression, the PC9 cell line demonstrates appropriate cell adhesion, a polarized epithelial phenotype, and reduced cell growth. These data link deficiencies both in E-cadherin or the E-cadherin-associated cofactors (the catenins) with the de-differentiated, invasive phenotype.

1.2 Catenins: Regulators of Cadherin Function

In addition to the extracellular EC domains of the cadherins, which mediate cell-cell adhesion, cadherins also contain a conserved intracellular domain that binds a family of ubiquitously expressed cell-adhesion co-factors, collectively referred to as the catenins ($p120^{ctn}$, α-, β-, and γ-catenin)(98, 103). The classical catenins are comprised of two Armadillo-like catenins (β-, and γ-catenin) as well as the vinculin-like catenin, α-catenin. Deletion mutants of the E-cadherin extracellular domain (corresponding to the intracellular and transmembrane domains alone) act in a dominant negative manner on cell-cell adhesion. These E-cadherin mutants likely sequester catenins, resulting in a weakening of endogenous E-cadherin mediated cell-cell adhesion (41, 64). Several lines of evidence establish that the interaction between the catenins and the intracellular domain of E-cadherin are not only involved in regulating cell-cell adhesion, but cellular transformation and epithelial cell de-differentiation as well.

1.2.1 Classical Catenins: Cell Adhesion co-factors

The classical-catenin family is comprised of α-, β-, and γ-catenin (or plakoglobin) that were respectively described as 102, 88, and 80 kDa proteins associated with the cytoplasmic domain of E-cadherin (100). β-catenin and plakoglobin are characterized by 12 **Arm**adillo repeats (Arm), which share 76% identity between the two proteins (38). These Arm repeats are found in other catenins (i.e. the *Drosophila* homologue of β-

catenin, Armadillo, and the distantly related non-classical catenin, p120^{ctn}) and mediate a diverse range of protein-protein interactions.

Functionally, β-catenin/plakoglobin bind a distinct interface on the intracellular domain of E-cadherin, termed the **c**atenin **b**inding **d**omain (CBD, **Figure 2**) (103), in a mutually exclusive manner, and form a ternary complex with α-catenin (16). β-catenin has a higher affinity for the CBD, and therefore has been more extensively studied as a cell adhesion cofactor (1). Within this complex, α-catenin anchors the adhesion complex to the actin cytoskeleton and reinforces areas of cell adhesion (68, 103, 121). According to this model, β-catenin/plakoglobin act as protein bridges that anchor the cadherin cell-cell adhesion plaque to α-catenin and the actin cytoskeletal network. Disruption of the cadherin-catenin complex (i.e. uncoupling β-catenin/plakoglobin from E-cadherin by deleting the CBD) results in adhesion-deficient E-cadherin complexes (103). Adhesive activity of E-cadherin CBD deletion mutants can be reconstituted by directly fusing α-catenin to the intracellular E-cadherin domain (90). Rescue of adhesive function by α-catenin, however, requires the functional α-catenin-actin binding domain (90). These data confirm that the role of β-catenin/plakoglobin is to anchor the cadherin complex to the actin cytoskeleton via α-catenin.

Regulation of Cell-Cell Adhesion by the Catenins

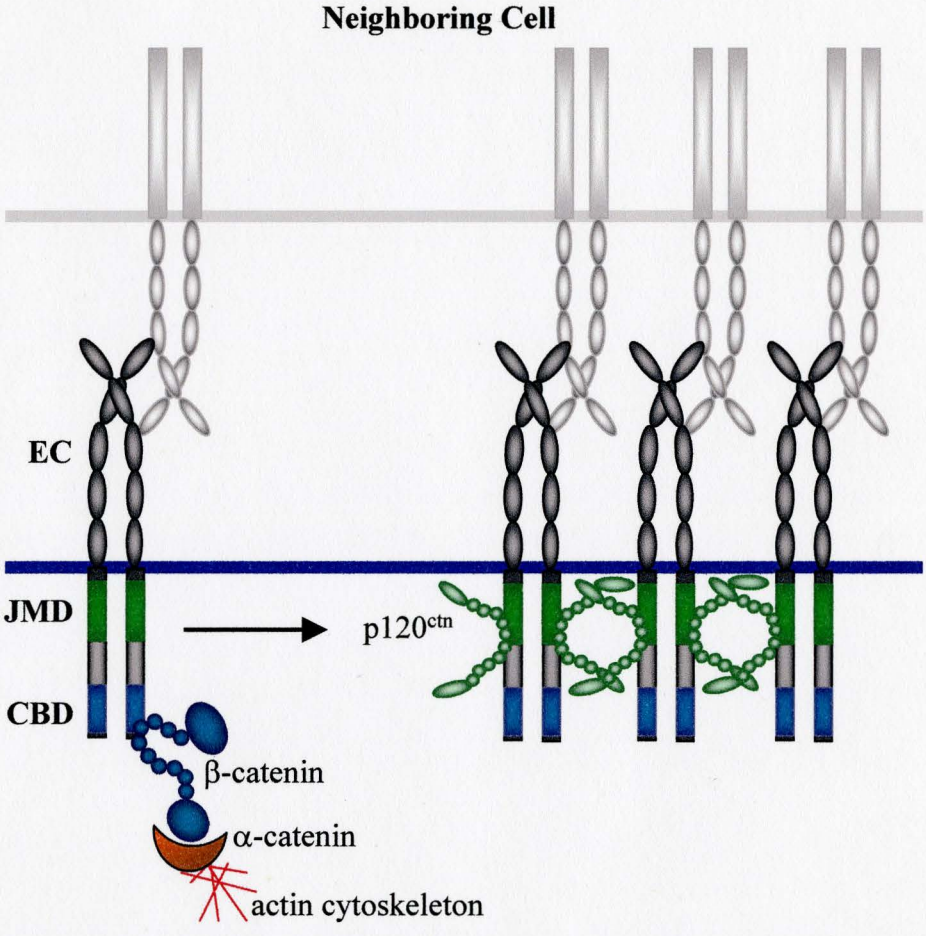


Figure 2: *Regulation of cell-cell adhesion by the catenins.* The **c**atenin **b**inding **d**omain (CBD) of E-cadherin associates with the actin cytoskeleton via β- and α-catenin, strengthening cell-cell adhesion. The **j**uxta**m**embrane **d**omain (JMD) of E-cadherin associates with $p120^{ctn}$ and mediates lateral clustering, which promotes strong cell-cell adhesion.

Disruption of the cadherin-catenin complex not only perturbs cell-cell adhesion, but also contributes to neoplastic transformation. In rat colorectal tumours, membrane-associated β-catenin is decreased and free cytoplasmic/nuclear β-catenin levels are increased (139). Membranous β-catenin/plakoglobin levels are also preferentially excluded from the invasive front of squamous cell carcinomas (76), providing an inverse correlation between membranous β-catenin and the invasive phenotype.

Moreover, nuclear levels of β-catenin/plakoglobin increase in transformed/de-differentiated tumour cells, and mutant oncogenic forms of β-catenin translocate to the nucleus and activate gene transcription (110). These data confirm that membrane-bound β-catenin/plakoglobin is inversely correlated with the invasive and metastatic phenotype and provide a link between the gene-regulatory and transforming properties of β-catenin/plakoglobin.

1.2.2 β-catenin: Gene Regulatory Roles and the Wg/Wnt Pathway

β-catenin indirectly anchors the cadherin complex to the actin cytoskeleton at the cell surface (16). In addition, β-catenin also plays a distinct gene regulatory role in the nucleus; ventral overexpression of β-catenin in *Xenopus laevis* results in the induction of a secondary body axis (1, 42, 68). Cadherin overexpression sequesters catenins at the membrane and reduces nuclear and free cytosolic pools of β-catenin. Concomitantly, the ability of β-catenin to induce the formation of a secondary body axis in *Xenopus laevis* (37) is inhibited. These data, confirm that the nuclear- and

cadherin-associated roles of β-catenin are both antagonistic and functionally distinct. Early experiments in which anti-β-catenin antibodies were injected into *Xenopus laevis* embryos demonstrated that β-catenin inactivation induces a secondary body axis phenotype, reminiscent of studies involving the misexpression of the canonical **w**ingless/**Wnt** (Wg/Wnt) pathway members (81), implicating β-catenin in the transduction of Wnt signals. Furthermore, the Arm repeats of β-catenin interact canonical Wg/Wnt signaling pathway transcription factors (8, 54), and this interaction is necessary for the induction of a secondary body axis phenotype by β-catenin (88).

1.2.3 The Canonical Wnt Pathway and Nuclear Roles for β-catenin

Wnt-1 was initially identified as a putative oncogene which enhances **m**ouse **m**ammary **t**umour **v**irus (MMTV)-mediated tumour formation (96), and later identified as the murine ortholog of the *Drosophila* wingless (Wg) protein. Signaling via the canonical Wnt signaling pathway **(Figure 3)** has emerged as a key regulator of β-catenin stability and cell proliferation. Soluble Wnt can bind the **F**rizzled (Fz) family of transmembrane receptors (150, 153). The presence or absence of this ligand-receptor association affects a signaling cascade, which subsequently regulates the formation of a β-catenin destruction complex.

Cadherin-Catenin Adhesion Complex Signaling

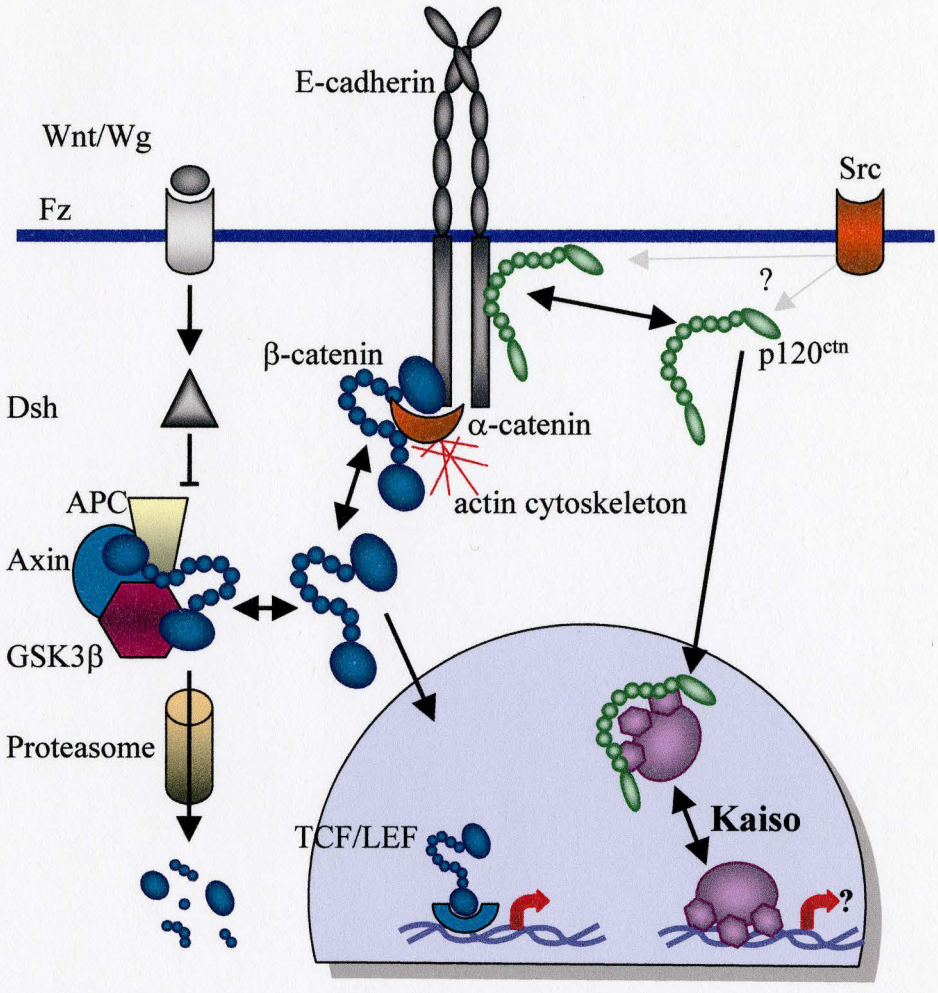


Figure 3: *Cadherin-catenin adhesion complex signaling.* Canonical Wnt signaling triggers formation of a nuclear β-catenin-TCF/LEF complex which results in tumourigenesis. The non-classical catenin p120^{ctn} can also associate with a transcription factor, Kaiso. However, the biological consequences of the Kaiso-p120^{ctn} interaction and/or Kaiso-mediated transcriptional regulation remain elusive.

In the absence of a Wnt signal, free cytosolic β-catenin complexes with **a**denomatous **p**olyposis **c**oli (APC), **g**lycogen **s**ynthase **k**inase (GSK)-3, and Axin (125, 137, 161, 163). GSK-3 is required to phosphorylate β-catenin and target it for proteasomal degradation. Overexpression of either a kinase-deficient GSK-3 mutant or a β-catenin mutant lacking GSK-3 phosphorylation sites results in constitutive β-catenin activity, confirming that GSK-3 phosphorylation regulates β-catenin activity (162). However, GSK-3 does not directly bind to β-catenin, but requires APC and Axin in order to form a functional "destruction complex". APC associates with Axin and β-catenin in order to recruit GSK-3 and target β-catenin for proteasomal degradation (124, 125). Phosphorylation of β-catenin via GSK-3 results in the ubiquitination of β-catenin and subsequent proteasomal degradation (47, 65).

In the presence of a canonical Wnt signal, β-catenin is stabilized, and formation of the destruction complex is inhibited by the cytosolic proteins **G**SK-3 **b**inding **p**rotein (GBP) and **Dish**evelled (Dsh). Following Wnt-Fz association, Dsh inhibits GSK-dependent phosphorylation of β-catenin, and protects β-catenin from proteasomal degradation (65). GBP binds to GSK-3, promotes the dissociation of the destruction complex, and reproduces the secondary body axis phenotype when overexpressed in *Xenopus* (74, 161). Currently, it is believed that Dsh, APC, GSK-3, and GBP form a quaternary complex that cannot associate with β-catenin. Consequently, free cytosolic β-catenin levels rise and translocate to the nucleus, where β-catenin interacts with the **T**-**c**ell **f**actor/**L**ymphoid **e**nhancer **f**actor (TCF/LEF) family of transcription factors (8, 54,

88, 110). β-catenin then acts as a transcriptional co-activator and promotes transcription of target genes such as *matrilysin*, *cyclinD1*, *Id2*, and *myc* which are involved in the regulation of cell adhesion and cell proliferation (12, 23, 49, 122, 132). Canonical Wnt signaling thus acts as a master regulator of cell proliferation by promoting accumulation of nuclear β-catenin. Mutations in members of the destruction complex, such as APC, occur early in tumourigenesis (111), resulting in nuclear accumulation of β-catenin, which correlates with cellular transformation (76, 111, 139). Thus, it is not surprising that constitutive activation of the Wnt/β-catenin pathway plays a causal role in human colorectal tumourigenesis (89).

1.2.4 p120^{ctn}: A Distinct Cell Adhesion co-factor

The intracellular domain of E-cadherin is characterized by its catenin binding domain (which associates with the classical catenins α-, β-, and γ-catenin), and the **j**uxta**m**embrane **d**omain (JMD)(**Figure 2**), which binds to the non-classical catenin p120^{ctn} (143, 156). p120^{ctn} was initially described as a Src substrate (118), and subsequently characterized as an Armadillo-catenin and a component of the E-cadherin cell-cell adhesion complex (116). p120^{ctn} binds to the JMD of E-cadherin, and does not associate with APC or α-catenin (26) implying that p120^{ctn} functions in a manner which is distinct from β- or γ-catenin.

Much controversy remains over whether p120^{ctn} enhances or perturbs cadherin-mediated cell-cell adhesion (4, 98, 101, 102, 143, 156). Deletions of JMD of E-cadherin

disrupt lateral cadherin clustering (156)(**Figure 1**), which is required for strong cell-cell adhesion (155, 156) and the inhibition of cell motility and invasion (19). This implies that $p120^{ctn}$, which associates with the JMD, induces cadherin clustering and strengthens cell-cell adhesion (**Figure 2**). Indeed, uncoupling $p120^{ctn}$ from the JMD results in a reduction of cell-cell adhesion and poor association between the actin cytoskeleton and sites of E-cadherin adhesive contacts (143). Moreover, recent studies in SW48 colorectal carcinoma cells demonstrate that exogenous $p120^{ctn}$ restores defects in epithelial cell morphology and E-cadherin function (56). Overexpression of $p120^{ctn}$ fails to alter E-cadherin mRNA levels but dramatically enhances E-cadherin half-life. These data strongly implicate $p120^{ctn}$ in the stabilization of E-cadherin protein levels and activation of E-cadherin-mediated adhesiveness, and support the hypothesis that $p120^{ctn}$ enhances cell-cell adhesion.

Contrary to evidence suggesting that $p120^{ctn}$ enhances cell-cell adhesion, other studies conclude that $p120^{ctn}$ disrupts adhesion and acts as a negative regulator of E-cadherin. For example, deletion of either the E-cadherin JMD or N-terminal deletions of $p120^{ctn}$ (which perturb Src-mediated tyrosine phosphorylation of $p120^{ctn}$) both result in enhanced cell aggregation (4, 98, 101, 102), suggesting that $p120^{ctn}$-E-cadherin association negatively regulates cell-cell adhesion. While discrepancies in the literature regarding the role of $p120^{ctn}$ in regulating E-cadherin function remain, additional roles for $p120^{ctn}$ in regulating the actin cytoskeleton and gene expression are emerging. These cytoskeletal/nuclear roles may help explain current conflicting theories of how $p120^{ctn}$ regulates E-cadherin function.

p120^{ctn} regulates members of the Rho-family GTPases (Rho, Rac, and Cdc42). Rho GTPases mediate polymerization of the actin cytoskeleton, which associates with E-cadherin complexes and positively regulates cell-cell adhesion. Previous studies confirmed that dominant active forms of Rho and Rac enhance cell-cell adhesion, while dominant negative Rho and Rac mutants inhibit cell-cell adhesion (13, 140). More recently, p120^{ctn} emerged as an activator of Cdc42 and Rac (45, 94) as well as an inhibitor of RhoA (3), providing a link between p120^{ctn}, the actin cytoskeleton, and the regulation of cell-cell adhesion. p120^{ctn} may thus regulate cell-cell adhesion directly via E-cadherin regulation, and indirectly via regulation of the actin cytoskeleton.

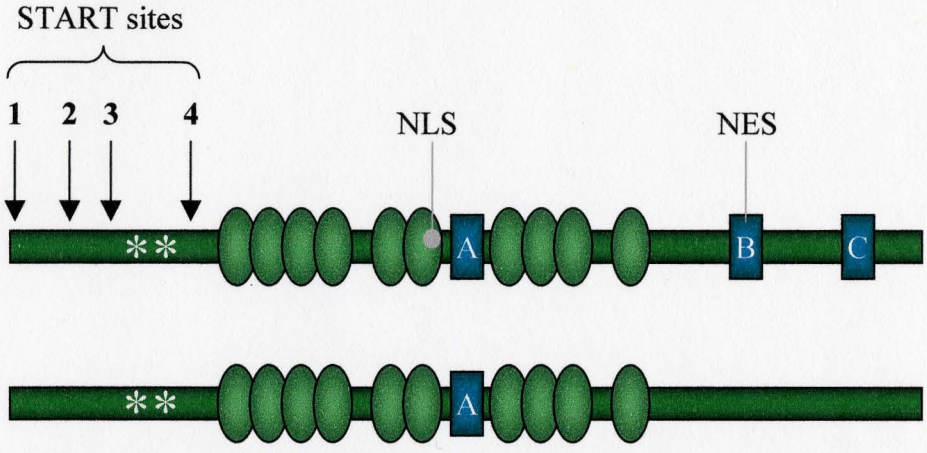
One possible explanation for the conflicting data regarding the role of p120^{ctn} in regulating cell-cell adhesion is as follows: differences in the Src-phosphorylation profile of p120^{ctn} and/or changes in nuclear activities of p120^{ctn} may alter the activity of p120^{ctn} (25, 61, 102). These possibilities were not addressed or controlled for in previous studies, and disruption of the interaction between p120^{ctn} and E-cadherin was assumed to abolish the effects of p120^{ctn} on cell-cell adhesion. In light of recent data, it is now clear that this assumption may be oversimplified. E-cadherin JMD deletion mutants may "force" p120^{ctn} to associate with Rho family GTPases and regulate cell-cell adhesion via a separate pathway. These JMD mutants could also promote the nuclear translocation of p120^{ctn} where it may exert separate effects on gene expression that impact cell adhesion in a possibly confounding manner. The possibility thus exists that p120^{ctn} may both positively and negatively regulate cell-cell adhesion, depending on which pathways (cadherin-associated, cytoskeletal, or nuclear) p120^{ctn} is acting upon.

1.2.5 p120^{ctn}: Possible Roles in Gene Regulation

The non-classical catenin p120^{ctn} has been reported to translocate to the nucleus (25, 61, 146), although the role of p120^{ctn} in the nucleus remains unknown. p120^{ctn} isoforms containing exon A (isoforms 1A, 2A, and 3A, **Figure 4**) translocate to the nucleus and induce a "branching phenotype", characterized by exaggerated cellular processes. In contrast, p120^{ctn} isoforms containing exon B (isoform 1AB), which carries a **n**uclear **e**xport **s**ignal (NES), or lack N-terminal Src phosphorylation sites (isoform 4A) are excluded from the nucleus and fail to induce a branching phenotype (2). Furthermore, mutation of a putative NLS in p120^{ctn} isoform 1A diminishes p120^{ctn} nuclear localization and abrogates the ability of p120^{ctn} to induce a branching phenotype (61). Thus, nuclear localization of p120^{ctn} appears to be necessary for certain biological activities of p120^{ctn}.

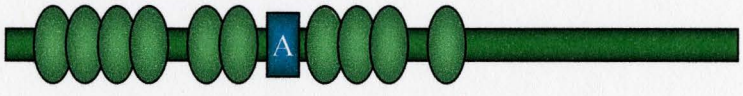
While the nuclear activities of p120^{ctn} are not yet fully understood, evidence indicates that the nuclear role for p120^{ctn} is involved in tumourigenesis. Reduced membranous staining of p120^{ctn} correlates with poor patient survival and increased bladder tumour stage and grade (138). Heterogeneous or reduced-membranous p120^{ctn} staining and tumour progression also correlates with colorectal (60, 133), breast (33), and poorly differentiated endometrial tumours (87). Therefore, altered subcellular localization of p120^{ctn} correlates with tumour progression, indicating that its subcellular localization is integral to its biological function, much like β-catenin.

Isoforms and Nomenclature of p120^{ctn}



p120^{ctn} Isoform 1A- contains N-terminal Src phosphorylation sites but lacks NES found in exon C: **can translocate to the nucleus**

p120^{ctn} Isoform 1AC- contains N-terminal Src phosphorylation sites and NES found in exon C: **rapidly exported from the nucleus**



p120^{ctn} Isoform 4A- lacks N-terminal Src phosphorylation sites: **fails to translocate to the nucleus**

Figure 4: *Isoforms and nomenclature of p120^{ctn}.* Four different p120^{ctn} isoforms are produced through the use of four alternate translational start sites (designated 1, 2, 3, and 4). Three exons (A, B, and C) are also differentially included in the mRNA via alternative splicing. Thus, a possible 32 variants of p120^{ctn} (isoform 1, 2, 3, or 4 with exon(s) A, B, C, AB, AC, BC, ABC, or neither A, B or C) exist. Note that exon B contains an nuclear export signal (2).

Previous studies demonstrated that $p120^{ctn}$ does not interact with APC (26), implying that the nuclear activities of $p120^{ctn}$ are not linked to the canonical Wnt signaling pathway. In order to gain further insight into the cellular role of $p120^{ctn}$, a yeast-two-hybrid screen was performed to identify interaction partners for $p120^{ctn}$. This screen identified a novel transcription factor, Kaiso, which specifically interacts with $p120^{ctn}$, providing the first direct evidence of a nuclear role for $p120^{ctn}$ in signal transduction (27).

1.3 Kaiso

1.3.1 Kaiso: the POZ-ZF Transcription Factor Family

Kaiso is a novel member of the BTB/POZ (**B**road complex, **T**ramtrak, **B**ric à brac/ **Po**x virus and **z**inc finger) ZF (**z**inc **f**inger) family of transcription factors (hereafter POZ-ZF proteins), strongly implicated in development and cancer (21, 22, 157, 158). The POZ-ZF family of transcription factors is characterized by a hydrophobic amino-terminal POZ protein-protein interaction domain and a carboxy terminal C_2H_2 ZF DNA-binding domain. Related POZ-ZF family members include **p**romyelocytic **l**eukemia **z**inc **f**inger (PLZF) and **B**-**c**ell **l**ymphoma-**6** (Bcl-6), which are implicated in promyelocytic leukemia (20, 21) and non-Hodgkin's lymphoma (157, 158), respectively. Both PLZF and Bcl-6 bind to sequence-specific DNA sequences and repress transcription as homo- or hetero-dimers (31, 51, 83, 84) by recruiting HDAC co-repressors (28, 32, 52, 55).

Similarly, Kaiso has been implicated as a transcriptional repressor when expressed as a chimeric Gal4 fusion protein (63). Like other POZ-ZF proteins, Kaiso

homodimerizes (25), and may heterodimerize with the POZ-ZF transcription factors Znf131 and **H**ypermethylated **i**n **c**ancer (HIC-1) via its POZ domain (Daniel Lab, unpublished data). However, whereas PLZF and Bcl-6 bind a single consensus DNA-binding site (18, 73, 128), Kaiso is a bi-modal DNA-binding protein. Initially, we characterized a sequence-specific (TCCTGCNA) consensus **K**aiso-**b**inding **s**ite (KBS) requiring a six nucleotide core sequence (CTGCNA) for Kaiso-DNA binding (27). Kaiso is also a member of the **M**ethyl **C**pG binding **p**rotein **1** complex (MeCP1) and bind methylated CpG dinucleotides (27, 112). Currently, no other POZ-ZF transcription factors bind DNA in both a sequence-specific and methylation-dependent manner. However, the **m**ethylated **D**NA **b**inding **p**rotein (MDBP) associates with a methylated DNA-binding sequence and a sequence-specific DNA binding sequence (149). Unlike Kaiso, however, the methylation-dependent and sequence-specific MDBP binding sites are identical with the substitution of a methyl-CpG site by a TG sequence (which mimics a MG moiety).

Kaiso is therefore not the only known bi-modal methylation-dependent and sequence-specific DNA-binding protein. The lack of homology between the methylation-specific and sequence-specific Kaiso-DNA binding sites, however, has not been reported before. Recent studies have shown that DNA-binding of the POZ-ZF transcription factors factor **b**inding to **I**ST (FBI-1) and **GAGA** binding factor (GAGA) is extremely flexible, which may account for the ability of Kaiso to bind two different sequences that demonstrate no homology to one another (36, 109).

We previously mapped both sequence-specific and methylation-dependent Kaiso-DNA binding activity to zinc fingers 2 and 3 (27). Yeast-two-hybrid analysis also previously demonstrated that the apparent $p120^{ctn}$ binding site encompasses the Kaiso-DNA binding domain (25)**(Figure 5)**. Therefore, $p120^{ctn}$ could associate with the Kaiso-DNA binding interface and sterically hinder DNA-association. This would result in Kaiso forming mutually exclusive complexes with either DNA or $p120^{ctn}$ and $p120^{ctn}$ could: i) inhibit the DNA-binding activity of Kaiso, and/or ii) regulate the transcriptional properties of Kaiso.

Putative Kaiso target genes have been identified on the basis of sequence-specific **K**aiso-DNA **b**inding **s**ites (KBS) in their cognate promoters. These target genes include known β-catenin target genes, such as *c-myc*, *matrilysin*, *cyclinD1*, and *Id2*, which are associated with cell proliferation (*c-myc*, *cyclinD1, Id2*) and cell motility (*matrilysin*). This finding implies that the putative Kaiso-$p120^{ctn}$ signaling pathway may act in either a synergistic or antagonistic manner to the β-catenin/Wnt signaling pathway.

Furthermore, many target genes thus far identified which carry KBS elements in their promoters are proto-oncogenes, implicating sequence-specific Kaiso-mediated transcriptional repression in the inhibition of tumourigenesis. In accordance with this theory, a human cancer-profiling array demonstrated that Kaiso mRNA is down regulated in 50% of ovarian tumours and 32% of breast tumours, relative to neighboring non-transformed tissue (Daniel Lab, unpublished data). Together these data suggest that Kaiso may act as a tumour suppressor and repress transcription of Wnt-regulated oncogenes in a sequence-specific manner.

Schematic Representation of Kaiso and Constructs Used

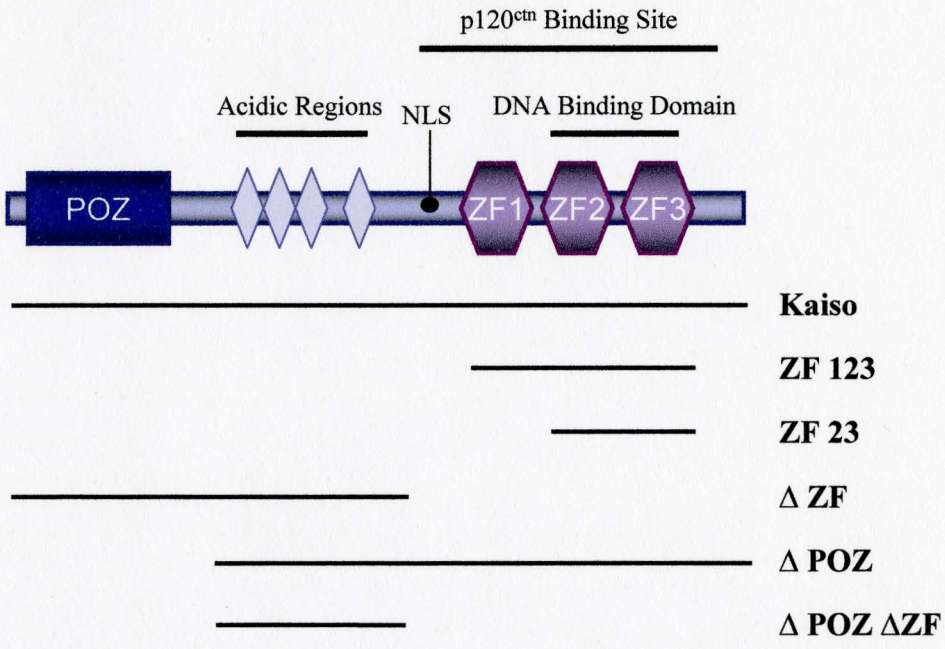


Figure 5: *Schematic representation of Kaiso and Constructs used.* A schematic representation of the functional domains of Kaiso is shown. Kaiso possesses an N-terminal POZ domain and three C-terminal ZF motifs. Deletion mutant analysis suggests that zinc fingers 2 and 3 are necessary and sufficient for both sequence-specific and methylation-dependent DNA-binding activity (26). Note that the p120^{ctn}-binding site overlaps with the DNA-binding site.

1.3.2 Transcriptional Repression via POZ-ZF Transcription Factors

PLZF and Bcl-6 are transcriptional repressors that recruit **H**istone **deac**etylase (HDAC) complexes via their POZ domains. Evidence suggests that dimerization of the POZ domain forms a charged pocket which interacts with HDAC co-repressors such as **N**uclear **co-r**eceptor (NCoR), **S**ilencing **m**ediator of the **r**etinoid and **t**hyroid hormone receptor (SMRT), and **m**ammalian **Sin3** (mSin3) (75, 84). These modular co-repressors then act as a scaffold which recruits histone-binding proteins, HDAC(s), and HDAC activating proteins (67). Subsequently, the N-terminal tails of local histones become deacetylated, resulting in the local formation of heterochromatin and silencing of gene expression.

Until recently, HDAC recruitment by the POZ domain was thought to represent a ubiquitous mechanism of transcriptional repression via POZ-ZF family members. However, the POZ-ZF transcriptional repressor HIC-1 does not repress transcription in an HDAC-dependent manner (30). Therefore, Kaiso may repress transcription as a HIC-1/Kaiso heterodimer. This mechanism of transcriptional repression would be expected operate via an HDAC-independent manner, since HIC-1 mediated transcriptional repression is insensitive to HDAC inhibitors (30).

Conversely, evidence also suggests that Kaiso may repress transcription via an HDAC-dependent manner. Analysis of the POZ domains of PLZF and Bcl-6 implicated the highly conserved D35 and R49/K49 residues in directly contacting HDAC the co-repressors NCoR and SMRT and forming a charged pocked that was integral to transcriptional repression (84). Residues at position 33 and 47 in the POZ domain were

also identified as important residues involved in transcriptional repression and co-repressor recruitment. While Kaiso diverges from Bcl-6 and PLZF at position 33 (F instead of L), positions 35, 47, and 49 are perfectly conserved, indicating that Kaiso may recruit HDAC co-repressors to repress transcription.

Furthermore, Kaiso interacts via its POZ domain with the mammalian insulator element and transcriptional repressor, **C**CTC-binding **f**actor (CTCF) (Daniel Lab, unpublished data). CTCF interacts with mSin3 and represses transcription in an HDAC-dependent manner (77). Therefore, it is possible that Kaiso exists in a complex with CTCF and represses transcription in an HDAC-dependent manner. Similarly, Kaiso interacts with members of the MeCP1 complex, and silences gene transcription in a methylation dependent manner (112). Members of the MeCP1 complex also interact with mSin3 and repress transcription via an HDAC-dependent mechanism (10). Thus, Kaiso interacts with HDAC-dependent transcriptional repressors, and therefore may repress transcription as a component of these complexes.

Lastly, the presence of acidic sequences in Kaiso, which are associated with transcriptional activation, imply that Kaiso could act as a transcriptional activator (86). Indeed, a recent study suggests that Kaiso may activate transcription via the **r**eceptor **a**ssociated **p**rotein of the **syn**apse (Rapsyn) promoter (123), although the role of the POZ domain and the acidic domain of Kaiso were not addressed in this study. Hence, multiple lines of evidence suggest that Kaiso may associate with the sequence-specific KBS and; i) Repress transcription in an HDAC-independent manner, ii) Repress transcription in an HDAC-dependent manner, or iii) Activate transcription of target genes **(Figure 6)**.

Alternative Possibilities for the Transcriptional Role(s) of Kaiso

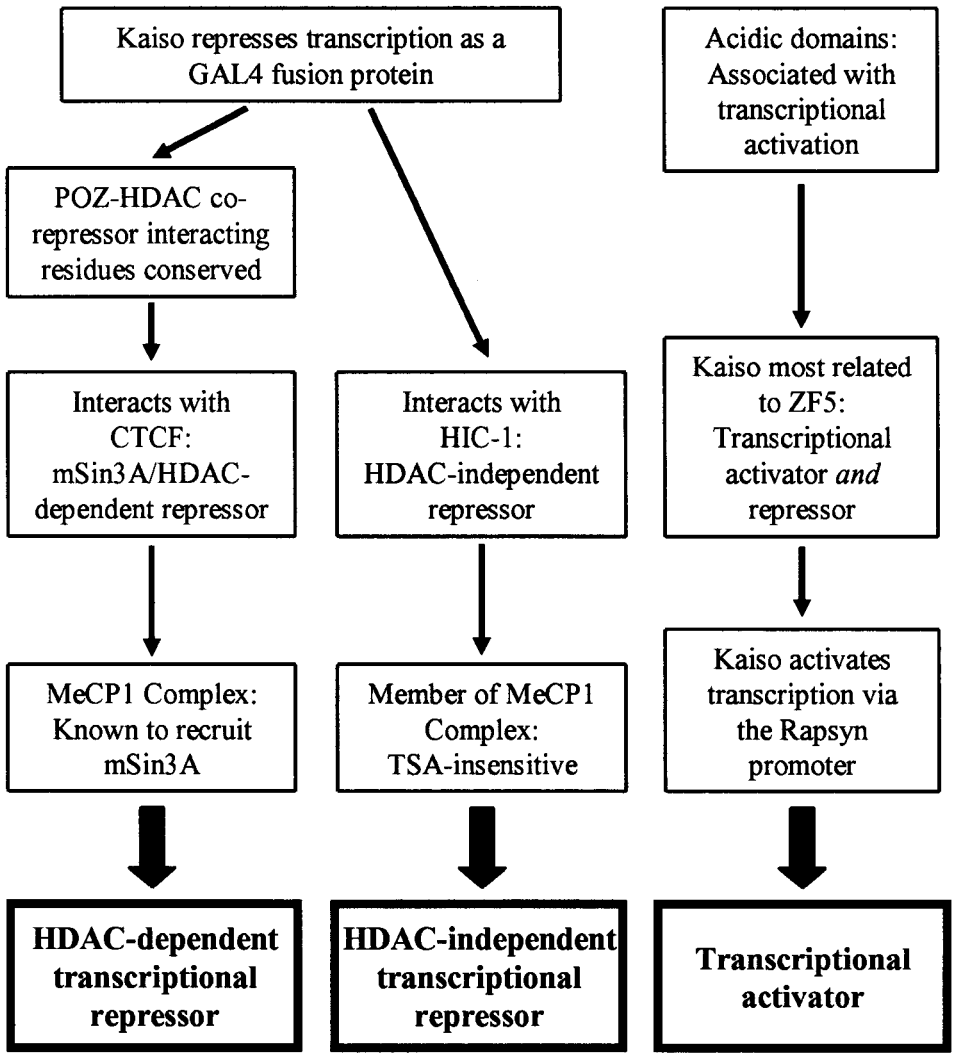


Figure 6: *Alternative possibilities for the transcriptional role(s) of Kaiso.* Current evidence implicates Kaiso as an HDAC-dependent transcriptional repressor, an HDAC-independent transcriptional repressor, and/or a transcriptional activator.

1.3.3 Current Understanding of the Function of Kaiso: Connection Between Non-Canonical Wnt Signaling and p120^{ctn}?

The connection between canonical Wnt signaling, β-catenin, and the regulation of cell proliferation is well established (49, 132, 142). However, the molecular aspects of the Kaiso-p120^{ctn} signaling pathway remain elusive. Early experiments established that p120^{ctn} and Kaiso are not likely to be involved in canonical Wnt signaling (25, 105). However, data suggest that the putative Kaiso-p120^{ctn} signaling pathway is either antagonistic or synergistic to the canonical Wnt signaling pathway. In addition, non-canonical Wnt signaling pathways (Wnts-5A, -4, and –11) inhibit the axis-inducing and cell-fate properties of the canonical Wnt signaling pathway (Wnts-1, -3A, -8, and -8b) (144). This raises the possibility that Kaiso/p120^{ctn} influence Wnt/β-catenin signaling indirectly by acting on the non-canonical Wnt signaling pathway.

Non-canonical Wnt signaling is transduced via the Wnt 5A family (Wnts-5A, -4, and –11) of secreted glycoproteins and the Frizzled family of transmembrane receptor proteins. Subsequently, the pertussis-toxin sensitive Gβγ trimeric G-protein becomes activated, which activates **p**rotein **k**inase **C** (PKC), and intracellular calcium (Ca^{2+}) release (129). The PKC and Ca^{2+} pathways then result in elevated **Ca**lcium/cal**m**odulin-dependent protein **k**inase **II** (CamKII), Cdc42, and **J**un **N**-terminal **k**inase (JNK) activity, which promote convergent extension (107), cardiogenesis (104), ventral cell fates (70), inhibits dorsal cell fates (58), and inhibits β-catenin signaling (69, 144) **(Figure 7)**.

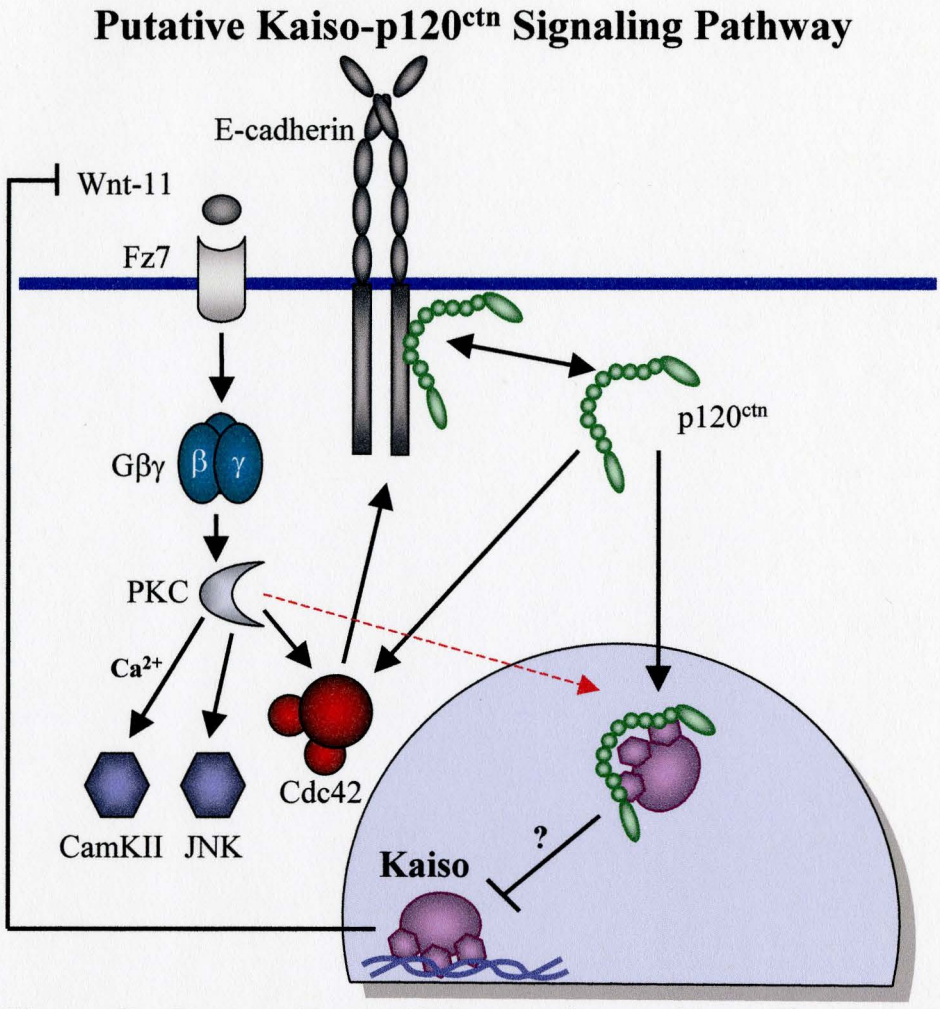


Figure 7: *Putative Kaiso-p120^{ctn} signaling pathway.* The Wnt-11 pathway mediates morphogenetic cellular movements by activating Cdc42, CamKII, and JNK pathways. Cytosolic p120^{ctn} also activates Cdc42 and alters cell motility, or translocates to the nucleus and inhibits Kaiso-mediated repression of Wnt-11 signaling. Interestingly, PKC stimulates p120^{ctn} translocation to the nucleus (red dashed arrow)(149), which could promote the inhibition of Kaiso-mediated down-regulation of Wnt-11.

In this manner, non-classical Wnts alter morphogenetic cellular movements, cell fate decisions, and inhibit β-catenin signaling via a mechanism that is distinct from the canonical Wnt signaling pathway. Conversely, canonical Wnt signaling may inhibit cardiogenesis (79), and promote dorsal cell fates (14), further supporting an antagonistic relationship between canonical and non-canonical Wnt signaling. Therefore, a dynamic balance between non-canonical Wnt and canonical Wnt signals appears to determine cell fate decisions and morphogenetic cellular movements in the developing vertebrate (69).

Current evidence suggests that Kaiso/p120^{ctn} activity may antagonize or enhance β-catenin/Wnt-1 signaling, and that these influences may be mediated indirectly via non-canonical Wnt signaling. To this end, Kaiso depletion experiments were performed in *Xenopus laevis*. These experiments demonstrate that reduction of Kaiso results in an upregulation of Wnt-11, gastrulation defects (incomplete blastopore closure and deficient mesodermal involution), failure to produce a proper neural fold, and failure to undergo convergent extension (62). The developmental defects caused by Kaiso depletion are rescued by exogenous Kaiso expression as well as dominant negative Xwnt-11 or Xdsh constructs. Therefore, Kaiso inhibits non-canonical Wnt signaling in *Xenopus*, which affects morphological cell movements. In addition, if Kaiso-p120^{ctn} and Kaiso-DNA complexes are mutually exclusive, p120^{ctn} may play a role in modulating Kaiso-mediated Wnt-11 inhibition (**Figure 7**).

PKC acts downstream of Gβγ to transduce non-canonical Wnt signals and affect morphogenesis. Furthermore, PKC activation alters the phosphorylation state of p120^{ctn}

(114) and increases levels of nuclear p120^{ctn} (146). Taken together with the finding that p120^{ctn} may inhibit Kaiso-DNA binding and transcriptional regulation, the possibility exists that PKC acts as a direct effector of non-canonical Wnt signaling (via activation of Cdc42, JNK, and CamKII), as well as an indirect activator of non-canonical Wnt signaling (via promoting nuclear p120^{ctn} accumulation, which inhibits Kaiso-mediated repression of Wnt-11 signaling). However, the effects of p120^{ctn} on the transcriptional properties of Kaiso remain to be elucidated.

1.4 Hypothesis and Project Rationale

Prior to the initiation of these experiments, Kaiso was characterized as a novel POZ-ZF transcription factor that specifically bound to the non-classical catenin p120^{ctn} *in vivo* (25). Furthermore, through analogy with the β-catenin/LEF/Tcf signaling pathway, it appeared that Kaiso and p120^{ctn} may act as part of a novel signal transduction pathway (**Figure 7**). To this end, Kaiso was identified as a bi-modal DNA-binding protein which associated with a sequence-specific DNA-binding site as well as methylated CpG dinucleotides (27, 112). p120^{ctn} inhibits both sequence-specific and methylation-dependent DNA-binding *in vitro* (27). The transcriptional properties of Kaiso, however, were poorly defined, and the role of p120^{ctn} on Kaiso-mediated transcriptional regulation was unknown. These data facilitated the formation of the following hypothesis:

Hypothesis: Kaiso is a transcriptional repressor whose transcriptional properties are regulated via p120^{ctn}.

Materials and Methods

2.1 *Protein Expression and Purification*

Bacterial colonies were inoculated into 10 mL of LB ampicillin (100 μg/mL) and grown with shaking at 37°C for 16 hours followed by inoculation into 90 mL fresh LB ampicillin (100 μg/mL) and further incubated at 37°C with shaking for 2 hours. IPTG was then added to 1 mM and cells grown at 37°C with shaking for 2.5 hours. Cells were pelleted at 3505 xg for 20 minutes at 4°C and the supernatants removed. Pellets were frozen at –80°C for 16 hours, thawed and lysed in 10 mL 0.1% Nonidet-P40 in sterile PBS, and sonicated on ice for 1 minute. Lysates were centrifuged at 14000 rpm for 10 minutes at 4°C and incubated with rotation for 1 hour at 4°C with 800 μL of GST beads in 0.1% Nonidet-P40 in sterile PBS. Beads were washed 3 times with 6 mL of lysis buffer (25 mM HEPES, 100 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% Nonidet-P40, 5% glycerol, and 1 mM DTT at pH 7.5). Protein was eluted from the beads with 500 μL of 10 mM glutathione in 1M Tris pH 8 and lightly agitated for 10 minutes at room temperature. Beads were pelleted at 3500 rpm for 5 minutes at 4°C and the supernatant was removed and stored at –80°C. Aliquots of each protein sample were run for 2 hours at 100 volts on a 12% acrylamide gel to confirm purification.

2.2 *Electrophoretic Mobility Shift Assays (EMSA)*

50 ng of oligonucleotide probes were incubated with 1 μL of $\gamma^{32}P$ dATP (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec), 1 μL of T4 polynucleotide

kinase, 1X T4 polynucleotide kinase buffer, and 6 μL of H_2O at 37°C for 45 minutes. The reaction was stopped with 25 mM EDTA (pH 8.0) and the DNA was purified using a Chromaspin TE-10 (Clontech Laboratories, Inc. Palo Alto, California) column at 1000 xg for 5 minutes. 50 000 cpm of probe were then incubated with 3 μg of poly dI-dC, 5 μg BSA, 9.8% glycerol, 500 ng protein, 25 mM HEPES, 100 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% Nonidet-P40, and 1 mM DTT at room temperature for 25 minutes and on ice for 30 minutes to allow specific protein / DNA binding. The preparation was then loaded on a non-denaturing 4% acrylamide 0.5X TBE gel and electrophoresed at 190 volts for 2.5 hours before transfer to Whatmann paper. The gel was dried for one hour at 80°C and visualized via autoradiography.

Cold competition assays (competition with unlabelled probe) utilized 20 ng, 40 ng, and 100 ng of unlabelled probe added to the 1 ng of purified, labeled probe prior to protein / DNA binding conditions, such that unlabelled probe was present in 20, 40, and 100 times excess, respectively. The $p120^{ctn}$ competition assay utilized 0 ng, 2500 ng, and 5000 ng of GST-$p120^{ctn}$ or 5000 ng of GST protein such that $p120^{ctn}$ was present in 0, 5, and 10 fold excess or GST was present in 10 fold excess relative to Kaiso protein, respectively.

2.3 Cell Growth and Treatment

HCT116, MCF-7, Cos-1, and 3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California) supplemented with 10% Fetal

Bovine Serum (Hyclone, Logan, Utah), 1% penicillin/streptomycin (Invitrogen, Carlsbad, California) and 0.4% fungizone and grown at 37°C with 5% CO_2.

Trichostatin A (TSA) sensitivity experiments were performed by adding 6 μL of 100μM TSA (Sigma-Aldrich, St. Louis, Missouri) in ethanol to each well of a 6-well plate such that TSA was present at 300 nM levels for 8 hours prior to luciferase assay. Sodium butyrate (NaB) sensitivity experiments were performed by adding 5μL of 2M NaB to each well of a 6-well plate such that NaB was present at a 5mM level for 8 hours prior to luciferase assay. Alternatively, 5μL of H_2O or 6 μL of ethanol was added to each well as a solvent alone control.

2.4 Cell Seeding

24 hours prior to transfection, confluent 100 mm plates were washed twice with 5 mL of PBS and incubated at 37°C, 5% CO_2 with 1 mL of 0.25% trypsin in 1 mM EDTA (Invitrogen, Carlsbad, California). Cells were resuspended vigorously, and 9 mL of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, California) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, Utah), 1% penicillin/streptomycin (Invitrogen, Carlsbad, California) and 0.4% fungizone was added. Cells were shaken vigorously and counted on a hemacytometer (Hausser, Horsham, Pennsylvania). Cells were then seeded onto 6 well dishes (VWR Canlabs, Mississauga, Ontario) as follows; HCT116- 8 x 10^5 cells per well, 3T3- 2.5 x 10^5 cells per well, Cos-1-

3 x 10^5 cells per well, and MCF-7- 4 x 10^5 cells per well. Total volume was brought up to 2 mL per well with supplemented DMEM.

2.5 Transient Transfections

In a 1 mL tube (DiaMed, Mississauga, Ontario) 800 ng of reporter construct (either pGL3 Control or 4xKBS in pGL3 Control) was added to 100 µL of un-supplemented DMEM, 5 µL of Superfect transfection reagent (Qiagen Inc., Mississauga, Ontario) and 200 ng of effector (Kaiso in pCDNA3, p120 1A in pRc/RSV, ΔR3-11 in pRc/RSV, Kaiso Antisense in pLXSN, Kaiso in *pSilencer*™ (Ambion), or backbone vector alone). Transfection complexes were then vortexed at full speed for 15 seconds and allowed to form at room temperature for 20 minutes. Following transfection complex formation, each tube was brought up to 1 mL with supplemented DMEM and added drop wise to one well containing cells previously washed with 2 mL of PBS. Cells were incubated at 37°C, 5% CO_2 for 3 hours, and were subsequently washed twice with 2 mL PBS and incubated with supplemented DMEM before being analyzed via luciferase assay.

2.6 Luciferase Assays

Cells were washed twice with 2 mL of PBS per well. After aspirating the last wash, 350 µL of Passive Lysis Buffer (PLB) was added to each well (Promega, Madison, Wisconsin) and incubated with vigorous shaking at room temperature for 20 minutes.

Lysates were vigorously resuspended and 20 μL of each well were assayed for luciferase expression on a Lumat LB 9501 Berthold Luminometer (Fisher Scientific, Toronto, Ontario). Note that all bars on graphs representing luciferase levels are representative of the mean of three data points, and each trial was performed five times. Data variance is depicted by standard deviation (vertical lines).

2.7 Immunoprecipitations / Immunoblots

Confluent, 100 mm^2 plates were washed twice with 5 mL of PBS followed by incubation on ice with lysis buffer containing 0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM Sodium orthovanadate, and 1 mM EDTA for 5 minutes. Cells were harvested with a rubber cell scraper, transferred into an eppendorf tube and sonicated on ice for 30 seconds. The suspension was microfuged at 14 000 rpm at 4°C for 5 minutes and the supernatant was transferred to another tube. Lysates were quantitated by Bradford assay and equal quantities of total protein were immunoprecipitated. Four μg of monoclonal antibody was added to the lysate and mixed end-on-end at 4°C for 1 hour. For HDAC co-immunoprecipitations and *Fluor de Lys* immunoprecipitations, lysates were pretreated with 20 μL of Sepharose A beads to block nonspecific protein binding to the Sepharose A beads. The suspension was centrifuged at 14 000 rpm and the supernatant was transferred to a new tube.

Sepharose A beads in lysis buffer (0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM Sodium orthovanadate, and 1

mM EDTA) were incubated with 9 μg of rabbit anti-mouse bridge antibody for 30 minutes at 4°C. Forty μL of sepharoseA::bridge antibody bead suspension was added to the lysate and incubated end-over-end at 4°C for 1 hour. Beads were pelleted at 14 000 rpm at 4°C and washed 4 times with 800 μL of lysis buffer (0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM Sodium orthovanadate, and 1 mM EDTA) on ice. Beads were incubated in 60 μL of 2x Laemmli Sample Buffer (0.004% bromophenol blue. 50 mM Tris (pH 6.8), 1% SDS, 5% sucrose, and 120 mM β-mercaptoethanol) and samples were boiled for 5 minutes. Beads were pelleted at 4 000 rpm for 30 seconds, and the supernatant was loaded on a 7% denaturing polyacrylamide gel.

After SDS-PAGE, proteins were transferred from the gel to a nitrocellulose membrane using Hoeffer SemiPhor (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec) semi-dry transfer apparatus and transfer buffer (20% methanol, 0.075% SDS, 190 mM glycine, and 2 mM Tris). The protein was transferred to the membrane at 60 amps per gel for 2 hours. The nitrocellulose membrane was then pre-blocked for 5 minutes at room temperature in 20 mL of 3% (w/v) milk in 1x TBS pH 7.4 (1mM Tris and 15mM NaCl pH 7.4). The blot was then incubated with specified amounts of primary antibody and incubated at 4°C overnight with agitation. The milk and antibody solution was removed from the membrane and five washes with water were performed prior to one 5 minute wash in 5 mL of 1x TBS pH 7.4 (described in (66)). One μL of peroxidase-conjugated secondary antibody (donkey anti-mouse for monoclonal primary western blot

antibodies or goat anti-rabbit antibody for polyclonal primary Western blot antibodies) in 40 mL of 3% (w/v) milk in 1x TBS pH 7.4 was added and incubated at room temperature for 2 hours with mild agitation. The nitrocellulose membrane was then washed five times with water followed by one 5 minute wash in 1x TBS pH 7.4. The membrane was then processed using the enhanced chemi-luminescence (ECL) system (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec) and exposed to film. Note that immunoprecipitations of proteins shown in **Figure B-1** were performed on nuclear extracts, as performed in (66), rather than whole cell lysates.

2.8 Antibodies

The following antibodies were used for immunoprecipitation procedures; Kaiso (4 μg of 6F monoclonal antibody)(24), p120 (2 μg of 15D2 monoclonal antibody and 2 μg 2B12 monoclonal antibody for the detection of both ΔR3-11, p120 1A)(152), HDAC-1 (20 μL of H-51 polyclonal antibody, Santa Cruz Biotechnology), and mSin3a (20 μL of KA-20 polyclonal antibody, Santa Cruz Biotechnology).

For immunoblotting, the following antibodies were used at the following concentrations; Kaiso (1 μL of polyclonal anti-Kaiso in 10 mL of 3% milk), p120 (5 μg 15D2 monoclonal antibody and 5 μg of 2B12 monoclonal antibody in 10 mL of 3% milk), HDAC-1 (20 μL of H-51 polyclonal antibody in 10 mL 3% milk), and mSin3A (20 μL of KA-20 polyclonal antibody in 10 mL 3% milk).

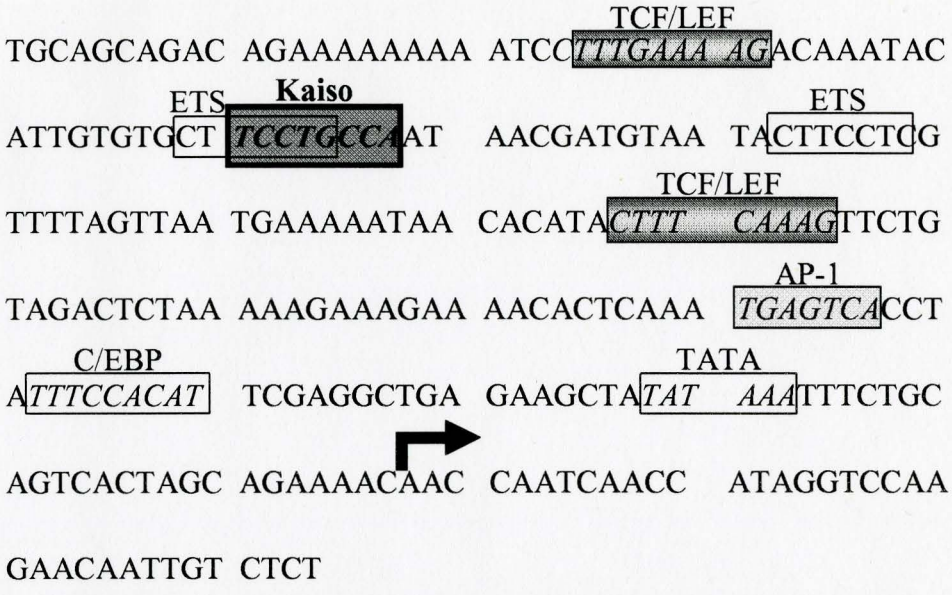
Results

3.1 Characterization of Kaiso as a transcriptional repressor of the 4xKBS cis-element

3.1.1 Evaluation of the KBS as a candidate cis-element

Previous studies identified a sequence-specific DNA binding site for Kaiso *in vitro* (27). This recognition site, also referred to as the **K**aiso **b**inding **s**ite (KBS), was discovered via **c**yclic **a**mplification and **s**election of **t**argets (CAST) analysis. Visual analysis identified this sequence in the promoters of the human and murine *matrilysin* genes. The sequence of the human and murine promoters as well as binding sites for known transcription factors is shown in **Figure 8A and 8B**, respectively. Previous studies also demonstrated that Kaiso binds to oligonucleotide probes derived from the **h**uman (*Hmat*) and **m**urine (*Mmat*) ***matrilysin*** promoters (27). Further analysis indicated mutational flexibility within the first two bases of the KBS; mutation of the first two bases (**TC**CTGCNA to **GA**CTGCNA) in the KBS does not abrogate Kaiso-DNA binding. However, mutation of any of the remaining bases abolished Kaiso-DNA binding entirely (27). Kaiso therefore requires a CTGCNA core recognition sequence to bind DNA *in vitro*. In addition to a sequence-specific DNA-binding activity, Kaiso also possesses a methylation-dependent DNA-binding activity (112) and binds to a sequence found in the **me**thylated murine *S100A4/**metastasin*** promoter (*me-mts*). The sequence of this methylation-specific DNA-binding site is shown and compared to the KBS in **Figure 8A and 8B**.

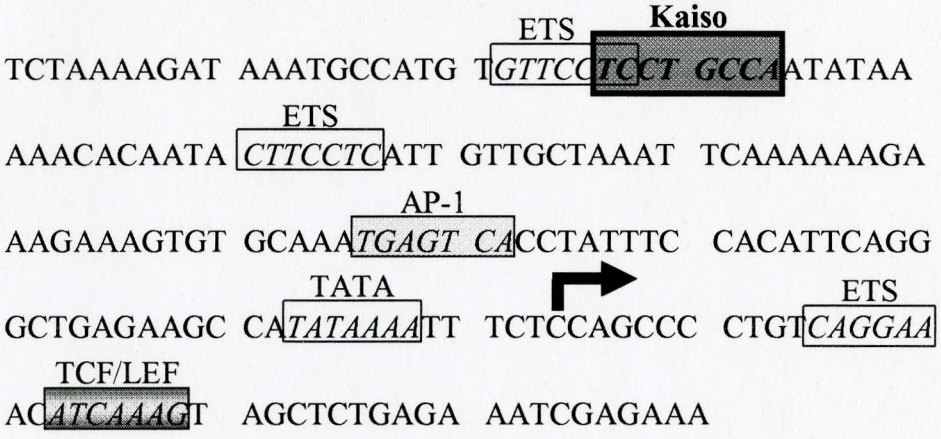
Human *matrilysin* Promoter



***Hmat* Probe:**	GTGCT**TCCTGC**CA**A**TAACG
-296 Hmat pro:	TGCT**TCCTGC**CA**A**TAA
-296 Hmat pro mut:	TGCT**TCCTGT**C**C**ATAA
***me-mts* Probe:**	AGCAGC**MGMG**CCCAA**MG**CTGGGAG

Figure 8A: *Human matrilysin promoter.* The sequence of the human *matrilysin* promoter is shown above. Known DNA-binding sites for transcription factors are shown as well as a putative Kaiso-binding site. The sequence of the sequence-specific *Hmat* and methylation-dependent *me-mts* oligonucleotide probes and the –296 Hmat pro *cis*-elements are also shown.

Murine *matrilysin* Promoter



***Mmat* Probe:** GTTCC**TCCTGC**AA**T**ATAAAAAC

***Me-mts* Probe:** AGCAGC**MGMG**CCCAA**MG**CTGGGAG

Figure 8B: *Murine matrilysin promoter.* The sequence of the murine *matrilysin* promoter is shown above. Known DNA-binding sites for transcription factors are shown as well as a putative Kaiso-binding site. The sequence of the sequence-specific *Mmat* and methylation-dependent *me-mts* oligonucleotide probes are also shown. Note that the sequence of the Kaiso binding site is conserved between the human and murine *matrilysin* promoters and that it overlaps with the ETS-binding site in both cases.

To determine the relative affinity of Kaiso for the *me-mts* and *Mmat/Hmat* probes *in vitro*, competitive **e**lectrophoretic **m**obility **s**hift **a**ssays (EMSA) were performed. In these assays, Kaiso was incubated with labeled *me-mts* or *Mmat/Hmat* probe and increasing concentrations of unlabelled competitor probe (*Mmat/Hmat* or *me-mts*, respectively).

Methylation-dependent (*me-mts*) DNA-binding activity was significantly inhibited by 40-fold excess of unlabeled *Hmat* or *Mmat* probe (**Figure 9A**), while an *Mmat* variant (*Mut3*) carrying a mutation (TCCTGCNA to TCATGCNA) which abolishes Kaiso-DNA binding ability (27) did not significantly effect methylation-dependent DNA binding activity. Methylation-dependent DNA binding is therefore significantly diminished by 40-fold excess of KBS-derived oligonucleotides.

Conversely, sequence-specific (*Mmat*) DNA-binding activity was not significantly inhibited at 40-fold excess of unlabeled *me-mts* competitor probe (**Figure 9B**). However, sequence-specific DNA-binding activity was abolished at 60-fold excess *me-mts*, but not by 60-fold excess of non-methylated *me-mts* (*mts*). These results demonstrate that Kaiso binds the *Mmat* probe with a higher affinity than the *me-mts* probe. It was thus reasoned that the KBS may be a *bona fide cis*-element that Kaiso could associate with *in vivo*.

***Mmat* and *Hmat* Competition of Methylation-Dependent Kaiso-DNA Binding**

Labelled Probe: ***me-mts***

Unlabelled Competitor:

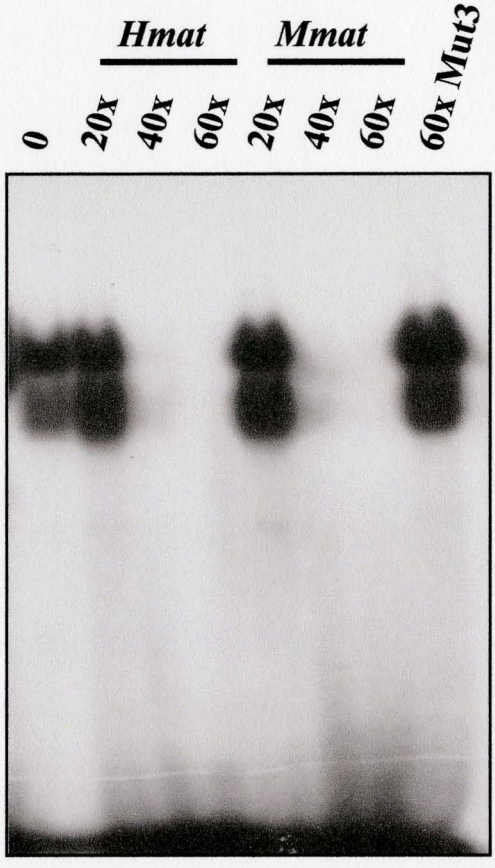


Figure 9A: *Mmat and Hmat competes for Kaiso-me-mts DNA-binding activity.* Kaiso-me-mts DNA-binding was significantly inhibited by 40-fold excess unlabelled *Mmat* or *Hmat* probe and completely abolished by 60-fold excess competitor. Sixty-fold excess of mutated *Mmat (Mut3)* failed to compete for Kaiso-*me-mts* DNA-binding activity.

***Me-mts* Competition of Sequence-Specific Kaiso-DNA Binding**

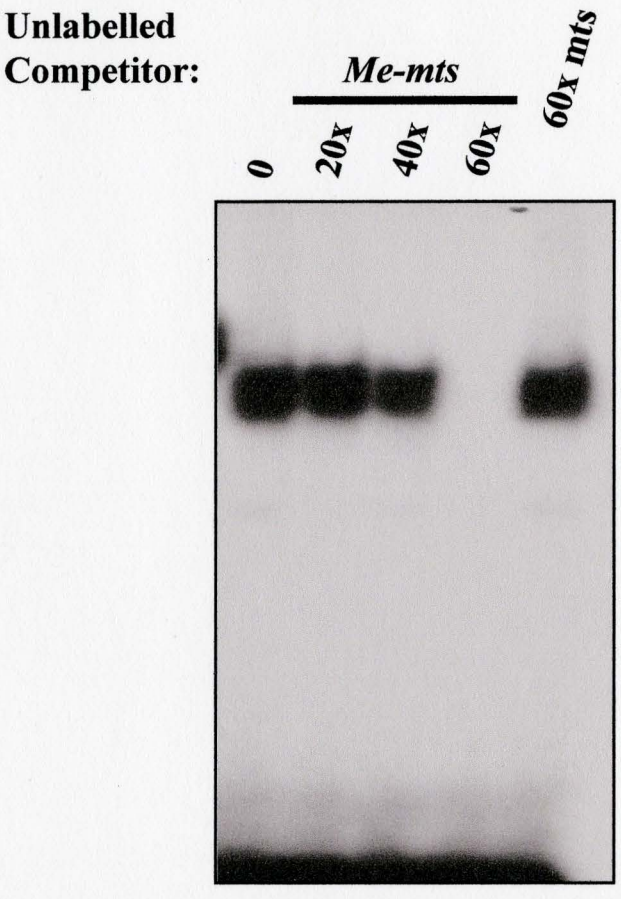


Figure 9B: *me-mts competes for Kaiso-Mmat DNA-binding activity.* Kaiso-*Mmat* DNA-binding was not significantly inhibited by 40-fold excess unlabelled *me-mts* probe but completely abolished by 60-fold excess competitor. Sixty-fold excess of non-methylated *mts* probe failed to compete for Kaiso-*Mmat* or Kaiso-*Hmat* DNA-binding activity.

3.1.2 Kaiso-mediated transcriptional repression of the 4xKBS element in artificial promoter assays

Kaiso represses transcription via a *me-mts* derived *cis*-element using artificial reporter assays (112). However, it is unknown whether Kaiso affected transcription via a KBS-derived *cis*-element. An artificial promoter consisting of four tandem copies of the KBS was cloned upstream of the *luciferase* gene in the pGL3 Control vector (4xKBS; see **Appendix C, Figure C-2**). Four copies of the KBS carrying a mutation which abrogates Kaiso-DNA binding (4xKBS CAmut; mutation of TCCTGCNA to TCATGCNA, **Appendix C, Figure C-3)**(27) was also cloned in the same manner.

When the 4xKBS plasmid was transfected into Cos-1 monkey fibroblasts, 2.5-fold lower levels of luciferase expression was observed when compared to the backbone pGL3 Control vector (**Figure 10**), indicating that either endogenous Kaiso or some other cellular factor represses transcription via the 4xKBS element. Ectopic Kaiso expression repressed luciferase expression via the 4xKBS approximately two-fold relative to the 4xKBS alone, confirming that transcriptional repression via the KBS was enhanced by exogenous Kaiso expression. Furthermore, Cos-1 cells were transfected with the 4xKBS CAmut to determine the specificity of the above results. While luciferase expression from the 4xKBS was 2-fold lower than the pGL3 Control backbone vector, 4xKBS CAmut expression levels were similar to pGL3 Control both in the presence and absence of ectopic Kaiso expression (**Figure 11**). These results demonstrate that the 4xKBS acts as a repressive *cis*-element and Kaiso can repress this *cis*-element in Cos-1 cells.

Kaiso Represses Transcription via the 4xKBS *cis*-element

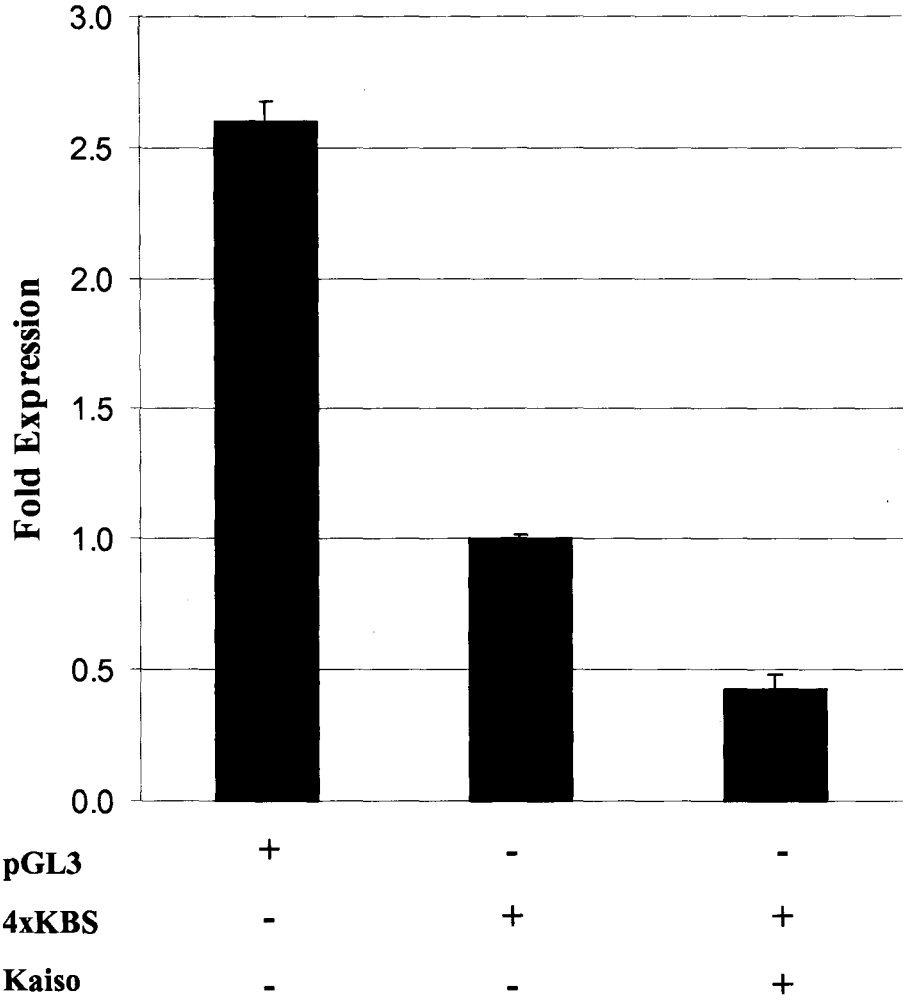


Figure 10: *Kaiso represses transcription via the 4xKBS cis-element.* In Cos-1 cells, the 4xKBS behaves as a repressive *cis*-element, since addition of the 4xKBS reduced luciferase expression by 2.5-fold (contrast pGL3 Control to 4xKBS). Exogenous Kaiso (4xKBS + Kaiso) repressed luciferase expression to less than 50% of baseline (4xKBS).

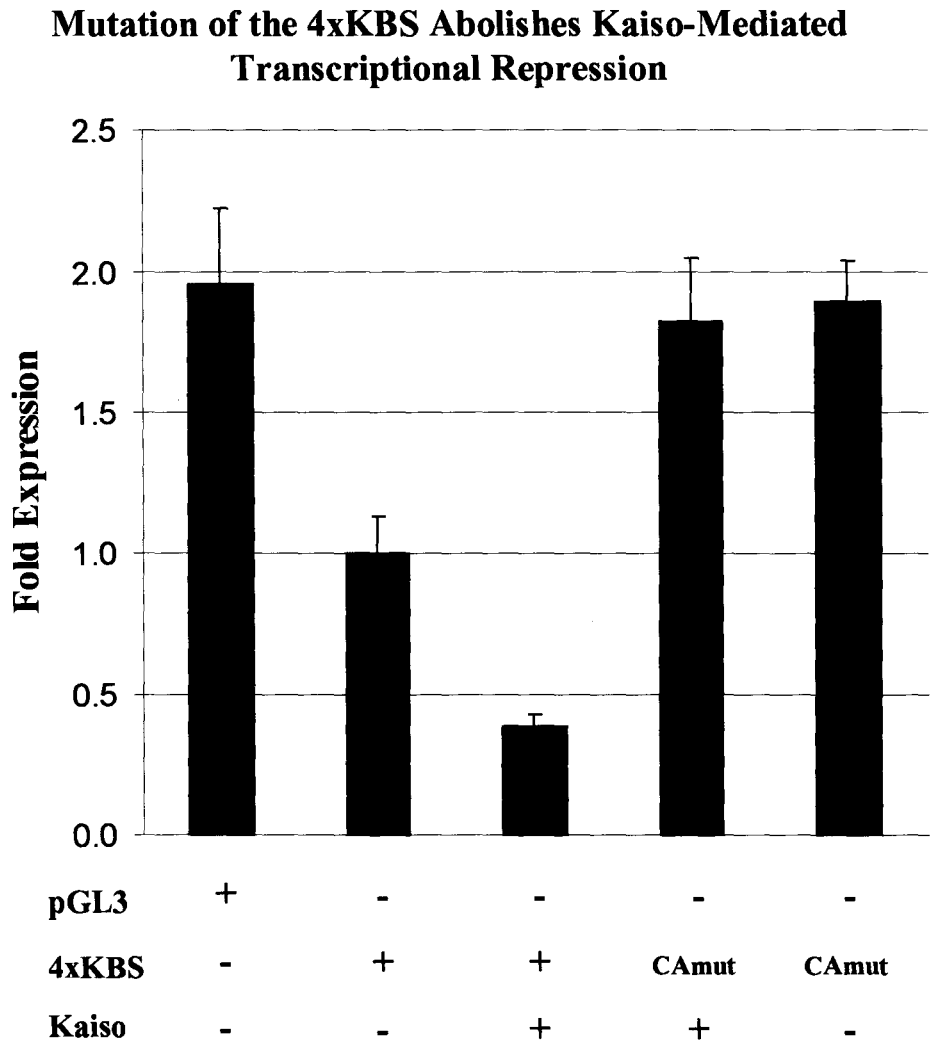


Figure 11: *Mutation of the 4xKBS abolishes Kaiso-mediated transcriptional repression.* Both endogenous Kaiso and exogenous Kaiso can repress transcription of the 4xKBS WT. Mutation of the 4xKBS which abrogate the Kaiso-DNA interaction (4xKBS CAmut) resulted in luciferase expression levels comparable to that of the pGL3 Control vector alone.

3.1.3 Specificity of Kaiso-mediated transcriptional repression via the 4xKBS element

To determine whether Kaiso could repress transcription via single or duplicate copies of the KBS, a 1x KBS and a 2xKBS were cloned into the pGL3 Control vector and transfected into Cos-1 cells. Exogenous Kaiso was capable of repressing the 4xKBS by approximately 2.5-fold relative to the 4xKBS alone. Kaiso also repressed luciferase expression via the 1xKBS and 2xKBS by approximately 2-fold (**Figure 12**). Thus, Kaiso-mediated transcriptional repression of the 4xKBS is not an artifact of tandem copies of an artificial promoter, and Kaiso only requires one KBS site to repress transcription in this system.

To confirm that Kaiso-mediated transcriptional repression via the 4xKBS was not an artifact in Cos-1 cells, ectopic Kaiso was expressed in A431 (epidermoid carcinoma), A431DE (epidermoid carcinoma deficient in E-cadherin), HeLa (human cervical adenocarcinoma), and SW620 (human colorectal adenocarcinoma) cells. Kaiso repressed transcription via the 4xKBS in all cell lines tested at least 2.5-fold relative to the 4xKBS alone (**Figure 13**). Furthermore, increasing amounts of exogenous Kaiso was expressed in the presence of the 4xKBS to establish a dose-response relationship between Kaiso and transcriptional repression of the 4xKBS. While 100 ng of Kaiso in pCDNA3 repressed transcription via the 4xKBS by 30%, 600 ng of Kaiso repressed transcription by 80% (relative to the 4xKBS alone; see **Figure 14**).

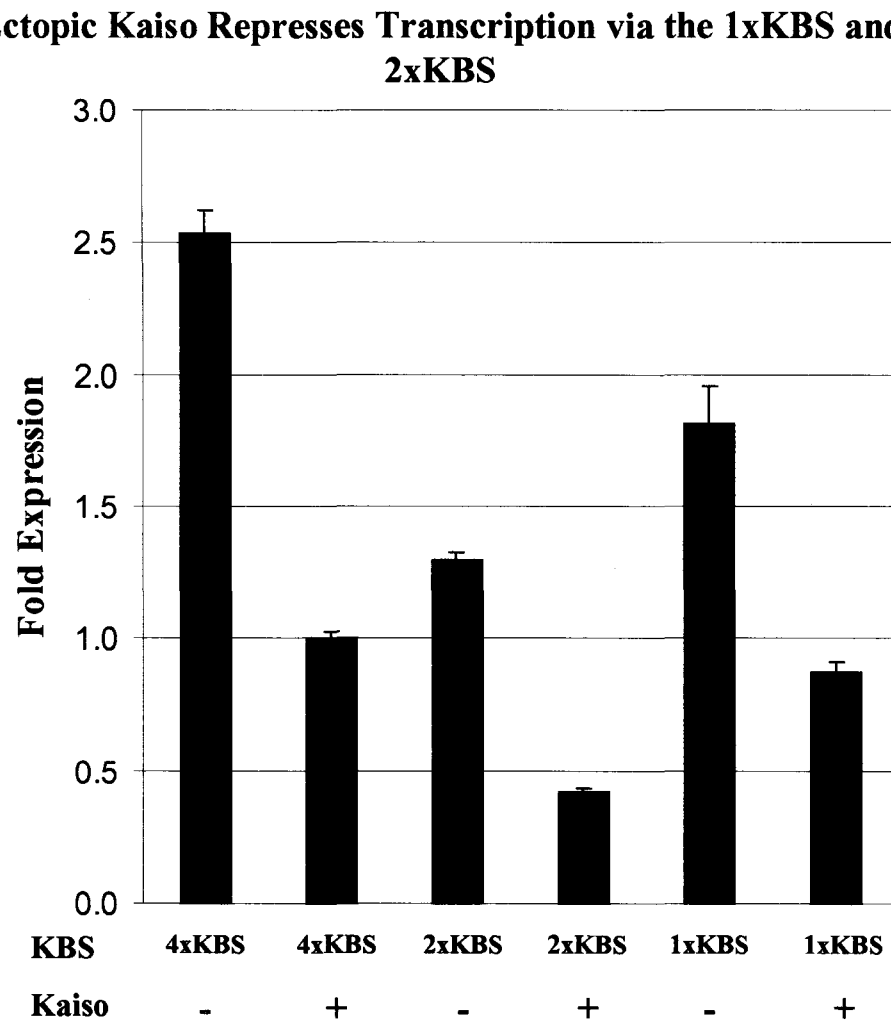


Figure 12: *Kaiso represses transcription via the 1xKBS and 2xKBS.* Ectopic Kaiso represses transcription of the 1xKBS, 2xKBS, and 4xKBS greater than 2-fold, confirming that Kaiso requires only one KBS to repress transcription.

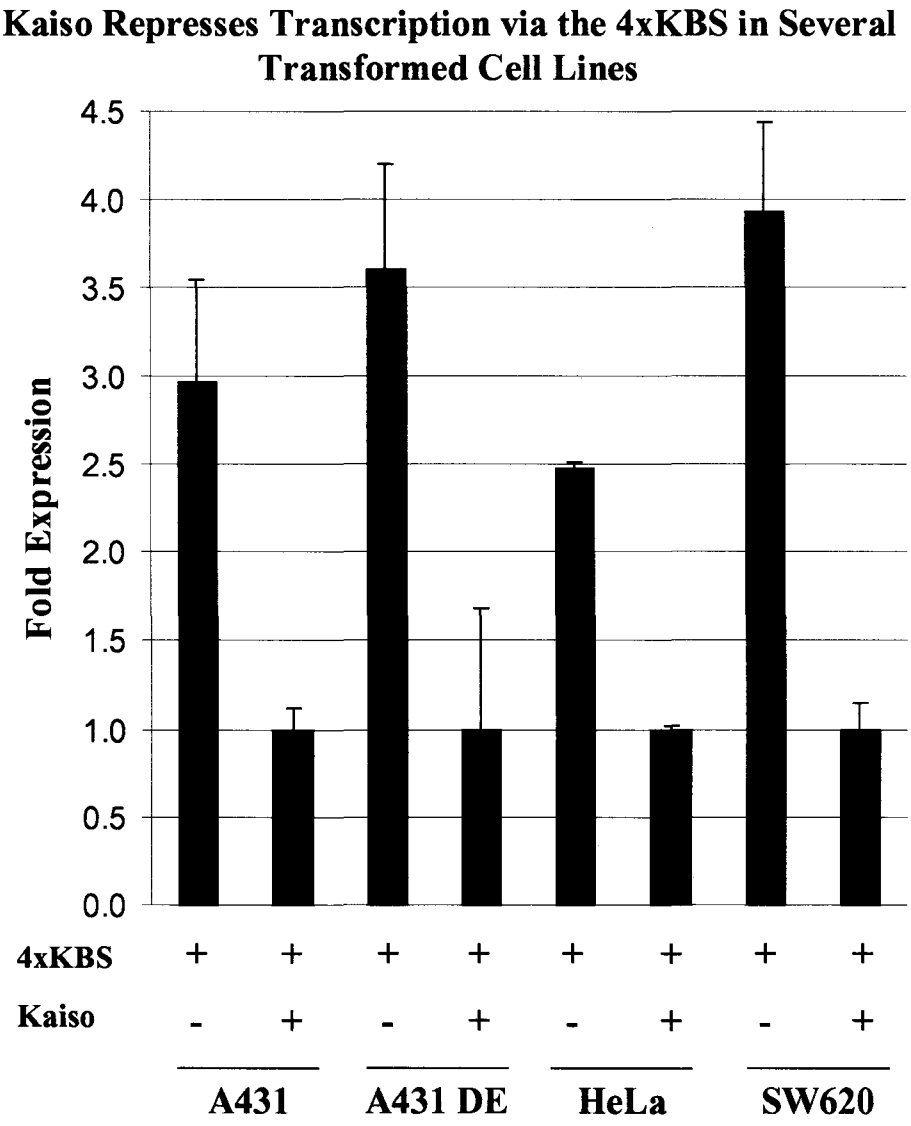


Figure 13: *Kaiso represses transcription via the 4xKBS in several transformed cell lines.* The 4xKBS plasmid was transfected into the indicated cell lines either alone (4xKBS) or with exogenous Kaiso (4xKBS + Kaiso). In all cell lines, exogenous Kaiso repressed transcription by at least 2.5-fold.

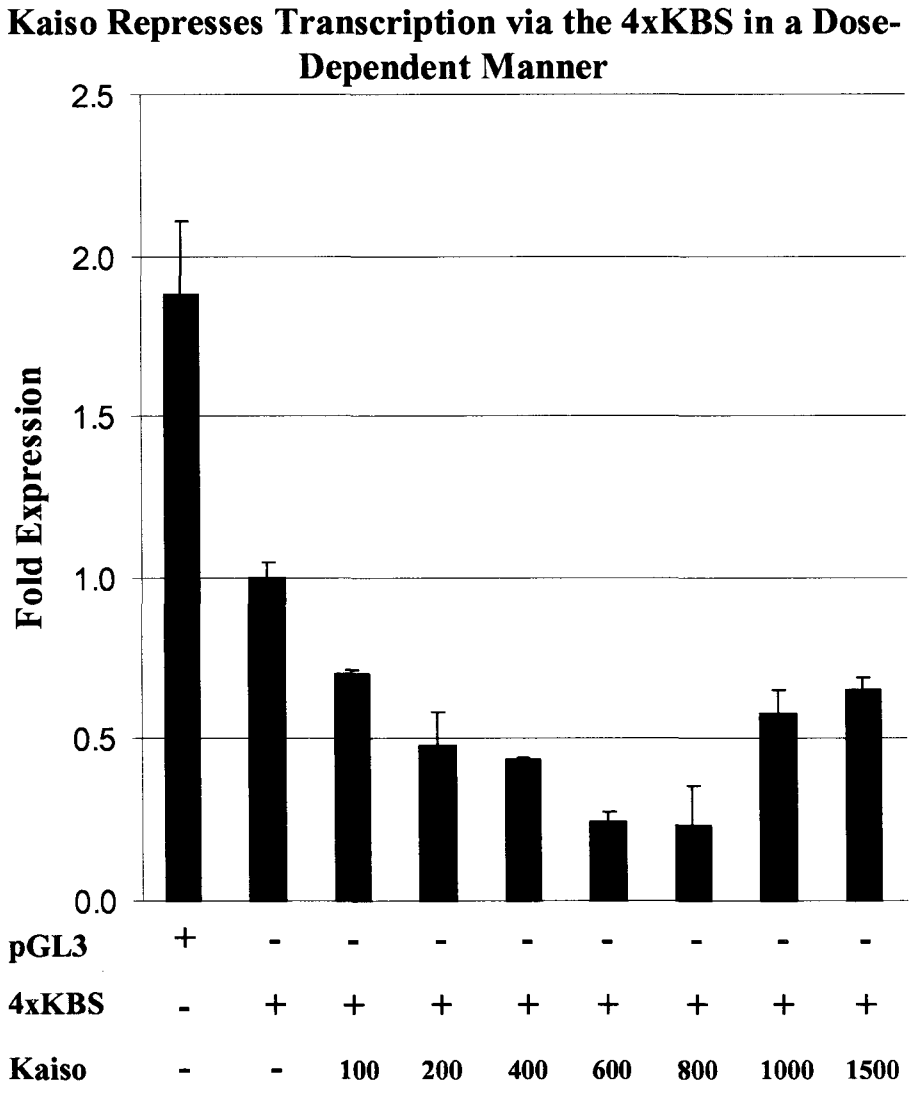


Figure 14: *Kaiso represses transcription via the 4xKBS in a dose-dependent manner.* Increasing amounts of exogenous Kaiso was co-transfected with the 4xKBS in Cos-1 cells. While 600ng of Kaiso in pCDNA3 repressed transcription by approximately 80% (relative to the 4xKBS alone), 1 μg of exogenous Kaiso repressed trancription by more than 40%.

Moreover, levels of exogenous Kaiso in excess of 800 ng (1μg and 1.5 μg of Kaiso in pCDNA3) repressed transcription less potently than 600 ng or 800 ng of exogenous Kaiso. These data indicate that levels of exogenous Kaiso in excess of 800 ng may squelch transcriptional repression of the 4xKBS artificial promoter, resulting in lower levels of Kaiso-mediated transcriptional repression.

To definitively establish a causal relationship between Kaiso and transcriptional repression via the 4xKBS, endogenous levels of Kaiso were artificially depleted by either an antisense Kaiso construct, or an **RNA** interference (RNAi) based approach (to reduce levels of Kaiso with a higher level of specificity). For the RNAi-based approach, an oligonucleotide insert homologous to Kaiso was cloned into the *pSilencer*^{TM} vector (Ambion). This insert is not homologous to any known nucleotide sequences other than human and murine Kaiso mRNA. The resulting *pSilencer*^{TM} Kaiso vector produces small interfering **RNA** (siRNA) sequences that specifically target Kaiso mRNA for degradation. The reduction in levels of Kaiso protein expression was confirmed via immunoblot analysis **(Appendix B, Figure B-1)**. Significantly decreased luciferase expression levels were concomitant with the reduction of Kaiso protein expression via either the antisense Kaiso approach (**Figure 15A**) or the *pSilencer*^{TM} Kaiso RNAi-based approach (**Figure 15B**). Together, these data establish a causal relationship between Kaiso and transcriptional repression of the 4xKBS plasmid. These data also verify that Kaiso represses transcription in a bi-modal manner; Kaiso can repress transcription not only via a methylation-specific *cis*-element, but also via the sequence-specific *cis*-element (TCCTGCNA).

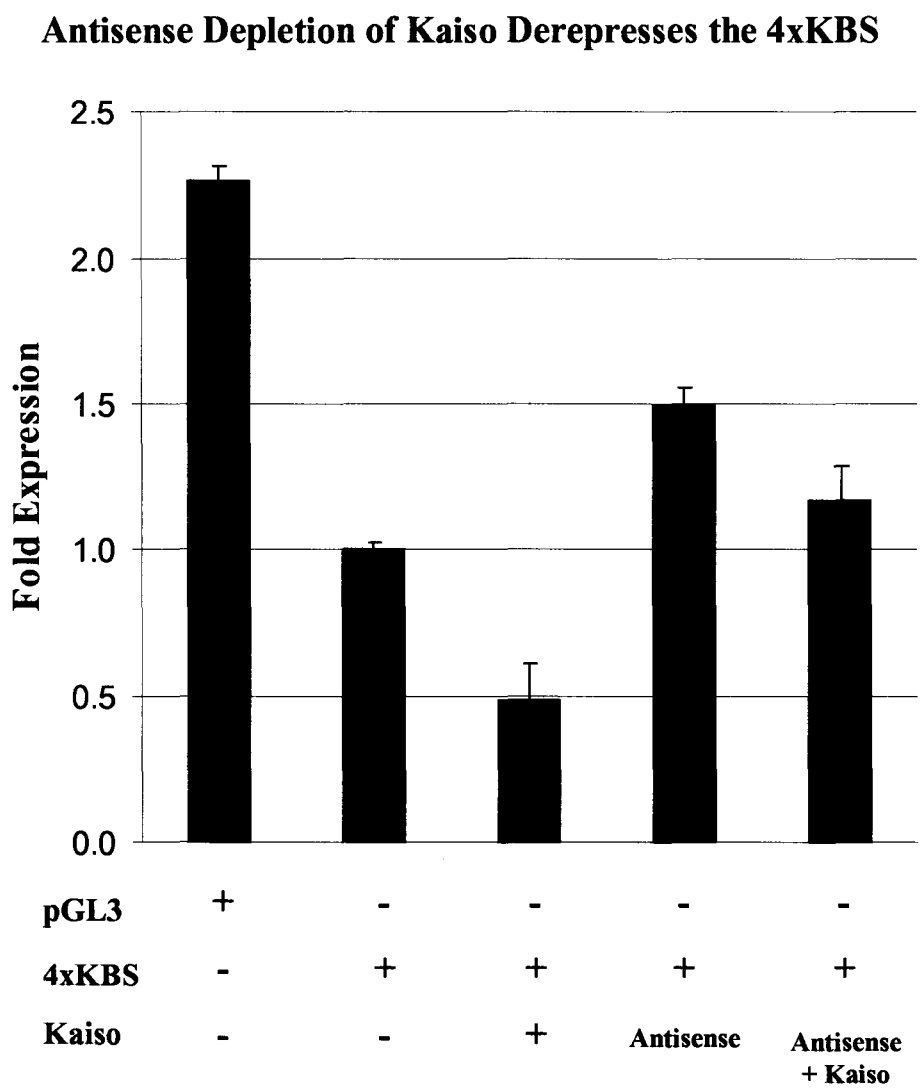


Figure 15A: *Antisense Depletion of Kaiso derepresses the 4xKBS.* Co-transfection of Antisense Kaiso partially de-repressed the expression of luciferase via the 4xKBS (4xKBS + Antisense and 4xKBS + Kaiso + Antisense) in Cos-1 fibroblasts.

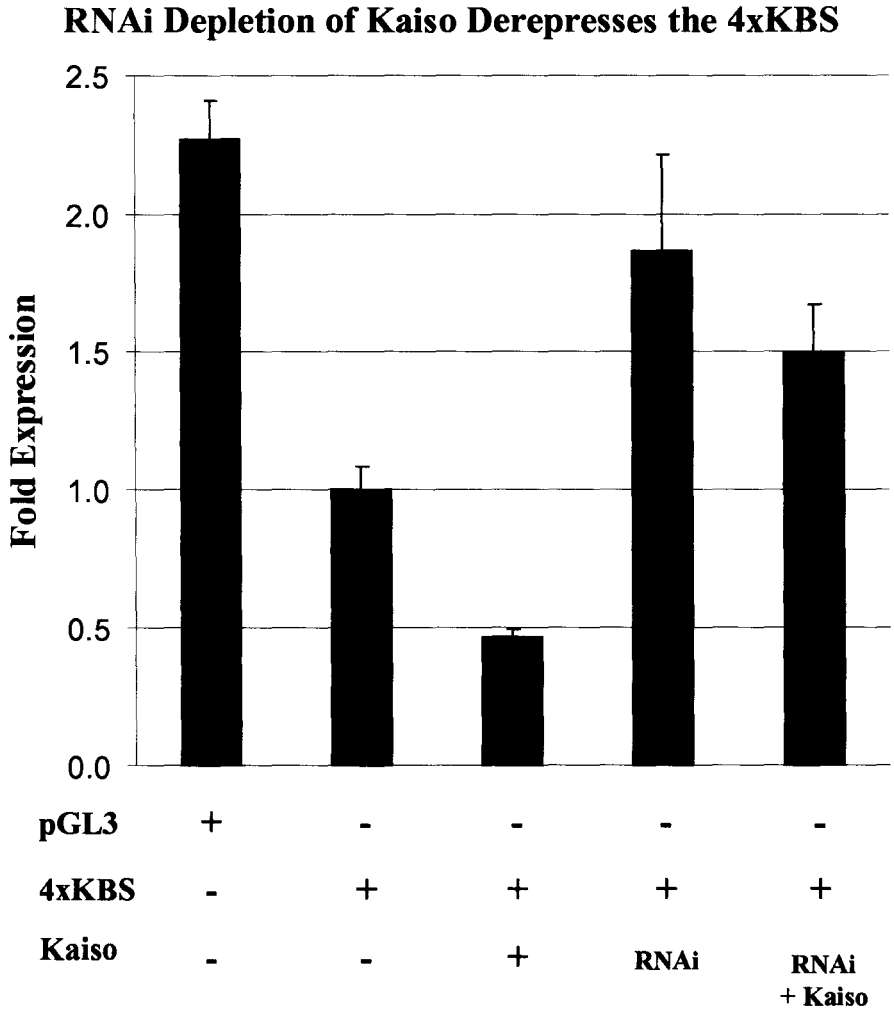


Figure 15B: *RNAi depletion of Kaiso derepresses the 4xKBS.* Co-transfection of *pSilencer* (Ambion) Kaiso de-repressed the expression of luciferase via the 4xKBS (4xKBS + pSilencer Kaiso and 4xKBS + Kaiso + pSilencer Kaiso) in Cos-1 fibroblasts.

3.2 *The catenin p120^{ctn} inhibits Kaiso-mediated transcriptional repression*

3.2.1 p120^{ctn} inhibits Kaiso-DNA binding in vitro

Having established Kaiso as a transcriptional repressor of the KBS *cis*-element, it was of interest to determine the effects of p120^{ctn} on the transcriptional properties of Kaiso. Interestingly, the DNA-binding domain of Kaiso and the p120^{ctn}-binding domain of Kaiso overlap (**Figure 5**). This hints that Kaiso-DNA and Kaiso-p120^{ctn} complexes are mutually exclusive, implying that p120^{ctn} could directly inhibit Kaiso-DNA binding. In order to test this hypothesis, EMSA analysis was performed in the presence of increasing quantities of GST-p120^{ctn} or GST alone. While 10-fold excess GST-p120^{ctn} significantly inhibited GST-ΔPOZ from binding to the *Mmat* probe *in vitro* (**Figure 16A**), 10-fold excess GST-p120^{ctn} completely inhibited GST-ΔPOZ from binding to the *me-mts* probe *in vitro* (**Figure 16B**). Therefore, p120^{ctn} inhibits both sequence-specific and methylation-dependent DNA-binding *in vitro*.

As a negative control, GST-p120^{ctn} was run alone in order to account for non-specific DNA-binding activity. GST-p120^{ctn} failed to bind DNA, indicating that p120^{ctn} does not complex with Kaiso target DNA sequences, but rather binds to Kaiso to inhibit DNA-binding activity. It is important to note that GST-p120^{ctn} failed to inhibit the DNA-binding activity of the ZF 123 and ZF 23 Kaiso constructs (see **Figure 5** for a schematic representation of ZF 123 and ZF 23).

GST-p120^{ctn} Inhibits Sequence-Specific Kaiso-DNA Binding

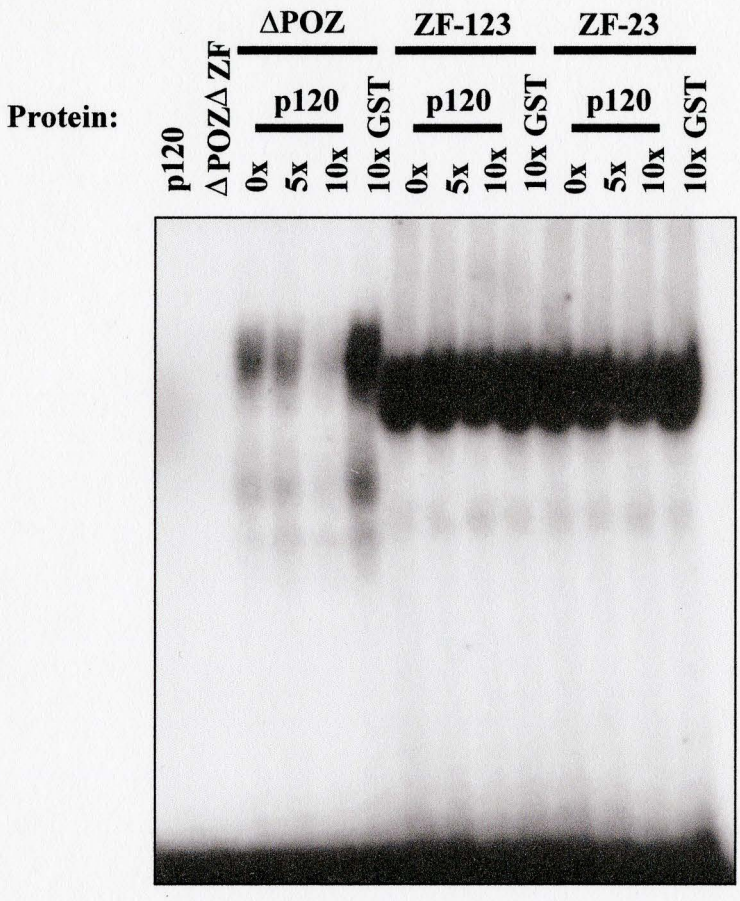


Figure 16A: *GST-p120^{ctn} inhibits sequence-specific DNA-binding.* Increasing amounts of GST-p120^{ctn} was incubated with Kaiso-*Mmat* complexes *in vitro*. Ten-fold excess of GST-p120^{ctn} significantly inhibited ΔPOZ-DNA binding, while GST alone did not inhibit DNA binding activity. GST-p120^{ctn} alone did not exhibit any DNA-binding activity with the *Mmat* oligonucleotide.

GST-p120^{ctn} Inhibits Methylation-Dependent Kaiso-DNA Binding

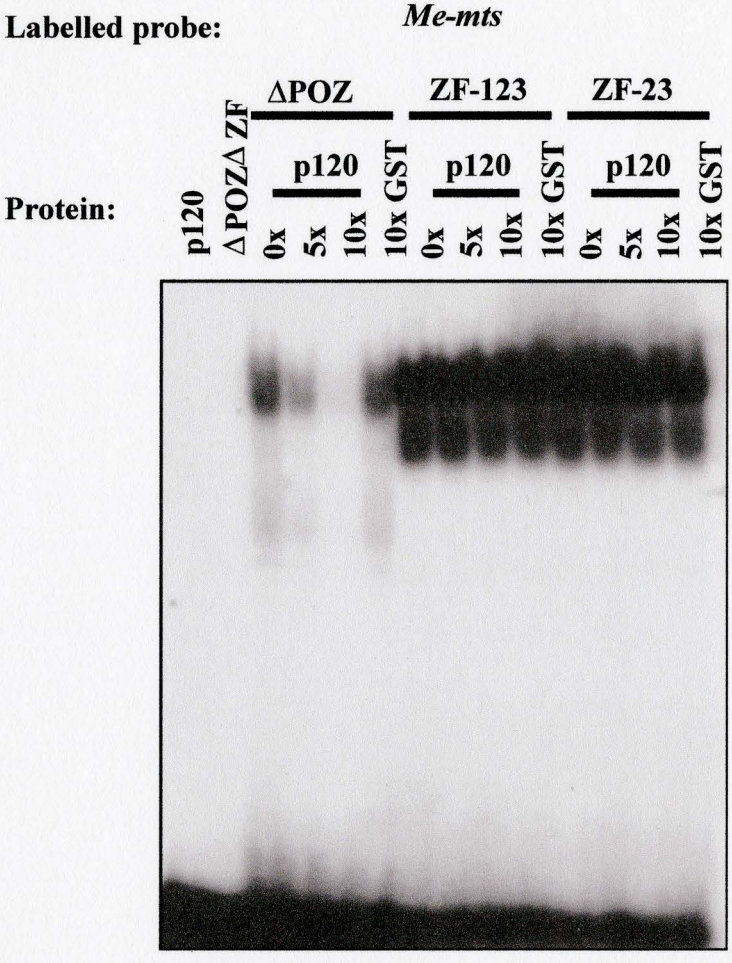


Figure 16B: *GST-p120^{ctn} inhibits methylation-dependent DNA-binding.* Increasing amounts of GST-p120^{ctn} was incubated with Kaiso-*me-mts* complexes *in vitro*. Five-fold excess of GST-p120^{ctn} significantly inhibited ΔPOZ-DNA binding, while 10-fold GST-p120^{ctn} completely abolished DNA binding activity. Note that GST alone did not inhibit DNA-binding activity and GST-p120^{ctn} did not exhibit any DNA-binding activity with the *me-mts* oligonucleotide.

One explanation for the finding that GST-p120^{ctn} failed to inhibit ZF 123 and ZF 23 DNA-binding activity is that these Kaiso deletion mutants may lack the necessary p120^{ctn}-binding site (ref. (25), **Figure 5**). These results verify that p120^{ctn} specifically inhibits ΔPOZ Kaiso-DNA binding activity in both a sequence-specific and a methylation-dependent manner. These data also imply that p120^{ctn} may act to inhibit Kaiso-DNA binding activity and inhibit Kaiso-mediated transcriptional repression.

3.2.2 p120^{ctn} inhibits Kaiso-mediated transcriptional repression

To determine the effects of p120^{ctn} on Kaiso-mediated transcriptional repression, exogenous p120^{ctn} was included in artificial promoter assays. While exogenous Kaiso repressed transcription of the 4xKBS by approximately 2-fold (**Figure 17**), co-expression of p120^{ctn} inhibited Kaiso-mediated transcriptional repression by approximately 90% (compare 4xKBS + Kaiso and 4xKBS + Kaiso + p120). Repression of the 4xKBS by endogenous Kaiso was also inhibited by exogenous p120^{ctn} (compare 4xKBS and 4xKBS + Kaiso). Furthermore, artificial promoter assays were performed to establish a dose-response relationship between the amount of p120^{ctn} transfected and the inhibition of Kaiso-mediated transcriptional repression. Transfection of 50 ng of p120^{ctn} de-repressed Kaiso-mediated transcriptional repression by 1.4-fold and 300 ng of p120^{ctn} inhibited transcriptional repression by 3.4-fold (**Figure 18**). Levels of transfected p120^{ctn} in excess of 300 ng failed to de-repress the 4xKBS beyond 3.5-fold, implying that 300 ng of p120^{ctn} is sufficient for maximal activity.

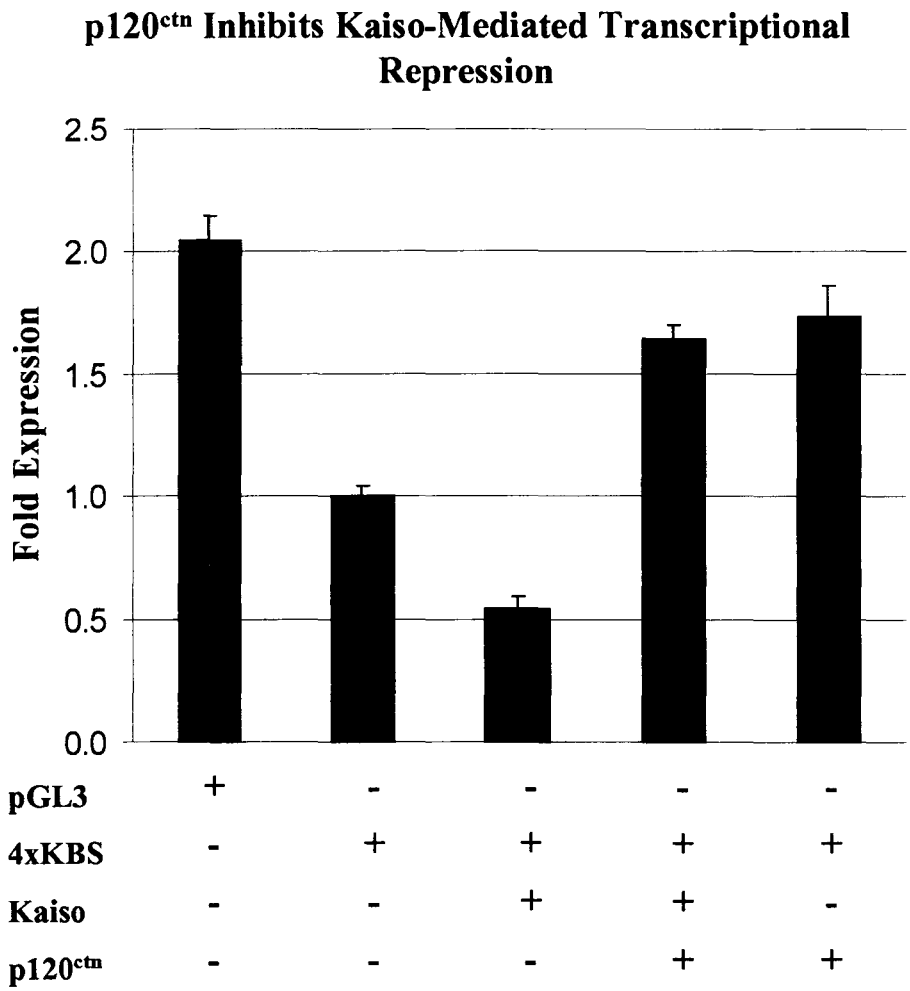


Figure 17: *p120^{ctn} inhibits Kaiso-mediated transcriptional repression.* While exogenous Kaiso represses transcription of the 4xKBS plasmid (4xKBS + Kaiso) by approximately two-fold, exogenous p120^{ctn} blocks Kaiso-mediated repression by approximately 90% (4xKBS + Kaiso + p120).

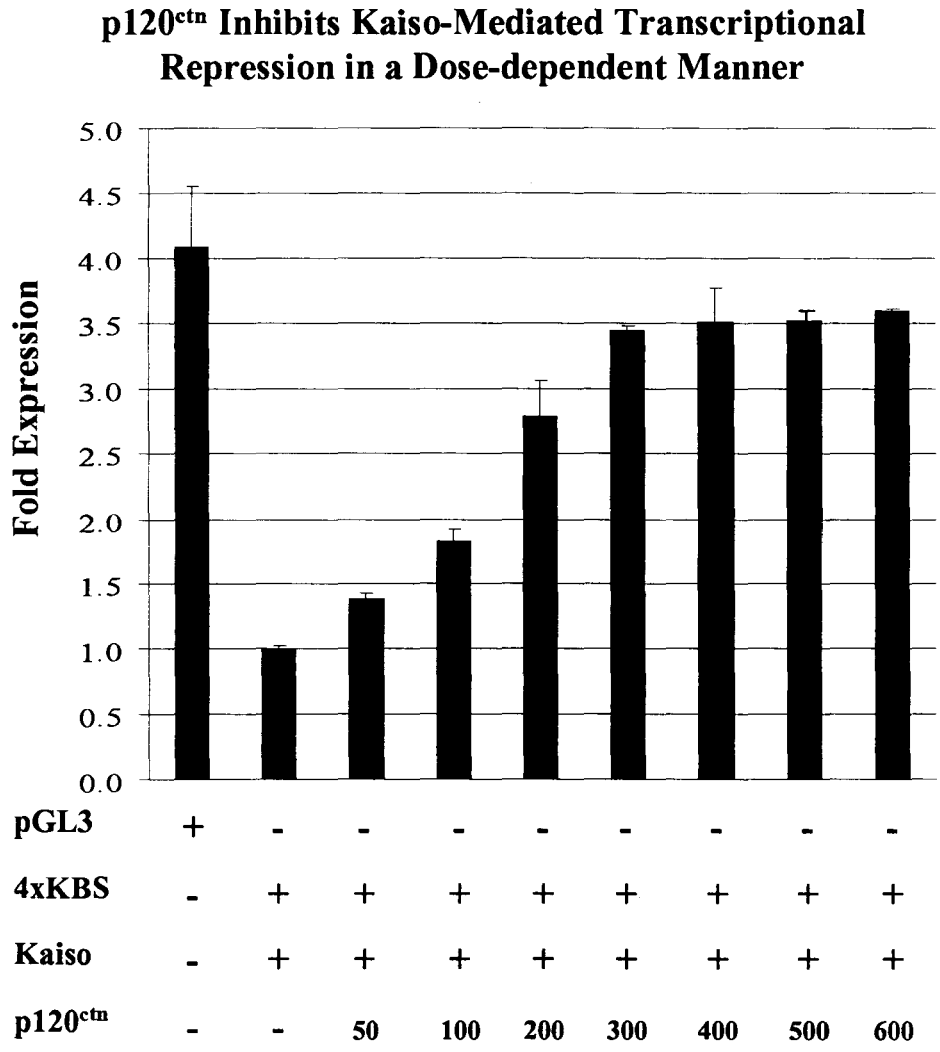


Figure 18: *p120ctn inhibits Kaiso-mediated transcriptional repression in a dose-dependent manner.* Transfection of increasing levels of p120ctn expression vector inhibited Kaiso mediated transcriptional repression of the 4xKBS in a dose dependent manner. Levels of p120ctn in excess of 300 ng do not further de-repress the system.

3.2.3 The Kaiso-p120^{ctn} interaction is required for transcriptional de-repression via the 4xKBS element

EMSA analysis established that p120^{ctn} inhibits Kaiso-DNA binding activity (**Figures 16A and 16B**) and artificial promoter assays demonstrated that p120^{ctn} inhibits Kaiso-mediated transcriptional repression (**Figure 17**). These data suggest that p120^{ctn} directly inhibits Kaiso-mediated transcriptional repression by interacting with Kaiso and masking the Kaiso-DNA binding interface (**Figure 5**). Alternatively, p120^{ctn} could inhibit Kaiso-mediated transcriptional repression in an indirect manner. To test the hypothesis that p120^{ctn} interacts with Kaiso and directly inhibits transcriptional repression, the p120^{ctn} deletion mutant ΔR3-11 (lacking the Kaiso-interaction domain) was included in artificial promoter assays. Full length p120^{ctn} de-repressed Kaiso mediated transcriptional repression significantly, while ΔR3-11 did not significantly affect luciferase expression (**Figure 19**). Note that the expression of the ΔR3-11 and p120^{ctn} were confirmed by western blot analysis **(Appendix B, Figure B-1).** To further verify that the Kaiso-p120^{ctn} interaction is necessary for the inhibition of Kaiso-mediated transcriptional repression, a cell-line specific control for the Kaiso-p120^{ctn} interaction was utilized. The Kaiso-p120^{ctn} interaction is generally disrupted in fibroblast cell lines (Rat-1, Va-2, and NIH 3T3), yet prominent in epithelial cell lines (HeLa, HCT116, SW620). In NIH 3T3 cells, neither full-length p120^{ctn} nor ΔR3-11 could inhibit Kaiso-mediated transcriptional repression to a significant extent (**Figure 20**). These data confirm that the Kaiso-p120^{ctn} interaction is necessary for p120^{ctn} to inhibit Kaiso-mediated transcriptional repression.

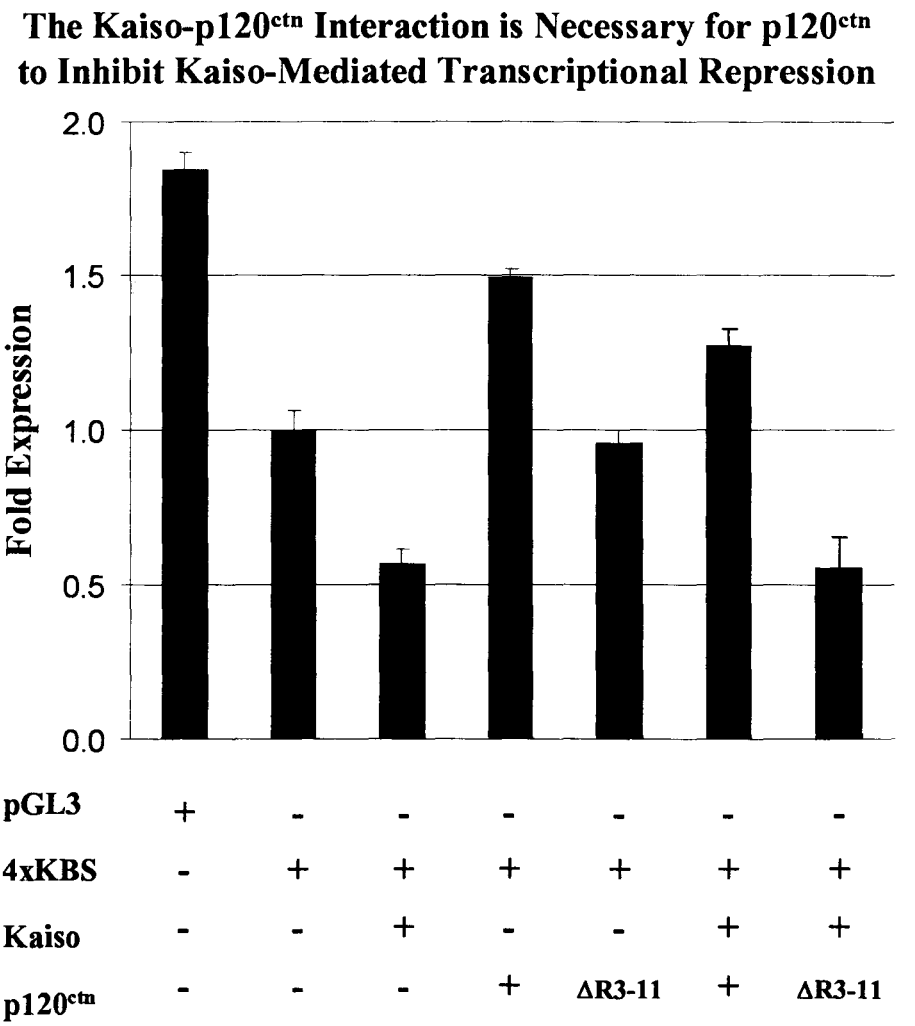


Figure 19: The *p120^{ctn}-Kaiso interaction is necessary for p120^{ctn} to inhibit of Kaiso-mediated transcriptional repression.* Full-length p120^{ctn} was capable of de-repressing the 4xKBS (4xKBS + p120 and 4xKBS + Kaiso + p120), while ΔR3-11 (4xKBS + ΔR3-11 and 4xKBS + Kaiso + ΔR3-11) did not significantly affect the luciferase expression.

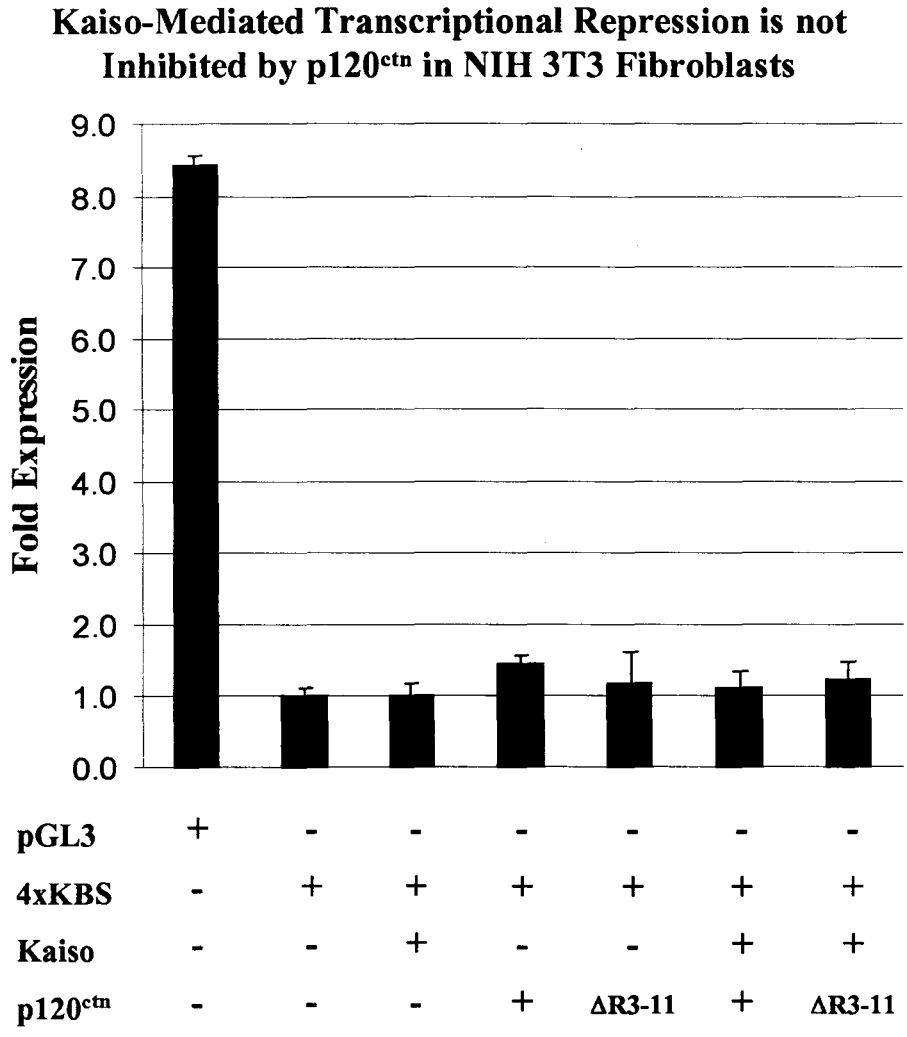


Figure 20: *p120^{ctn} does not inhibit Kaiso-mediated transcriptional repression in 3T3 fibroblasts.* Kaiso and p120^{ctn} do not interact in 3T3 fibroblasts. In this cell line, neither full-length p120^{ctn} nor ΔR3-11 (4xKBS + p120, 4xKBS + Kaiso + p120, 4xKBS + p120 ΔR3-11, and 4xKBS + Kaiso + p120 ΔR3-11) were capable of de-repressing luciferase expression.

3.2.4 Inhibition of Kaiso-mediated transcriptional repression requires p120^{ctn} nuclear localization

DNA-binding assays demonstrated that Kaiso-DNA binding was inhibited by GST-p120^{ctn} (**Figures 16A and 16B**) and artificial promoter assays confirmed that p120^{ctn} inhibits Kaiso-mediated transcriptional repression (**Figure 17**). p120^{ctn} could therefore either enter the nucleus and inhibit Kaiso-mediated transcriptional repression (via inhibiting Kaiso-DNA association), or bind to and sequester Kaiso in the cytoplasm. To distinguish between these distinct possibilities, p120^{ctn} carrying a point mutation in its NLS (p120 NLS Mut) was included in artificial promoter assays.

While wild-type p120^{ctn} can enter the nucleus and cause a branching phenotype (**Figure B-4**), p120^{ctn} NLS Mut neither enters the nucleus nor induces a branching phenotype (**Figure B-5**). Additionally, when overexpressed in Cos-1 cells, wild-type p120^{ctn} could de-repress luciferase expression levels in the presence of Kaiso. However, the NLS mutant could not de-repress luciferase expression **(Figure 21)**, confirming that the regulation of Kaiso-mediated transcriptional repression by p120^{ctn} is in fact a nuclear activity. Western blot analysis of the expression of luciferase assay effector constructs (**Appendix B, Figure B-1**) were done on nuclear Cos-1 cell extracts, confirming that these proteins were expressed in the appropriate subcellular compartment.

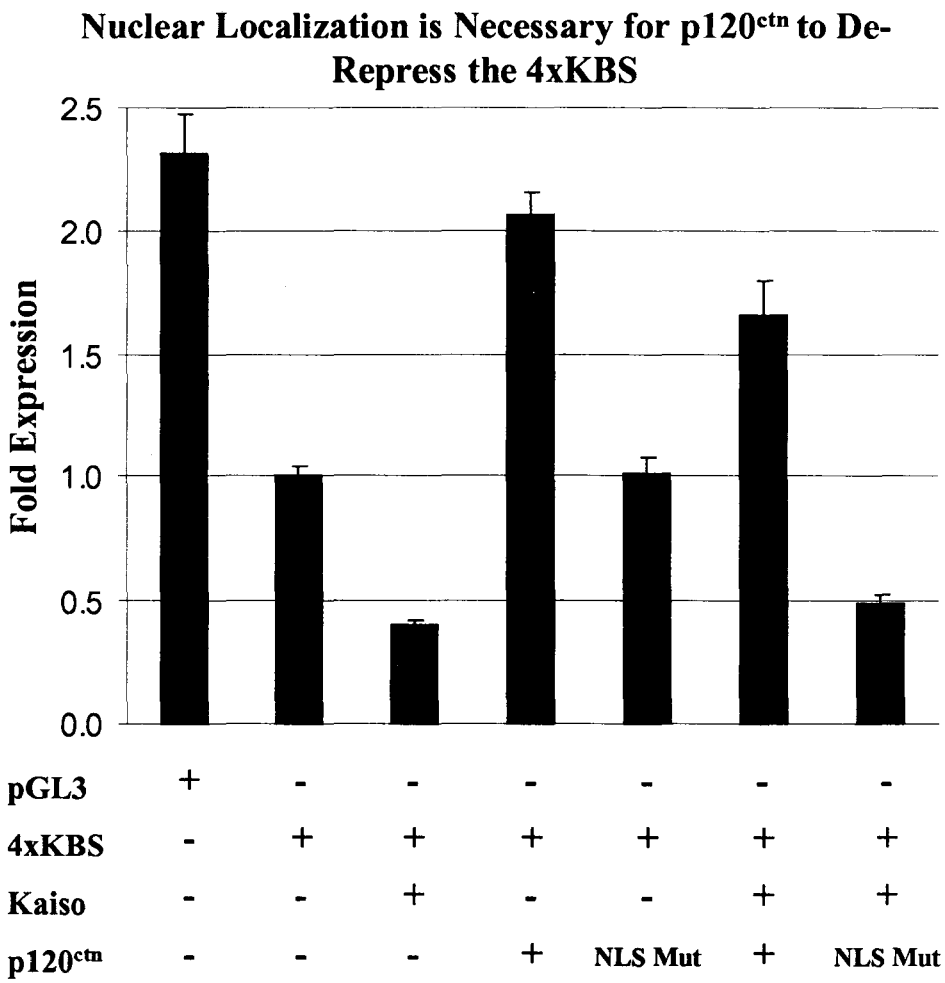


Figure 21: *Nuclear localization is necessary for p120^{ctn} to de-repress the 4xKBS.* Overexpression of wild-type p120^{ctn} de-repressed luciferase expression via the 4xKBS, while cytosolically-stranded p120^{ctn} (NLS Mut) failed to inhibit Kaiso-mediated transcriptional repression.

3.3 Kaiso recruits histone deacetylase activity to repress transcription

3.3.1 The POZ and ZF domains are required for Kaiso-mediated transcriptional repression via the 4xKBS element

Having established Kaiso as a repressor via the consensus KBS *cis*-element, further studies were performed to understand the mechanism of transcriptional repression utilized by Kaiso. The POZ-ZF transcriptional repressors Bcl-6 and PLZF silence gene expression by binding sequence-specific *cis*-elements with their ZF domains and subsequently recruit HDAC complexes via their POZ protein-protein interaction motifs (18, 73, 128). Therefore, it was hypothesized that Kaiso may also repress transcription by recruiting an HDAC complex via the POZ domain.

To investigate the mechanism of Kaiso-mediated transcriptional repression, Kaiso deletion mutants lacking the POZ (ΔPOZ) or zinc finger (ΔZF) domains were co-transfected with the 4xKBS reporter construct into Cos-1 cells. While full-length exogenous Kaiso could repress transcription via the 4xKBS by 2-fold, the ΔPOZ or ΔZF deletion mutants had no effect (**Figure 22**). These data demonstrate that the POZ and ZF domains are required for Kaiso-mediated transcriptional repression. Kaiso thus appears to repress transcription in a manner which may be mechanistically similar to other POZ-ZF proteins.

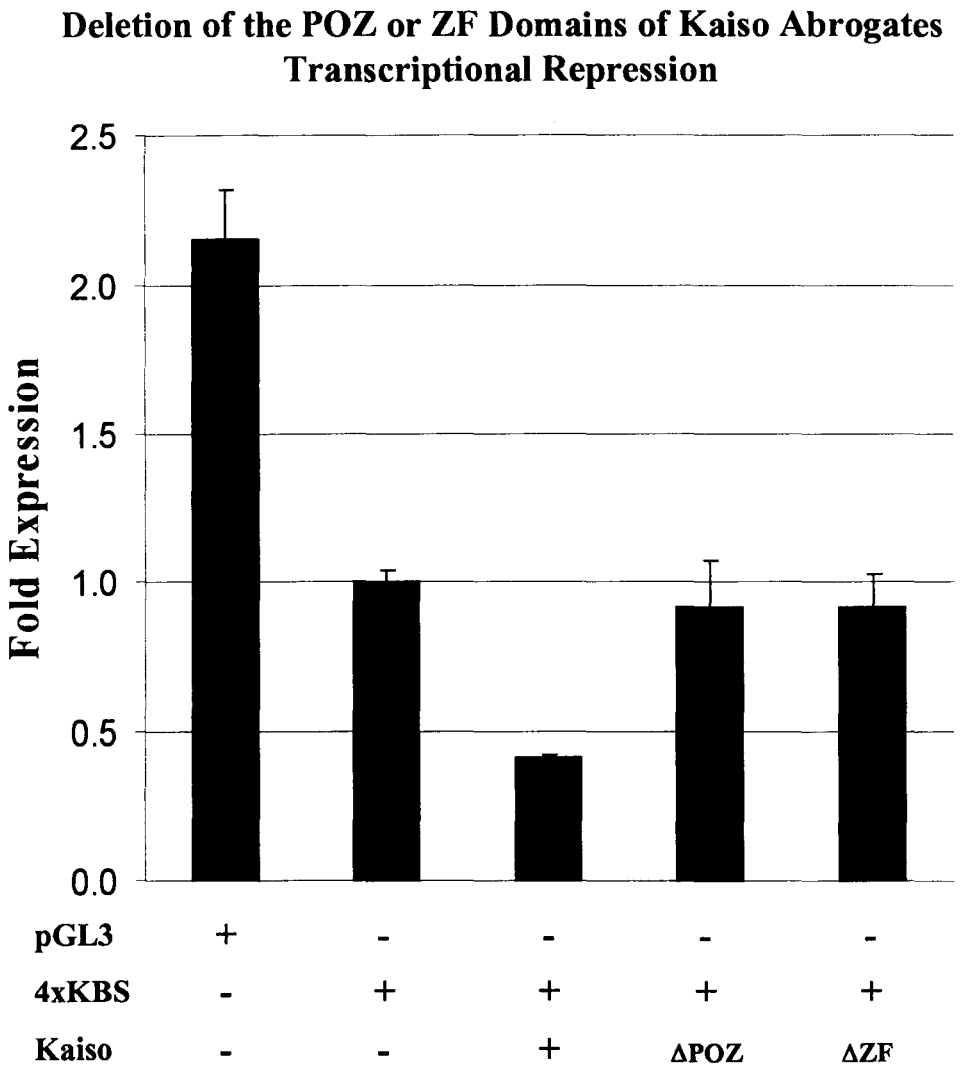


Figure 22: *Deletion of the POZ or ZF domains of Kaiso abrogates transcriptional repression.* In Cos-1 cells, endogenous Kaiso represses luciferase expression 4-fold relative to the empty vector (compare pGL3 Control to 4xKBS). Exogenous Kaiso (4xKBS + Kaiso) further repressed luciferase expression 2-fold relative to baseline 4xKBS expression, while overexpression of Kaiso deletion mutants lacking the POZ (ΔPOZ) or ZF (ΔZF) domains did not affect basal luciferase expression from the 4xKBS plasmid.

3.3.2 Kaiso co-immunoprecipitates with the HDAC co-repressor mSin3A

Both Bcl-6 and PLZF interact with the co-repressor mSin3A in order to recruit an HDAC complex (28, 32) which results in local chromatin condensation and transcriptional silencing. To determine whether Kaiso represses transcription in a similar manner, Kaiso was overexpressed in Cos-1 cells and lysates were subject to immunoprecipitation and with Kaiso (positive control), $p120^{ctn}$ (positive Kaiso-protein interaction control), mSin3A, HDAC-1, or 12CA5 (negative control) antibodies. Western blot analysis detected Kaiso protein in both $p120^{ctn}$ and mSin3A immunoprecipitates, indicating that Kaiso complexes with both $p120^{ctn}$ and mSin3A in Cos-1 cells **(Figure 23A)**. Notably, the Kaiso-mSin3A co-precipitation is significantly weaker than the Kaiso-$p120^{ctn}$ interaction. Immunoprecipitates performed with an irrelevant isotype control (12CA5) did not contain any Kaiso protein.

Cos-1 cell lysates were also subject to immunoprecipitation with mSin3A (positive control), HDAC-1 (positive mSin3A-protein interaction control), Kaiso, $p120^{ctn}$, or 12CA5 (negative control) antibodies. mSin3A protein was detected in mSin3A, HDAC-1, and Kaiso immunoprecipitates, but not in $p120^{ctn}$ or negative control (12CA5) immunoprecipitates (**Figure 23B**). These data confirm that Kaiso complexes with the mSin3A co-repressor *in vivo*, and further implies that Kaiso-mediated transcriptional repression may be mechanistically similar to HDAC-dependent Bcl-6 or PLZF transcriptional repression.

Kaiso and Sin3A Co-immunoprecipitate in Cos-1 Cells

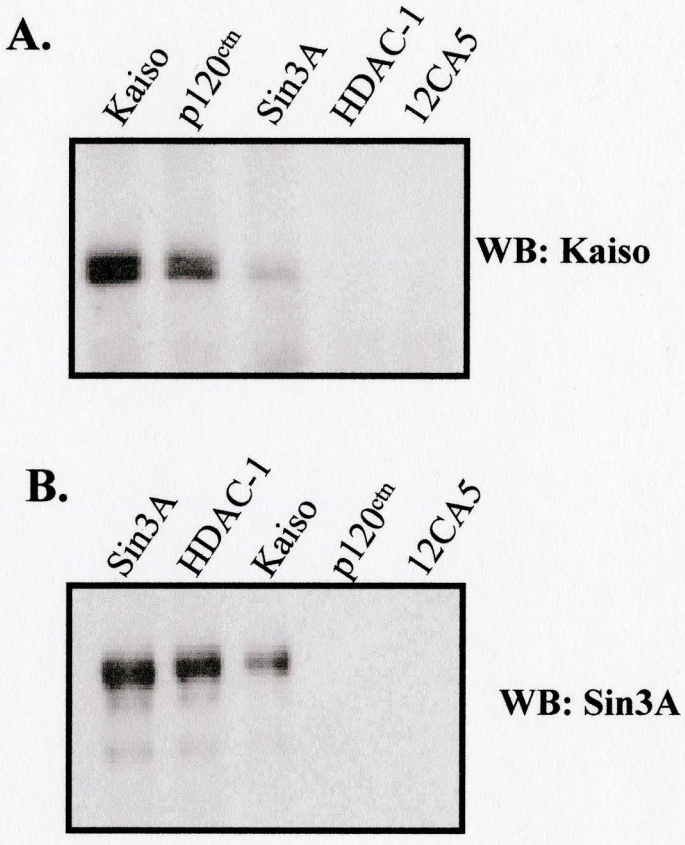


Figure 23: *Kaiso co-immunoprecipitates with Sin3A in Cos-1 cells.* Kaiso was overexpressed in Cos-1 cells and the indicated immunoprecipitates were performed. (A) Kaiso protein was detected via Western blot in Kaiso, p120^{ctn}, and Sin3A immunoprecipitates. (B) Conversely, Sin3A protein was detected in Sin3A, HDAC-1, and Kaiso immunoprecipitates, but not in p120^{ctn} immunoprecipitates. Neither Sin3A or Kaiso were co-precipitated by the irrelevant isotype control antibody (12CA5).

3.3.3 Kaiso represses transcription in an HDAC-dependent manner

To determine whether Kaiso-mediated transcriptional repression requires the recruitment of an HDAC complex, Kaiso immunoprecipitates were assayed for HDAC activity using the *Fluor de Lys*^{TM} (BioMol) assay. In this assay, the *Fluor de Lys*^{TM} substrate is incubated with putative sources of HDAC activity. Deacetylation of the *Fluor de Lys*^{TM} substrate subsequently results in the formation of a fluorophore. Thus, fluorescence is directly proportional to the HDAC activity present in a given sample.

Kaiso immunoprecipitates were incubated with the *Fluor de Lys*^{TM} substrate, and fluorescence was measured. 12CA5 immunoprecipitates were also assayed for HDAC activity as a negative control, and HDAC-1 immunoprecipitates served as a positive control. Kaiso immunoprecipitates from HCT116 (human colorectal carcinoma), HeLa (human cervical adenocarcinoma), Cos-1 (transformed monkey fibroblast), and NIH 3T3 (immortalized murine fibroblast) cells possessed extremely high levels of deacetylase activity when compared to 12CA5 immunoprecipitates (**Figure 24**). As a positive control, HDAC-1 immunoprecipitates from HCT116 cells were also assayed, which contained very high levels of HDAC activity. Note that HDAC activity present in Kaiso immunoprecipitates was similar to that of HDAC-1 immunoprecipitates, indicating that Kaiso associated with very high levels of deacetylase activity.

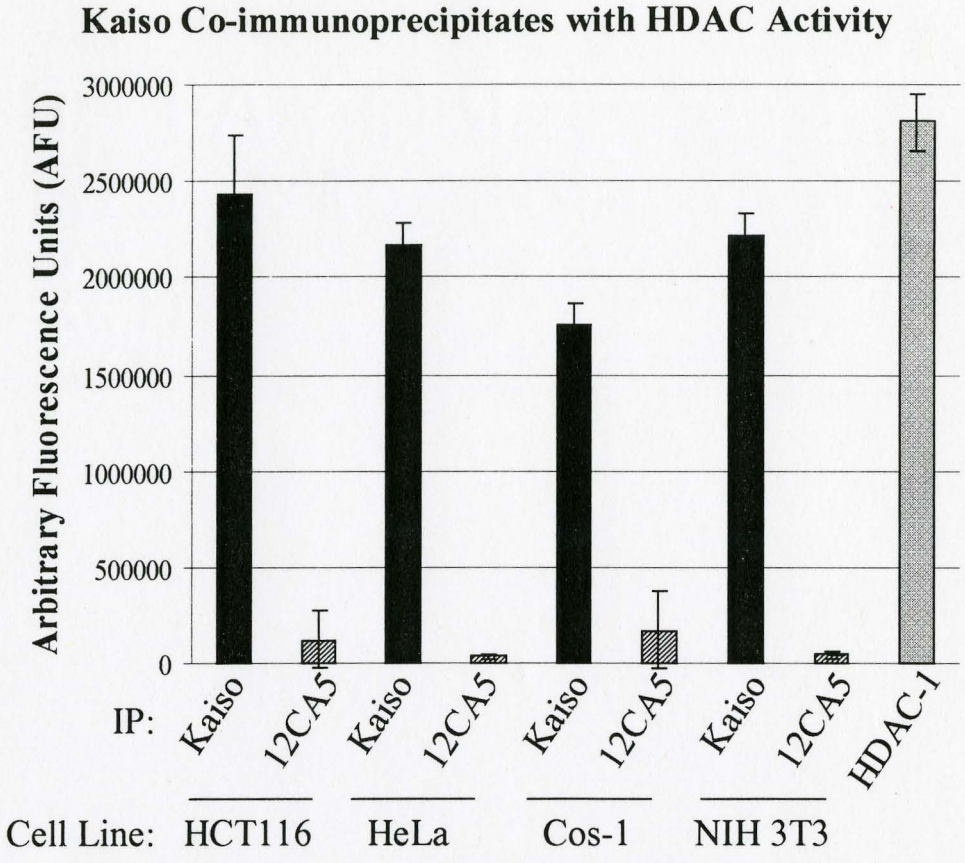


Figure 24: *Kaiso co-immunoprecipitates with HDAC activity-* Histone *d*eacetylase activity was measured via the *Fluor de Lys* assay (BioMol) in Kaiso immunoprecipitates from HCT116, HeLa, Cos-1, and NIH 3T3 cells (black bars). As a negative control, the irrelevant isotype control monoclonal antibody 12CA5 was used (hatched bars). Samples were compared to an HDAC-1 immunoprecipitates (grey bar) serving as a positive control. Histone deacetylase activity associated with Kaiso is greater than ten-fold above background levels, and was comparable to the HDAC control.

Since Kaiso co-precipitated with mSin3A (**Figure 23A and 23B**) and HDAC activity (**Figure 24**), it was hypothesized that Kaiso may recruit an HDAC complex to repress transcription. To test this hypothesis, the HDAC inhibitors Trichostatin A (TSA) and sodium butyrate (NaB) were included in artificial promoter assays. Cos-1 cells were treated with either 300 nM TSA or 5 mM NaB for 8 hours prior to luciferase assays. Kaiso-mediated transcriptional repression (4xKBS + Kaiso) was inhibited by 90% and 60% by TSA and NaB, respectively (**Figure 25**). Solvent alone did not affect Kaiso-mediated transcriptional repression, and neither HDAC inhibitor nonspecifically affected expression of the backbone vector alone (pGL3 Control + Kaiso).

Thus, Kaiso-mediated transcriptional repression requires the POZ domain (**Figure 22**), and Kaiso co-immunoprecipitates with the HDAC co-repressor mSin3A *in vivo* (**Figure 23A and 23B**). Kaiso also co-immunoprecipitates with HDAC activity (**Figure 24**), and Kaiso-mediated transcriptional repression is blocked by the pan-specific HDAC inhibitors TSA and NaB (**Figure 25**). These four lines of evidence confirm that Kaiso, like Bcl-6 and PLZF, acts as an HDAC-dependent transcriptional repressor.

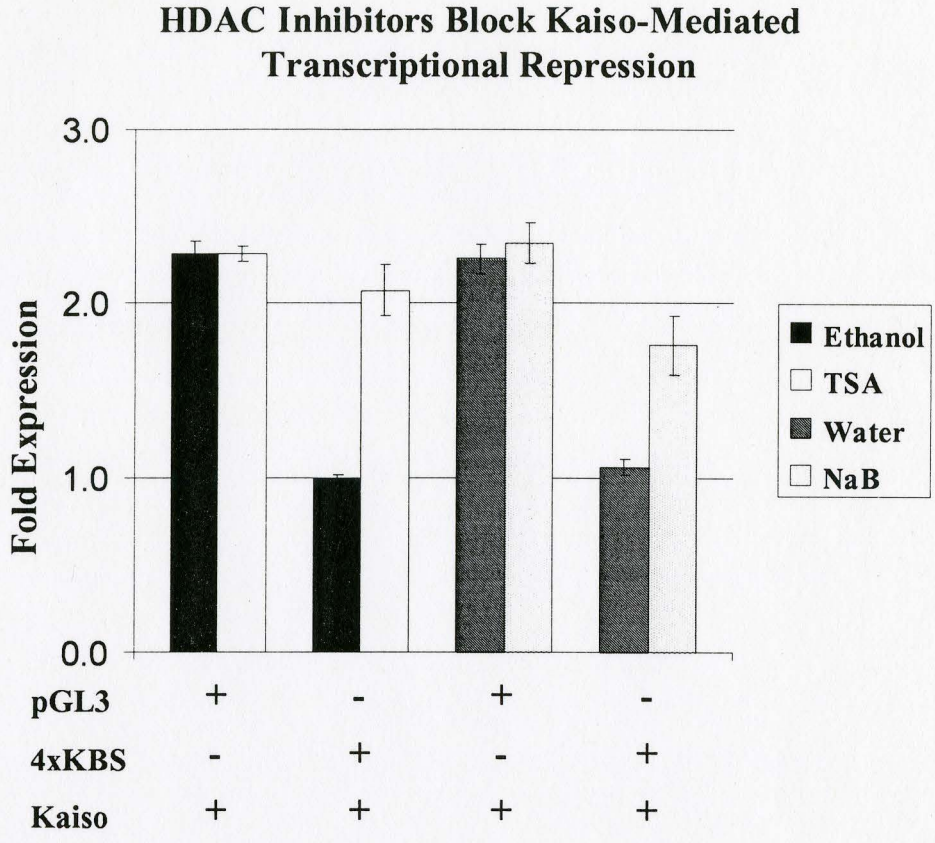


Figure 25: *HDAC inhibitors block Kaiso-mediated transcriptional repression.* A Kaiso-repressed system (4xKBS + Kaiso) is sensitive to 300 nM TSA (white bars) and 5mM sodium butyrate (NaB), but not solvent alone (black bars and black hatched bars). The backbone vector (pGL3 Control + Kaiso) is not significantly affected by either HDAC inhibitor.

3.4 p120^{ctn} inhibits HDAC-dependent Kaiso-mediated transcriptional repression via a matrilysin promoter derived cis-element

3.4.1 p120^{ctn} inhibits Kaiso-mediated transcriptional repression via a matrilysin promoter derived cis-element

Kaiso associates with *matrilysin* promoter-derived probes *in vitro* (27) and Kaiso represses transcription via a KBS *cis*-element which shares homology to the *matrilysin* promoter (**Figure 10**). It was therefore hypothesized that Kaiso may repress transcription via the *matrilysin* promoter (4xKBS)(**Figure 10**). To test this hypothesis, a 329 base pair fragment corresponding to nucleotides –296 to +33 of the human *matrilysin* promoter (-296 Hmat pro) was used in artificial promoter assays. While basal expression of the –296 Hmat pro construct was extremely high, exogenous Kaiso was capable of repressing luciferase expression by 7-fold **(Figure 26)**. Since p120^{ctn} inhibits Kaiso-mediated transcriptional repression of the 4xKBS *cis*-element (**Figure 17**), the effects of p120^{ctn} on the –296 Hmat pro were also determined. While wild-type p120^{ctn} was capable of de-repressing Kaiso-mediated transcriptional repression of the –296 Hmat pro construct by roughly 3-fold, both ΔR3-11 and the p120^{ctn} NLS Mut did not significantly affect luciferase expression. Mutation of the KBS site in the Hmat –296 pro element (**Figure 8A**) abrogated the ability of Kaiso to repress transcription (**Figure 27**), confirming that Kaiso-mediated transcriptional repression of the –296 Hmat pro was due to Kaiso binding the consensus TCCTGCNA site.

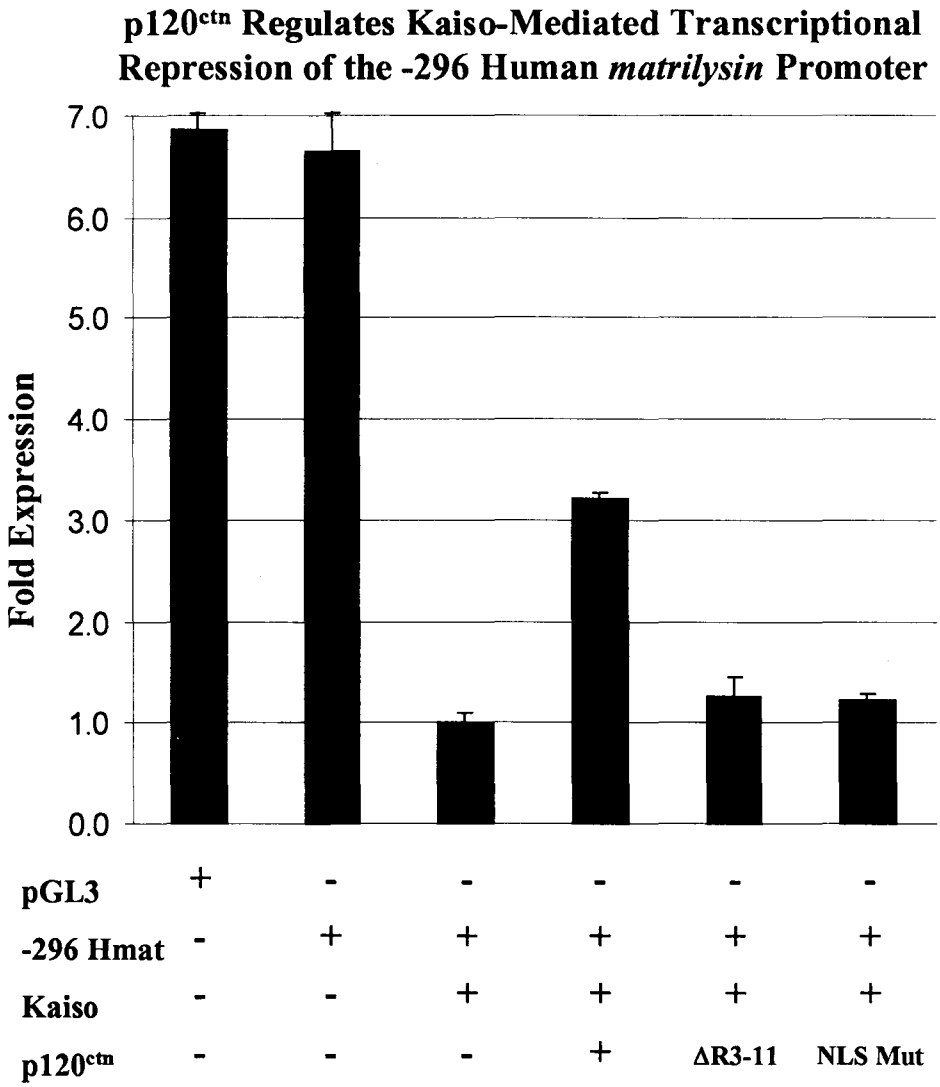


Figure 26: *p120ctn regulates Kaiso-mediated transcriptional repression of the -296 human matrilysin promoter.* Basal expression levels of the -296 *matrilysin* promoter was extremely high, and Kaiso repressed this expression level by over 6-fold. While p120ctn significantly de-repressed the -296 *matrilysin* promoter, neither ΔR3-11 or the p120ctn NLS Mut could inhibit Kaiso-mediated transcriptional repression.

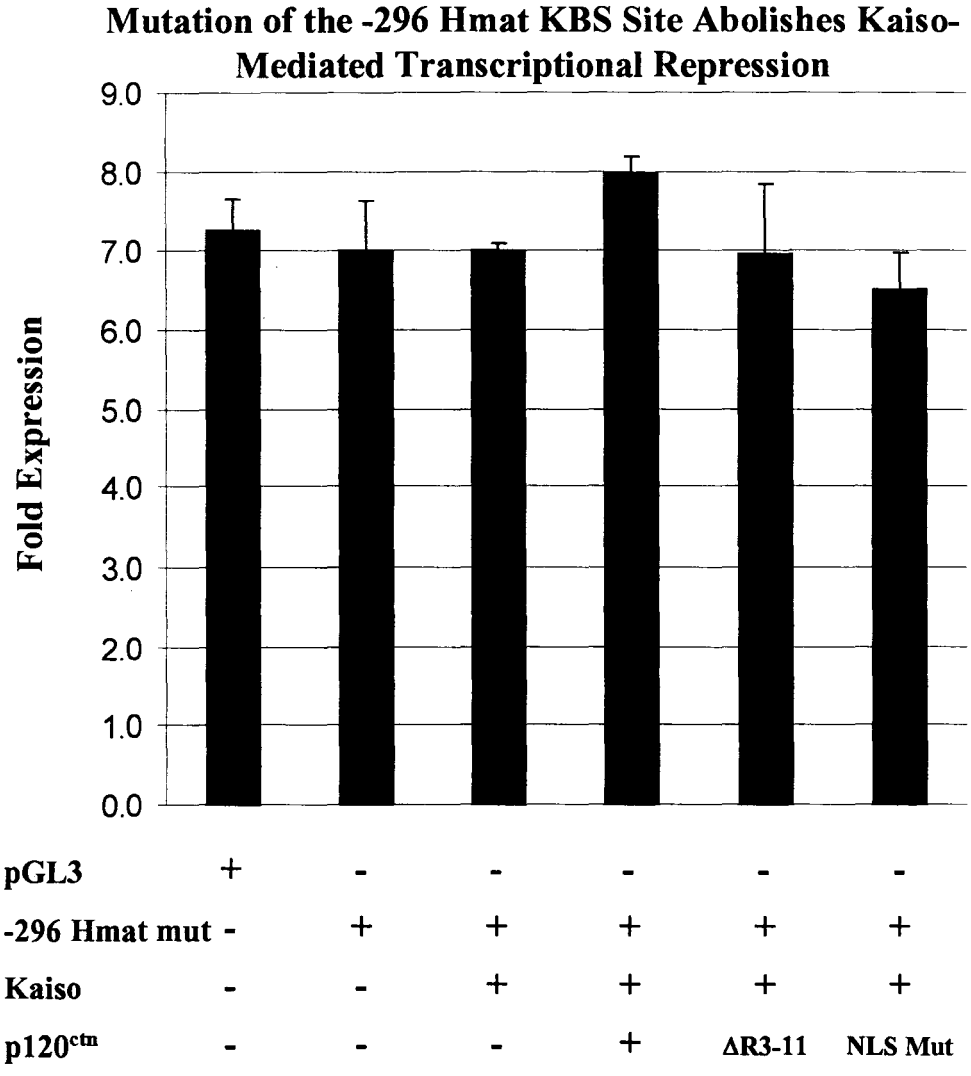


Figure 27: *Mutation of the -296 Hmat KBS site abolishes Kaiso-mediated transcriptional repression.* pGL3 containing the mutated -296 Hmat pro (-296 Hmat pro mut) element is not affected by the expression of Kaiso or p120^{ctn}, indicating that the KBS is necessary for Kaiso/p120^{ctn} regulation of the *matrilysin* promoter.

3.4.2 Kaiso represses transcription via the matrilysin promoter derived cis-element in an HDAC-dependent manner

Kaiso-mediated transcriptional repression via the 4xKBS *cis*-element is inhibited by the HDAC inhibitors TSA and NaB (**Figure 25**). Kaiso also interacts with the HDAC co-repressor mSin3A *in vivo* (**Figure 23**), and associates with deacetylase activity (**Figure 24**), suggesting that Kaiso represses transcription by recruiting an mSin3A-HDAC complex. Kaiso was also shown to repress transcription via an additional *cis*-element, the –296 Hmat pro (**Figure 26**). Artificial promoter assays were performed using the –296 Hmat pro in the presence of TSA and NaB to determine whether repression of the *matrilysin* promoter fragment was HDAC-dependent. Both TSA and NaB significantly inhibited Kaiso-mediated transcriptional repression of the –296 Hmat pro, but did not affect expression levels of the –296 Hmat pro in the absence of exogenous Kaiso **(Figure 28)**. This finding is consistent with the assumption that no known HDAC-dependent transcriptional repressors associate with the *matrilysin* promoter (**Figure 8A and 8B**). Therefore, Kaiso appears to repress transcription via the *matrilysin* promoter in an HDAC-dependent manner.

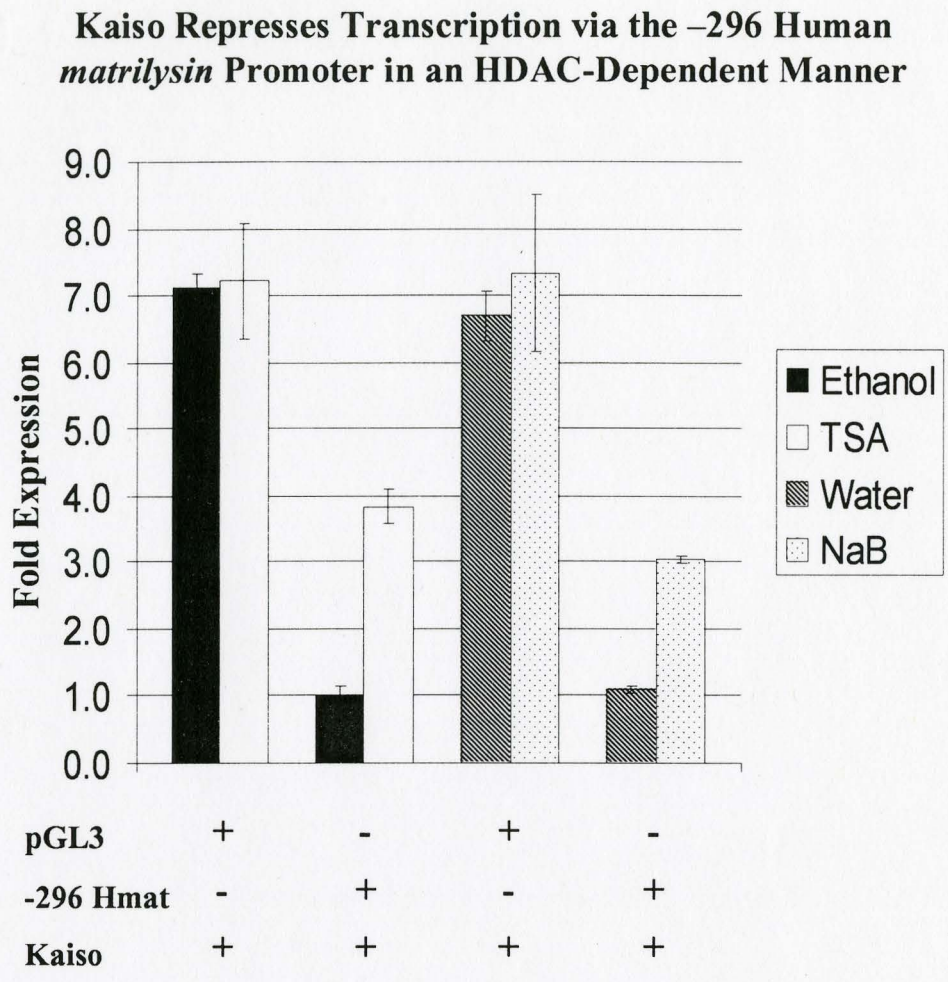


Figure 28: *HDAC inhibitors block Kaiso-mediated transcriptional repression of the -296 human matrilysin promoter fragment.* The HDAC inhibitors TSA (white bars) and NaB (spotted bars) partially inhibited Kaiso-mediated transcriptional repression via the -296 Hmat promoter fragment. Solvent alone (black bars and hatched bars, respectively) did not affect luciferase expression, and neither TSA nor NaB significantly affected -296 Hmat pro expression in the absence of Kaiso.

Discussion

4. *The Catenin p120^{ctn} Regulates Kaiso-Mediated Transcriptional Repression*

Kaiso was previously described as a bi-modal DNA-binding protein (27) and implicated as a methylation-specific transcriptional repressor (112), yet the nature of sequence-specific transcriptional regulation remained undefined (**Figure 6**). In this study, Kaiso is reported as a sequence-specific transcriptional repressor of the KBS *cis*-element (**Figures 10-15**) and the KBS element located within the *matrilysin* promoter (**Figure 26**). Furthermore, the cell adhesion co-factor and Kaiso interaction partner p120^{ctn} inhibits Kaiso-DNA binding *in vitro* (**Figure 16A and 16B**), and was implicated as an inhibitor of Kaiso-mediated transcriptional repression (27). p120^{ctn} inhibited Kaiso-mediated transcriptional repression via the KBS *cis*-element (**Figure 17**), and this activity requires the p120^{ctn}-Kaiso interaction (**Figures 19 and 20**) as well as the nuclear localization of p120^{ctn} (**Figure 21**). Lastly, co-immunoprecipitation and artificial promoter analysis demonstrated that Kaiso represses transcription in an HDAC-dependent manner (**Figures 24 and 25**) and associates with the mSin3A HDAC co-repressor (**Figure 23A and 23B**).

4.1 Kaiso is a sequence-specific transcriptional repressor

4.1.1 Implications of Kaiso as a sequence-specific transcriptional repressor

Human cancer profiling array analysis suggests that Kaiso plays a role in inhibiting tumourigenesis. Kaiso mRNA levels are reduced in 50% of ovarian tumours and 32% of breast tumours, relative to non-transformed tissue (Daniel Lab, unpublished data). This implicates Kaiso as a tumour suppressor, in which case Kaiso-mediated transcriptional repression of target genes would be selectively disrupted in transformed cells. Tumour suppressors that are transcription factors (i.e. p53) are often subject to disruption of their DNA-binding activity and/or genomic instability in transformed cells and cancers (35, 115). Therefore, it is possible that Kaiso-DNA association will be disrupted in transformed cells and cancers by mutation of KBS elements in the promoters of target genes or mutation of the Kaiso ZF domain.

PCR amplification of promoter DNA of KBS sites in putative Kaiso target gene promoters would be expected to reveal a high incidence of mutational instability of KBS sites. Mutational instability of KBS sites at multiple loci (e.g. *matrilysin* and *cyclinD1* promoter sites, both of which Kaiso can bind *in vitro*; (27) and **Figure B-6**) would be supportive of a tumour suppressor role for Kaiso. In addition, direct disruption of the Kaiso-DNA association could result from the mutation of the ZF domain of Kaiso. Indeed, DNA-binding regulatory domains of p53 are mutational "hot spots" in cancers (106). To detect mutations in the ZF domain of Kaiso, the ZF coding sequence of *kaiso*

could be PCR amplified from genomic DNA and sequenced. Comparison of the DNA sequences from human cancers and non-transformed neighboring tissue would reveal whether the DNA-binding domain of Kaiso is subject to mutation in human cancer. It is likely that other disruptions of the Kaiso-p120^{ctn} pathway will also contribute to cellular transformation (e.g. upregulation of nuclear p120^{ctn}, downregulation of Kaiso expression, and/or sequestering Kaiso/co-repressors away from target gene promoters). However, mutational instability of the KBS sites and/or the ZF domain of Kaiso would be expected to contribute to tumour progression if Kaiso is indeed a tumour suppressor.

4.1.2 Kaiso target genes: cross-talk between sequence-specific and methylation-dependent Kaiso-mediated transcriptional repression?

Exogenous Kaiso represses transcription via a 1x KBS construct (**Figure 12**), indicating that a single *cis*-element is sufficient for Kaiso-mediated transcriptional repression. Transcription factors that homodimerize often require inverted tandem repeats in order for a functional homodimer to associate with a DNA-recognition sequence. Although Kaiso homodimerizes (25, 63), tandem copies of the KBS *cis*-element are not required for Kaiso-mediated transcriptional repression in this system. This further supports the hypothesis that, Kaiso could repress transcription of putative target genes, such as *cyclinD1* (promotes cell-cycle progression), *Id2* (inhibits differentiation and promotes cell proliferation), and *c-myc* (promotes cell proliferation) that carry a single KBS in their cognate promoters.

Kaiso may also repress transcription of putative Kaiso target genes which contain methylation-sensitive Kaiso sites in their promoters (*Rb*, *Pgk*, *metastasin*, and *Xist*), involved in anti-tumourigenesis (17), anti-angiogenesis (71), metastasis (29), and taxol sensitivity/chemotherapy success (43, 53) respectively. While the two subsets of Kaiso target genes (KBS-regulated and methylation-dependent) are separate and distinct, it is interesting to note that both methylation-dependent and sequence-specific sites appear in the *E-cadherin* promoter (**Figure B-7** and (112)). Thus, either methylation-dependent or sequence-specific Kaiso-mediated transcriptional repression may mediate two separate signal transduction pathways. These two pathways could converge in a synergistic manner by repressing transcription of the *E-cadherin* gene, which is associated with an increase in cell division and inhibition of contact inhibition of cell growth (59). KBS-dependent transcriptional repression of the *E-cadherin* gene could transiently repress E-cadherin levels (i.e. during normal cell division), which would be affected by Kaiso and $p120^{ctn}$ expression levels.

Alternatively, DNA methylation directs HDAC-dependent transcriptional repression of local DNA, resulting in permanent silencing of methylated genetic material (72). Epigenetic modification of the *E-cadherin* promoter (e.g. DNA methylation) would result in permanent silencing of *E-cadherin* expression, disrupting cell adhesion and promoting cellular transformation. Indeed, *E-cadherin* promoter methylation is associated with irreversible transcriptional silencing as well as transformation and tumour progression (44).

Experiments assaying Kaiso-mediated transcriptional repression via the *E-cadherin* promoter in both a methylated and unmethylated context and/or carrying mutations in the KBS sites would clarify the potentially synergistic effects of sequence-specific and methylation-dependent Kaiso-mediated transcriptional repression. Artificial promoter assays using the *E-cadherin* promoter carrying mutations in the KBS sites (E-cad KBS mut), mutations in the CpG islands (E-cad CpG mut), and the wild-type E-cadherin promoter (E-cad WT) could provide insight into a synergistic/antagonistic role for KBS- and methylation-dependent Kaiso mediated repression of the E-cadherin promoter. According to the above model, exogenous Kaiso would be expected to repress transcription of the E-cad WT more potently than the E-cad KBS mut or E-cad CpG mut.

EMSA analysis could also support these findings by determining whether the E-cad WT promoter migrates more slowly when mixed with Kaiso *in vitro*. The E-cad WT promoter fragment would also be expected to associate with a higher ratio of Kaiso than E-cad KBS mut or E-cad CpG mut, assuming that Kaiso binds to the KBS- and methylated CpG sites of the *E-cadherin* promoter *in vitro*.

4.2 *Nuclear p120^{ctn} inhibits Kaiso-mediated transcriptional repression*

4.2.1 Links between p120^{ctn}, Src, Kaiso, and Cancer

It has become clear that malfunction of the catenins correlates with cellular transformation and the metastatic invasive phenotype (80, 89, 145). This may be due to aberrant nuclear signaling events, which alter the expression of genes that regulate cell adhesion and proliferation (12, 23, 49, 122, 132, 142). While nuclear roles for β-catenin in gene expression have been described, there have not been any nuclear roles for p120^{ctn} described to date. However, the interaction between p120^{ctn} and Kaiso (25, 135), as well as the finding that the Kaiso-p120^{ctn} binding site overlaps with the Kaiso-DNA binding site implies a nuclear role for p120^{ctn} in the regulation of gene expression.

EMSA analysis confirmed that p120^{ctn} inhibits both sequence-specific and methylation-dependent Kaiso-DNA binding (**Figure 16A and 16B**), and inhibits Kaiso-mediated transcriptional repression via the 4x KBS (**Figure 17**). The above data indicate that cellular events affecting the Kaiso-p120^{ctn} interaction would also modify the transcriptional properties of Kaiso and affect Kaiso target gene expression. Based on our preliminary data that Kaiso may act as a tumour suppressor (Daniel Lab, unpublished data), disruption of the Kaiso-p120^{ctn} signaling pathway would be expected to affect cellular transformation. Alterations of the Kaiso-p120^{ctn} signaling pathway that promote Kaiso-mediated transcriptional repression (i.e. increases in nuclear Kaiso levels and/or decreases in nuclear p120^{ctn} levels) would be expected to inhibit cellular transformation.

Alternatively, inhibition of Kaiso mediated transcriptional repression (i.e. reduction in nuclear levels of Kaiso and/or increase in the nuclear level of p120^{ctn}) would be expected to enhance tumourigenesis. Decreased membranous and increased nuclear p120^{ctn} staining is observed in human tumours, consistent with the hypothesis that alterations in the Kaiso-p120^{ctn} signaling pathway that reduce Kaiso-mediated repression would lead to cellular transformation (60, 87, 133).

Src phosphorylation of p120^{ctn} correlates with v-Src cellular transformation, and non-oncogenic Src mutants fail to phosphorylate p120^{ctn} (11, 119). While Src phosphorylation of p120^{ctn} correlates with transformation, human tumours also demonstrate reduced membranous/increased nuclear p120^{ctn} immunostaining (60, 80, 87, 133) and p120^{ctn} mutants lacking Src phosphorylation sites fail to enter the nucleus (2). These data imply that Src transformation increases the nuclear pool of p120^{ctn}, although a causal relationship between Src and p120^{ctn} nuclear localization has not yet been definitively established.

Src may thus promote nuclear accumulation of p120^{ctn}, and thereby inhibit the activity of Kaiso, a putative tumour suppressor. This provides a possible mechanism for Src-induced cellular transformation via p120^{ctn} and Kaiso. Further experiments are necessary to investigate the effects of constitutively active Src on p120^{ctn}-inhibition of Kaiso-mediated transcriptional repression and cellular transformation. For example, transfection of non-transformed cell lines with oncogenic forms of Src, followed by detection of the subcellular localization of p120^{ctn} (via immunofluorescence or subcellular fractionation and western blot) would reveal the effects of Src activity on

p120^{ctn} localization. Subsequent analysis of the effects of constitutively active Src on Kaiso-mediated transcriptional repression would elucidate whether Kaiso is downstream of Src in the Src-mediated cellular transformation pathway.

4.2.2 Nuclear p120^{ctn} and the non-canonical Wnt signaling pathway

Co-expression of cytosolically stranded p120^{ctn} (p120^{ctn} NLS Mut), carrying a mutation in the putative NLS (amino acids 622-623 KK-AA), did not inhibit Kaiso-mediated transcriptional repression, while wild-type p120^{ctn} did (**Figure 21**). These data establish that p120^{ctn} must enter the nucleus to inhibit the transcriptional properties of Kaiso, as opposed to p120^{ctn} binding and sequestering Kaiso in the cytoplasm and thus inhibiting Kaiso-mediated transcriptional repression.

Certain cell stimuli (i.e. PKC activity) promote the nuclear translocation of p120^{ctn} (146), but the effects of such stimuli on Kaiso-mediated transcriptional repression remain to be determined. Interestingly, while cytosolic p120^{ctn} activates Cdc42 (a downstream effector of non-canonical Wnt signaling), nuclear p120^{ctn} may inhibit Kaiso-mediated repression of non-canonical Wnt signaling (**Figure 7**). This putative Kaiso-p120^{ctn} signaling pathway predicts that p120^{ctn} may both directly (via Cdc42) and indirectly (by inhibiting Kaiso) activate non-canonical Wnt signaling.

To test the hypothesis that PKC activation results in p120^{ctn}-mediated inhibition of Kaiso-mediated transcriptional repression, PKC activators could be used in artificial promoter assays. Activation of PKC with **P**horbol 12-**m**yristate 13-**a**cetate (PMA) would be expected to promote nuclear accumulation of p120^{ctn} and inhibit Kaiso-mediated

transcriptional repression in artificial reporter assays. Activation of PKC, which acts as a central effector of non-canonical Wnt signaling by acting upstream of Cdc42, JNK, and CamKII (70, 104, 107), would be expected to indirectly inhibit Kaiso-mediated transcriptional repression. Therefore, activation of PKC would result in increased Wnt-11 expression, forming a positive feedback loop in which Wnt-11 activates PKC, which maintains increased Wnt-11 expression (**Figure 7**).

4.2.3 Kaiso, p120^{ctn}, and the branching phenotype

Interestingly, while activation of Cdc42 or Rac have been associated with the formation of filopodia (93) or lamellipodia/membrane ruffles, respectively (120), overexpression of p120^{ctn} results in the "branching phenotype" characterized by an abundance of membrane protrusions (117). Furthermore, nuclear translocation of p120^{ctn} is required for p120^{ctn} to induce the branching (2, 61), and p120^{ctn} must enter the nucleus to inhibit Kaiso-mediated transcriptional repression (**Figure 21**). It follows that Kaiso may therefore be involved in mediating the p120^{ctn}-induced branching phenotype.

Indeed, Kaiso is involved in negatively regulating the non-canonical Wnt signaling pathway, which promotes morphogenetic cell movements and activation of Rho-family GTPases involved in the extension of cellular processes (62, 93, 120). p120^{ctn} could thus induce the branching phenotype by entering the nucleus and inhibiting Kaiso from negatively regulating a signaling pathway (Wnt-11 pathway) that promotes the formation of cellular process extensions.

This theory could be experimentally investigated by overexpressing Kaiso and determining whether this inhibits the p120^{ctn}-induced branching phenotype. Expression of an NLS-fused p120^{ctn} mutant that cannot interact with Kaiso (NLS-ΔR3-11) would not be expected to induce a branching phenotype, assuming that Kaiso was involved in the induction of this activity. Conversely, siRNA directed against Kaiso would be expected to induce the branching phenotype if Kaiso does act downstream of p120^{ctn} to induce the branching phenotype. Such experiments could be performed in the future to investigate the role of Kaiso in regulating cellular process extension, and provide a link between Kaiso, p120^{ctn}, and the branching phenotype.

4.3 Kaiso is an HDAC-dependent transcriptional repressor

4.3.1 Mechanistic comparison of sequence-specific and methylation-dependent Kaiso-mediated transcriptional repression

Kaiso is a bi-modal DNA-binding protein which recognizes both sequence-specific KBS sites and methylation-dependent sites (27). Kaiso also represses transcription via the KBS in an HDAC-dependent manner (**Figure 23, 24, and 25**). However, methylation-dependent Kaiso-mediated transcriptional repression was not blocked by the pan-specific HDAC inhibitor TSA (112), indicating that Kaiso represses transcription via methylation-dependent *cis*-elements through a distinct, HDAC-independent mechanism. Together these data suggest that Kaiso acts as a bi-modal

transcriptional repressor that may act via two distinct pathways. The first pathway involves sequence-specific HDAC-dependent transcriptional repression in which Kaiso may silence β-catenin-responsive target genes such as *c-myc*, *cyclinD1*, *Id2*, and *matrilysin*. Kaiso-mediated repression of this pathway would implicate Kaiso as a tumour suppressor. This hypothesis is supported by a human cancer-profiling array that demonstrated that Kaiso mRNA is downregulated in 50% of ovarian tumours and 32% of breast cancer tumours, relative to neighboring untransformed tissue (Daniel Lab, unpublished data).

Conversely, a second, HDAC-independent pathway, involving methylation-dependent Kaiso-mediated transcriptional repression would result in the silencing of anti-tumourigenic genes including *Rb* (tumour suppressor), *Pgk* (anti-angiogenic), and *Xist* (expression correlates with successful taxol treatment and is upregulated by the tumour suppressor BRCA1) (17, 43, 53, 71). Kaiso-mediated transcriptional repression of this pathway would implicate Kaiso as an oncogene. Support for this hypothesis comes from preliminary studies using the MCF-7 mammary adenocarcinoma cell line. In a more aggressively growing subpopulation of MCF-7 cells (MCF-7-V) levels of Kaiso are highly elevated (Daniel Lab, unpublished data), providing a correlation between Kaiso expression and a more transformed phenotype. However, a better understanding of these data and the role of methylation-specific and sequence-specific transcriptional regulation are necessary before such conclusions can be definitively drawn.

Previous data suggests that $p120^{ctn}$ can inhibit sequence-specific and methylation-dependent Kaiso-DNA binding *in vitro* (27). However, a role for $p120^{ctn}$ in regulating

Kaiso-mediated transcriptional repression has only been verified at the transcriptional level in the context of the KBS (**Figure 17**). p120^{ctn} could therefore hypothetically inhibit sequence-specific Kaiso-DNA binding, but not methylation-dependent DNA-binding. Indeed, preliminary data failed to demonstrate a convincing role for p120^{ctn} in the inhibition of methylation-dependent Kaiso-mediated transcriptional repression (Egor Prokhortchouk, personal communication). In this case, the possibility exists that Kaiso acts as a strong sequence-specific tumour suppressor (since Kaiso binds KBS DNA with a higher affinity than methylated DNA), which is inhibited by p120^{ctn} (**Figure 29**).

Furthermore, Kaiso may act as a weak methylation–dependent oncogene. Therefore, in the absence of p120^{ctn}, the appropriate proto-oncogenes are "turned off" and the cell does not become transformed. In the presence of p120^{ctn} the Kaiso-mediated tumour suppressor pathway becomes inhibited, and by "default", Kaiso promotes cellular transformation by repressing methylation-dependent genes (*Rb*, *Pgk*, and *Xist*). This theory is consistent with the finding that nuclear p120^{ctn} is pronounced in transformed cells (60, 87, 133).

While such a dual-role for a single transcription factor in controlling tumourigenesis has never been reported, current data suggest such a pathway as a possibility. Further studies to investigate the biological consequences of the bi-modal nature of Kaiso-mediated transcriptional regulation are required before such conclusions can be more definitively drawn. Regardless, Kaiso is currently the only known POZ-ZF transcription factor, which represses two distinct subsets of target genes in either a sequence-specific or a methylation-dependent manner.

Hypothetical Role of Kaiso in Cellular Transformation

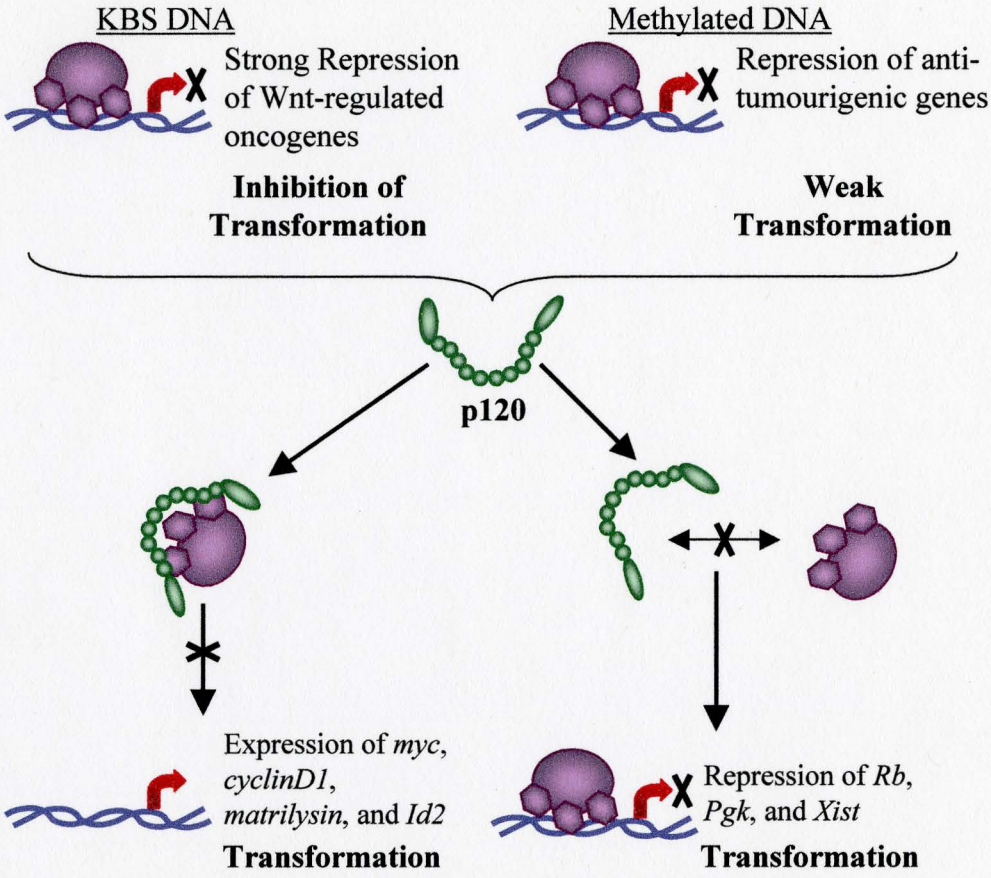


Figure 29: *Hypothetical role of Kaiso in cellular transformation.* Kaiso acts as a strong sequence-specific tumour suppressor by inhibiting expression of Wnt-regulated oncogenes (*left*). Kaiso also acts as a weak oncogene by suppressing expression of anti-tumourigenic genes (*right*). Under normal conditions, Kaiso represses cellular transformation. However, in the presence of p120, the tumour suppressive properties of Kaiso are inhibited. Alternatively, excessive DNA methylation (which correlates with tumourigenesis) could promote Kaiso-mediated repression of anti-tumourigenic genes and result in cellular transformation.

4.3.2 Comparison of mSin3A-, NuRD-, and Kaiso-HDAC complexes

Kaiso has been characterized as an HDAC-dependent transcriptional repressor. Currently, two major HDAC complexes have been extensively studied and are recruited by HDAC-dependent transcriptional repressors. The first is the Sin3A complex, which associates with several unliganded nuclear repressors (5). This complex operates in a modular fashion in which the transcription factor (i.e. Kaiso) provides the DNA-binding domain and recruits the Sin3A complex (HDAC-1, HDAC-2, Sin3, RbAp46, RbAp48, SAP18, and SAP30)(165), which deacetylates histones and represses transcription. The second prominent HDAC complex, the so-called **Nu**cleosome **r**emodeling and histone **d**eacetylase complex (NuRD), consists of CHD4 (or Mi-2), HDAC-1, HDAC-2, MTA-2, RbAp46, RbAp 48, and MBD3 (126, 148). The NuRD complex is believed to associate with methylated and non-methylated sequences in order to repress transcription in a deacetylase dependent manner. While the specific roles and differences between the NuRD and Sin3 HDAC complexes remains unclear, both complexes have been shown to be recruited by several transcription factors in order to silence gene transcription.

Kaiso associates with mSin3A (**Figure 23**), as well as NCoR (159), suggesting that Kaiso may recruit the Sin3 HDAC complex in order to silence gene transcription. In this case, we would expect Kaiso to co-immunoprecipitate with HDAC-1 or HDAC-2. However, repeated experiments failed to detect a Kaiso-HDAC-1/HDAC-2 interaction (Daniel Lab, unpublished data). Kaiso also does not interact with the Sin3A/NuRD integral histone-binding proteins **R**etino**b**lastoma-**a**ssociated **p**rotein **46** and **48** (RbAp46 and RbAp48) (159), suggesting that Kaiso does not associate with classical NuRD or Sin3A complexes, but rather a non-classical Sin3A-containing complex. In support of this, Kaiso complexes with HDAC-3 (a class I HDAC, with homology to HDAC-1/2 and the yeast RPD3 deacetylase) and the integral histone-binding proteins **T**ransducin (**b**eta)-like 1 (TBL1) and **TBL r**elated protein **1** (TBLR1) (159).

Therefore, Sin3A, NCoR and HDACs may comprise a larger, heterogeneous family of macromolecular transcriptionally repressive complexes than are currently recognized (i.e. classical Sin3A and NuRD complexes are not the only HDAC complexes in the nucleus). Moreover, Kaiso represses transcription of the MTA2 locus (159), which is a central member of the NuRD complex primarily responsible for activating HDACs in the NuRD complex (160). Through the silencing of MTA2 expression, Kaiso may diminish NuRD activity. This could lead to a large-scale shift in gene expression control during development and/or tumourigenesis by altering which multiprotein HDAC complexes are available in the nucleus to silence large subsets of genes (i.e. shifting the primary source of HDAC1 and HDAC2 activity from NCoR to Sin3A).

4.4 Kaiso represses transcription of the –296 matrilysin promoter element

4.4.1 Comparison of the –296 Hmat pro and 4xKBS cis-elements

Kaiso is a bi-modal HDAC-dependent transcriptional repressor that binds to KBS elements found in both the human and murine *matrilysin* promoter (27). Both human and murine KBS elements demonstrate perfect conservation of the core TCCTGCNA Kaiso-binding sequence between humans and mice, implying functional significance (**Figure 8A and 8B**). The spatial relationship between the KBS and the E26 transformation specific domain protein (ETS) binding site is also conserved, supporting the hypothesis that the KBS sites in the *matrilysin* promoter are functionally relevant. This also implies that Kaiso may regulate *matrilysin* expression.

Artificial promoter assays using the –296 Hmat pro fragment of the human *matrilysin* promoter verified that Kaiso could repress transcription via this *cis*-element (**Figure 26**). Further analysis confirmed that Kaiso represses transcription via the –296 Hmat pro in an HDAC-dependent manner (**Figure 28**), and that nuclear $p120^{ctn}$ could inhibit this transcriptional repression (**Figure 26**). Interestingly, while the 4xKBS acted as a potently repressive *cis*-element (compare pGL3 and 4xKBS, **Figure 10**), the –296 Hmat pro fragment did not act as a potent inhibitory *cis*-element (compare pGL3 and Hmat –296, **Figure 26**). This may be explained by the fact that the 4xKBS *cis*-element presumably only associates with the transcriptionally repressive Kaiso-HDAC complex,

while the –296 Hmat pro element associates with the Kaiso-HDAC complex as well as several activators of transcription (TBP, ETS, AP-1, and β-catenin-Tcf/LEF, **see Figure 8A and 8B**). Therefore, the 4xKBS acts as an exclusively repressive element, resulting in a dramatic reduction in luciferase expression, while the –296 Hmat pro acts as a primarily activating element, resulting in a high level of expression, similar to the pGL3 Control backbone vector alone.

Analysis of the –296 Hmat pro *cis*-element also demonstrated that $p120^{ctn}$ inhibits Kaiso-mediated transcriptional repression via the -296 Hmat pro by only approximately 50% (**Figure 26**). One possible explanation of these observations is that $p120^{ctn}$ inhibits Kaiso-co-repressor association *in vivo*, but not Kaiso-DNA binding *in vivo*.

In support of this, while $p120^{ctn}$ associates with Kaiso *in vivo*, it does not appear to associate with mSin3A (**Figure 23A and 23B**). Kaiso-$p120^{ctn}$ and Kaiso-mSin3A complexes may therefore be mutually exclusive, supporting the possibility that $p120^{ctn}$ may inhibit Kaiso-corepressor association *in vivo*, rather than Kaiso-DNA binding to diminish Kaiso-mediated transcriptional repression. Based on this assumption, $p120^{ctn}$ may inhibit HDAC-dependent Kaiso-mediated transcriptional repression via the -296 Hmat pro. Kaiso, however, could still bind to the KBS element of the *matrilysin* promoter (**Figure 30**), and block ETS association with the *matrilysin* promoter. Consequently, Kaiso could "repress" transcription via the *matrilysin* promoter by blocking ETS-mediated activation of the -296 Hmat pro element.

Model for Kaiso-mediated Repression via the –296 Hmat pro

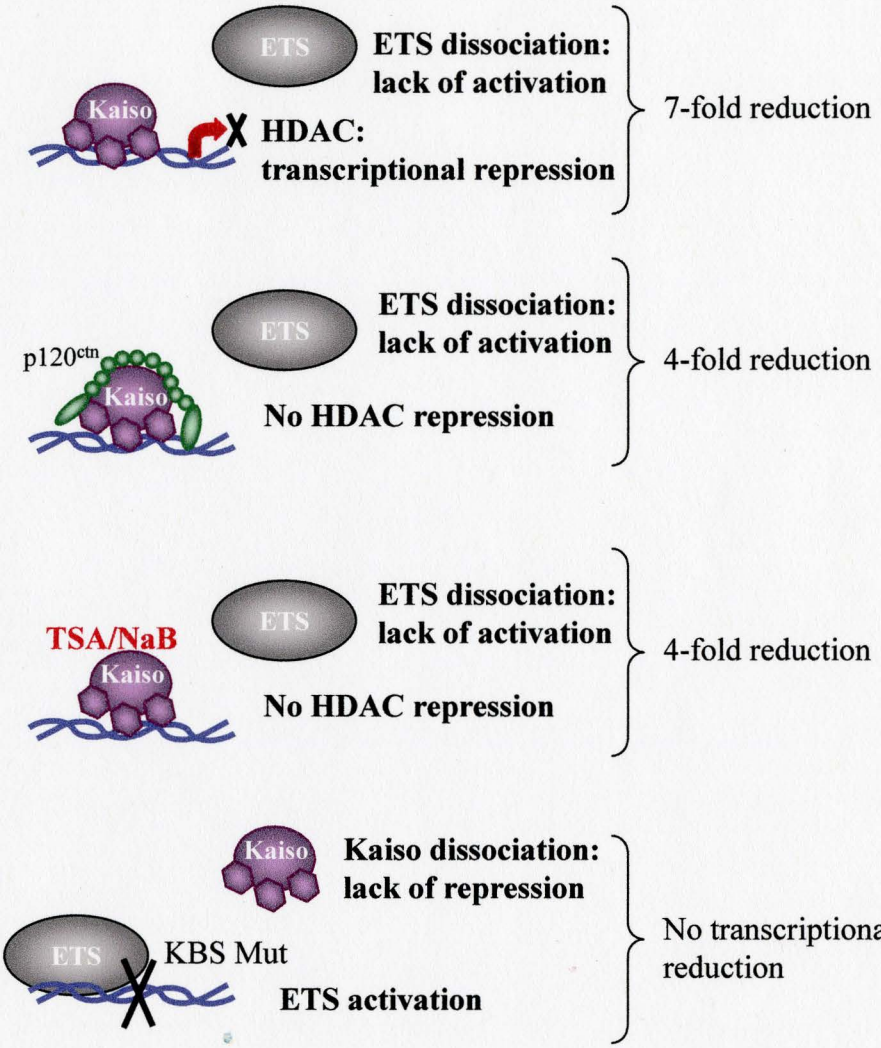


Figure 30: ***Model for Kaiso-mediated transcriptional repression via the –296 Hmat pro.*** p120^{ctn} may inhibit Kaiso-HDAC association but not Kaiso-DNA association *in vivo*. Thus, p120^{ctn} , TSA, and NaB would alleviate Kaiso-HDAC repression, but Kaiso may still hinder ETS-DNA association and inhibit activation of the *matrilysin* promoter.

While the effects of p120^{ctn} on Kaiso-DNA binding *in vivo* are not currently known, evidence presented here supports the hypothesis that p120^{ctn} inhibits Kaiso-co-repressor association but not Kaiso-DNA association *in vivo*. HDAC inhibitors, for example, presumably do not disrupt the Kaiso-DNA association can only de-repress transcription of the -296 Hmat pro by 50% (**Figure 28**). It is possible that while TSA and NaB block HDAC-dependent Kaiso-mediated transcriptional repression via the –296 Hmat pro *cis*-element, Kaiso still "represses" transcription by blocking ETS association with the promoter. In further support of this hypothesis, mutation of the KBS element (which disrupts association of Kaiso and the *matrilysin* promoter fragment) fully de-represses the -296 Hmat pro (**Figure 27**). This model could be further experimentally validated by determining whether increasing amounts of ETS and p120^{ctn} could completely abolish Kaiso-mediated transcriptional repression. Alternatively, HDAC inhibitors and/or p120^{ctn} would be expected to completely diminish Kaiso-mediated transcriptional repression of a –296 Hmat pro *cis*-element carrying a mutation in the ETS-binding site that overlaps with the KBS.

Analysis of the –296 Hmat pro also revealed that HDAC inhibition via TSA or NaB did not significantly de-repress expression via the –296 Hmat pro construct in the absence of exogenous Kaiso (**Figure 28**), which is consistent with the fact that to date no known HDAC-dependent transcriptional repressors (other than Kaiso) associate with the –296 Hmat pro fragment (**Figure 8A and 8B**). Analysis of the effects of HDAC inhibitors on the –296 Hmat pro verified that Kaiso specifically represses transcription of the *matrilysin* promoter via an HDAC-dependent manner. This finding further supports

the hypothesis that Kaiso may act in an antagonistic manner to the β-catenin-Tcf/LEF pathway by repressing transcription of genes activated by the canonical Wnt signaling pathway. Future studies utilizing constitutively active β-catenin mutants and the –296 Hmat pro promoter in conjunction with Kaiso could be performed to validate this hypothesis. In addition, qRT-PCR experiments assaying the level of *matrilysin* mRNA in the stable Kaiso-overexpressing and Kaiso-null stable cell lines created in our lab should facilitate the elucidation of *matrilysin* as a *bona fide* Kaiso target gene. Lastly, a **ch**romosomal **i**mmuno**p**recipitation (ChIP) using Kaiso and primers specific for the *matrilysin* promoter is currently underway, which will further validate *matrilysin* as a Kaiso target gene.

4.4.2 Functional implications of Kaiso-mediated transcriptional repression of the matrilysin promoter

In addition to the *matrilysin* promoter, the β-globin promoter also contains a KBS element approximately 60 nucleotides upstream of a CTCF binding site (Pierre DeFossez, personal communication). This indicates that Kaiso may also repress transcription of the *β-globin* gene, both implicating *β-globin* as a target gene and supporting the hypothesis that Kaiso may functionally associate with CTCF and regulate gene transcription. Thus, protein-protein interactions (Kaiso-CTCF and Kaiso-mSin3A) and DNA-sequence analysis (the *β-globin* promoter) imply that Kaiso and CTCF functionally associate in a transcriptionally repressive complex.

Interestingly, both CTCF and the *β-globin* promoter associate with nuclear **m**atrix **a**ssociated **r**egions (MARs), which are known to contain HDACs and other transcriptional co-repressors (34, 99, 164). It would therefore be of interest to determine whether Kaiso associates with MARs, as this would provide a greater mechanistic understanding of Kaiso-mediated transcriptional repression. Other POZ-ZF transcriptional repressors (i.e. PLZF) associate with the nuclear matrix, and cellular events which disrupt PLZF-MAR association abrogate transcriptional repression in a dominant negative manner (85).

To determine whether Kaiso is a MAR-associated protein, Triton X-100 soluble (free nuclear proteins) and insoluble (MAR-associated proteins) fractions could be immunoblotted for Kaiso protein. Cross-linking experiments using cisplatin (which preferentially cross-links MAR associated proteins to DNA) would be expected to cause Kaiso to precipitate with DNA-bound fractions of the nucleus if Kaiso is a MAR-associated protein. Furthermore, $p120^{ctn}$ (which inhibits Kaiso-mediated transcriptional repression) may inhibit the ability of Kaiso to localize to MARs. This hypothesis could be experimentally addressed by performing cisplatin cross-linking experiments and immunoblotting Triton X-100 fractions in the presence or absence of exogenous $p120^{ctn}$. These experiments would reveal whether Kaiso is indeed a MAR-associated protein, and would provide a more in-depth understanding of the effects of $p120^{ctn}$ on Kaiso-mediated transcriptional repression and the Kaiso-CTCF interaction.

MAR-associated proteins interact not only with HDAC(s) and HDAC co-repressors, but also with nuclear matrix proteins (82, 151). Atrophin-1 is a nuclear

matrix protein encoded by identified in a yeast-two-hybrid screen for interaction partners of the HDAC-dependent transcriptional repressor t(8;21) **e**ight **t**wenty-**o**ne (ETO-1) (151). Co-expression of ETO-1 and atrophin-1 in artificial promoter assays confirmed that atrophin-1 enhances HDAC-dependent transcriptional repression via ETO-1. Atrophin-1 may act as a docking platform for ETO-1 and HDAC transcriptional machinery, thus bringing ETO-1 and the HDAC complex together in the nuclear matrix, and facilitating transcriptional repression (151).

More recently, a Kaiso yeast-two-hybrid screen has been performed that identified **c**ollagen **t**riple **h**elix **r**epeat **c**ontaining protein **1** (Cthrc-1) as a Kaiso-interaction partner (Daniel Lab, unpublished data). This protein is predicted to possess a collagen triple-helix domain (characteristic of structural matrix proteins), and both C- and N-terminal domains of unknown function (GenBank accession number XM_128002). Yeast-two-hybrid analysis mapped the interaction with Kaiso interacts to the C-terminal domain of Cthrc-1. Therefore, it is tempting to hypothesize that Cthrc-1 may be a novel nuclear matrix protein that interacts with Kaiso and enhances Kaiso-mediated transcriptional repression. The collagen triple-helix domain may insert into the nuclear matrix, while the C-terminal domain acts as a docking platform for Kaiso and its associated repression machinery. To test this hypothesis, Cthrc-1 could be expressed in mammalian cells and detected by immunofluorescence to determine its subcellular localization. If Cthrc-1 localizes to the nucleus, Cthrc-1 and Cthrc-1 ΔC-term could be overexpressed in artificial promoter assays to determine whether it influences Kaiso-mediated transcriptional repression.

5. *Summary and Conclusion of Data*

Conclusion: Kaiso is an HDAC-dependent transcriptional repressor that is negatively regulated by p120^{ctn}

Kaiso acts as a sequence-specific transcriptional repressor of KBS-derived *cis*-elements (**Figure 10 and 26**). Kaiso-mediated transcriptional repression is sensitive to HDAC inhibitors (**Figure 25**) and Kaiso interacts with mSin3A (**Figure 23**). Hence, this report establishes that Kaiso is a sequence-specific HDAC-dependent transcriptional repressor. The finding that p120^{ctn} inhibits Kaiso-DNA binding *in vitro* (**Figure 16A and 16B**) and inhibits Kaiso-mediated transcriptional repression (**Figure 17**) provides the first evidence for the involvement of the non-classical catenin and adhesion co-factor p120^{ctn} in the regulation of gene expression. The identification of *bona fide* Kaiso target genes will greatly facilitate the understanding of the nature of the Kaiso-p120^{ctn} signaling pathway. However, many putative target genes identified on the basis of the presence of a KBS element in their promoter are also β-catenin/Wnt target genes (*matrilysin*, *cyclinD1*, *c-myc*, and *Id2*). This implies a connection between Kaiso and Wnt signaling. Indeed, p120^{ctn} and Kaiso may be part of a Wnt-11 positive feedback loop in which Wnt-11 activation may induce p120^{ctn} to inhibit Kaiso from repressing Wnt-11 signaling (**Figure 7**). While the biological consequences of Kaiso-mediated transcriptional regulation remain to be determined, this study provides a fundamental understanding of the properties of Kaiso-mediated transcriptional regulation and establishes Kaiso as a p120^{ctn}-regulated, HDAC-dependent transcriptional repressor.

Appendix A: Data Summary Tables

Table A-1: Summary of Results- Kaiso-Mediated Transcriptional Repression of the 4xKBS

Experiment	Trial Number	Result in Luciferase Expression
Exogenous Kaiso Repression	1	2-fold decrease
	2	2-fold decrease
	3	2.5-fold decrease
	4	2.5-fold decrease
	5	2.5-fold decrease
Mutation of the KBS: Fold De-repression	1	2-fold increase
	2	2.5-fold increase
	3	2.5-fold increase
	4	2-fold increase
	5	2-fold increase
Repression of the 1xKBS/2xKBS	1	2/1.5-fold decrease
	2	2/2-fold decrease
	3	1.5/2-fold decrease
	4	2/2-fold decrease
	5	2/2.5-fold decrease

Table A-1: Summary of Results- Kaiso-Mediated Transcriptional Repression of the 4xKBS (continued)

Experiment	Trial Number	Result in Luciferase Expression
Optimal Dose for Repression	1	600 ng Kaiso
	2	600/800 ng Kaiso
	3	800 ng Kaiso
	4	600/800 ng Kaiso
	5	800 ng Kaiso
Antisense De-repression of KBS	1	0.5-fold increase
	2	0.5-fold increase
	3	1.5-fold increase
	4	1-fold increase
	5	0.5-fold increase
pSilencer De-repression of KBS	1	1-fold increase
	2	1.5-fold increase
	3	1-fold increase
	4	1.5-fold increase
	5	1.5-fold increase

Table A-2: Summary of Results- HDAC Inhibitors on the 4xKBS

Experiment	Trial Number	Inhibition of Kaiso Repression
TSA Inhibition of Kaiso Repression	1	90%
	2	90%
	3	85%
	4	90%
	5	80%
NaB Inhibition of Kaiso Repression	1	60%
	2	80%
	3	70%
	4	80%
	5	70%

Table A-3: Summary of Results- p120^{ctn} Inhibition of Kaiso-Mediated Transcriptional Repression

Experiment	Trial Number	Result in Luciferase Expression
p120^{ctn} Inhibition of Kaiso Repression	1	2-fold increase
	2	1.5-fold increase
	3	2-fold increase
	4	2-fold increase
	5	2-fold increase
Optimal p120^{ctn} Dose for De-repression	1	300 ng p120
	2	300 ng p120
	3	300 ng p120
	4	300 ng p120
	5	400 ng p120
ΔR3-11 inhibition of Kaiso Repression	1	N/A*
	2	N/A*
	3	N/A*
	4	N/A*
	5	N/A*

Table A-3: Summary of Results- p120^{ctn} Inhibition of Kaiso-Mediated Transcriptional Repression (continued)

Experiment	Trial Number	Result in Luciferase Expression
3T3 Fibroblasts: p120^{ctn} Inhibition of Kaiso Repression	1	N/A*
	2	N/A*
	3	N/A*
	4	N/A*
	5	N/A*
p120^{ctn} NLS Inhibition of Kaiso Repression	1	N/A*
	2	N/A*
	3	N/A*
	4	N/A*
	5	N/A*

***Note:** N/A refers to the consistent failure of p120^{ctn} mutants (ΔR3-11 and NLS mut) in Cos-1 cells and wild-type p120^{ctn} in 3T3 cells to interfere with Kaiso-mediated transcriptional repression. These results are the anticipated outcome, consistent with the hypothesis that nuclear p120^{ctn} must interact with Kaiso in order to diminish transcriptional repression.

Table A-4: Summary of Results- Human matrilysin -296 Promoter

Experiment	Trial Number	Result in Luciferase Expression
Exogenous Kaiso Repression	1	7-fold decrease
	2	7.5-fold decrease
	3	6-fold decrease
	4	7-fold decrease
	5	6.5-fold decrease
p120^{ctn} De-repression	1	3-fold (42%) inhibition of repression
	2	4-fold (53%) inhibition of repression
	3	3-fold (50%) inhibition of repression
	4	3.5-fold (50%) inhibition of repression
	5	4-fold (62%) inhibition of repression
p120^{ctn} NLS Mut	1	N/A*
	2	N/A*
	3	N/A*
	4	N/A*
	5	N/A*

Table A-4: Summary of Results- Human matrilysin -296 Promoter (continued)

Experiment	Trial Number	Result in Luciferase Expression
ΔR3-11 De-repression	1	N/A*
	2	N/A*
	3	N/A*
	4	N/A*
	5	N/A*
Endogenous Kaiso Repression	1	N/A*
	2	N/A*
	3	N/A*
	4	N/A*
	5	N/A*
KBS Site Mutation	1	7-fold increase
	2	7.5-fold increase
	3	6-fold increase
	4	7-fold increase
	5	6.5-fold increase

***Note:** N/A refers to the consistent failure of $p120^{ctn}$ mutants (ΔR3-11 and NLS mut) in Cos-1 cells to interfere with Kaiso-mediated transcriptional repression. Furthermore, endogenous Kaiso does not appear to repress transcription via the -296 Hmat pro.

Table A-5: Summary of Results- Effect of HDAC Inhibitors on the Human matrilysin -296 Promoter

Experiment	Trial Number	Inhibition of Kaiso Repression
TSA Inhibition of Kaiso Repression	1	90%
	2	90%
	3	85%
	4	90%
	5	80%
NaB Inhibition of Kaiso Repression	1	60%
	2	80%
	3	70%
	4	80%
	5	70%

Appendix B: Supplementary Data

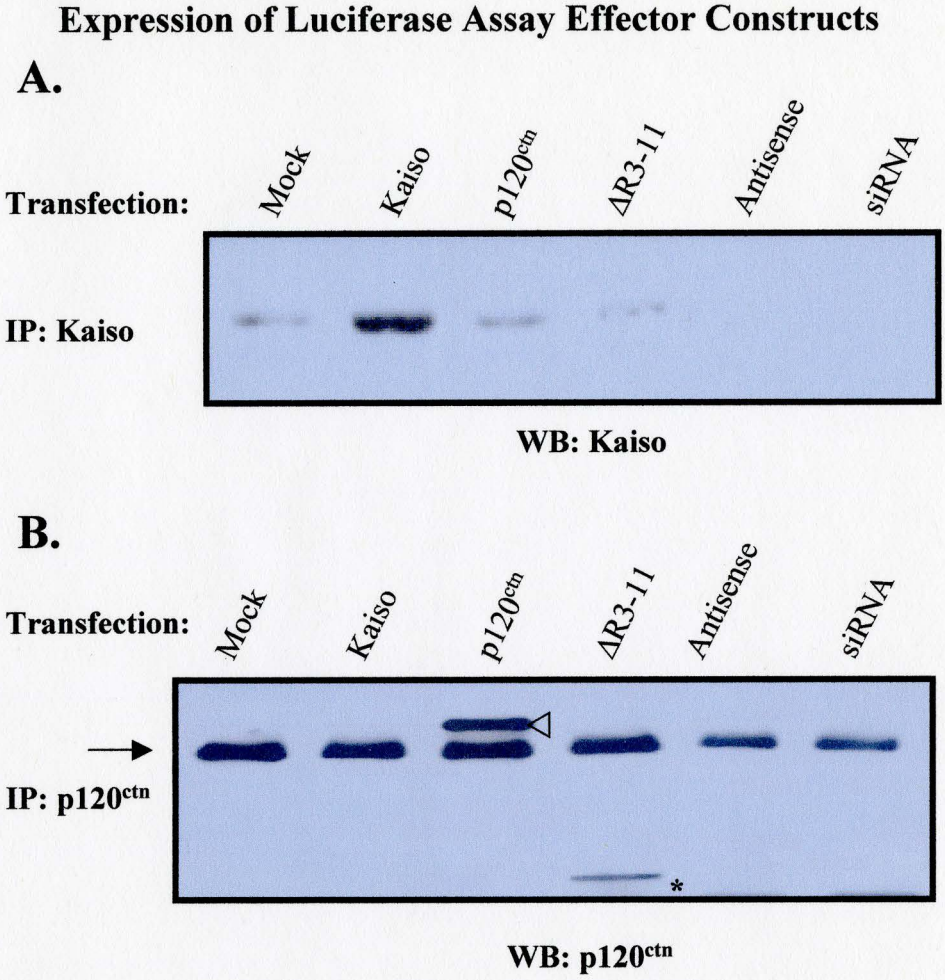


Figure B-1: *Expression of luciferase assay reporter constructs in Cos-1 cells.* (A) Both antisense and siRNA treatment dramatically reduced levels of Kaiso protein, while pCDNA3 Kaiso increased levels of Kaiso protein. (B) While endogenous p120^{ctn} levels remained constant (arrow), exogenous p120^{ctn} (open arrowhead) and ΔR3-11 (asterisk) expression was confirmed.

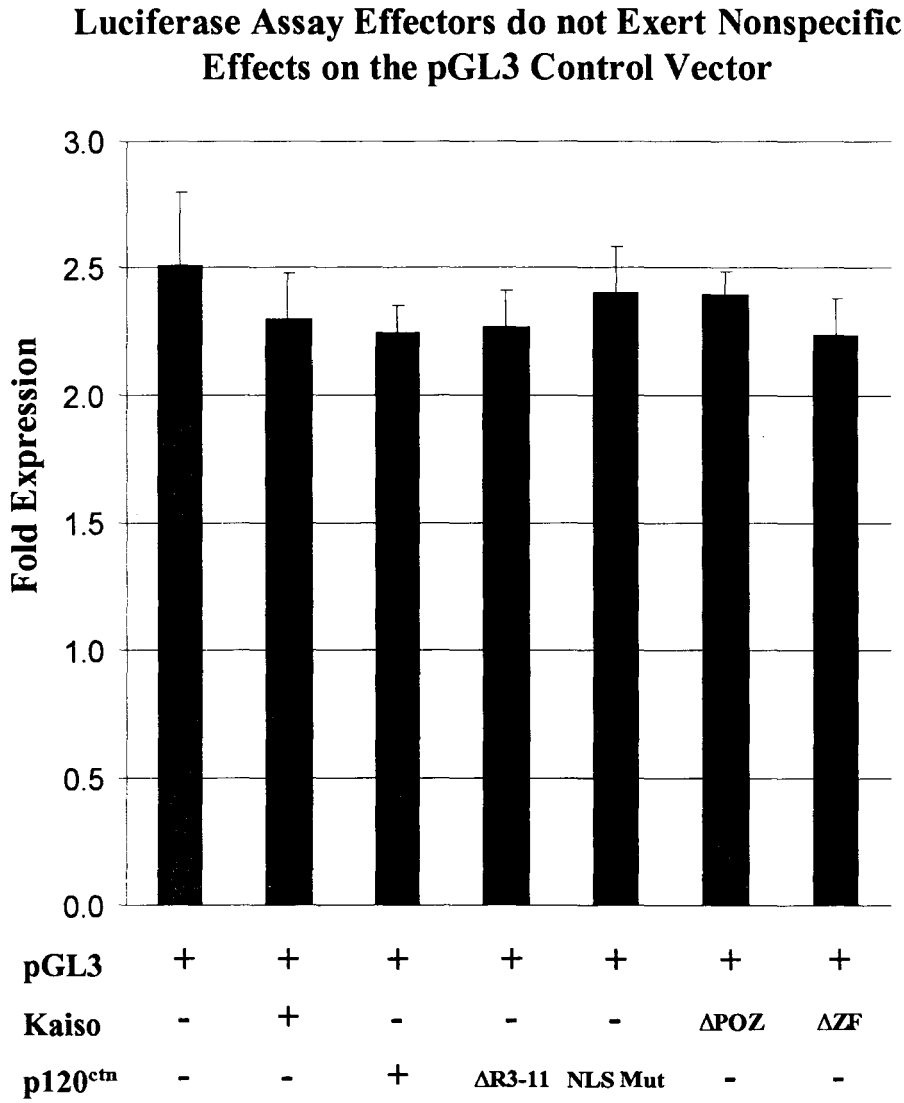


Figure B-2: *p120^{ctn} and Kaiso effectors do not affect pGL3 Control expression levels.* All Kaiso and p120^{ctn} deletion mutants were co-expressed with pGL3 Control. None of the experimental conditions used significantly affected the expression levels of pGL3 Control.

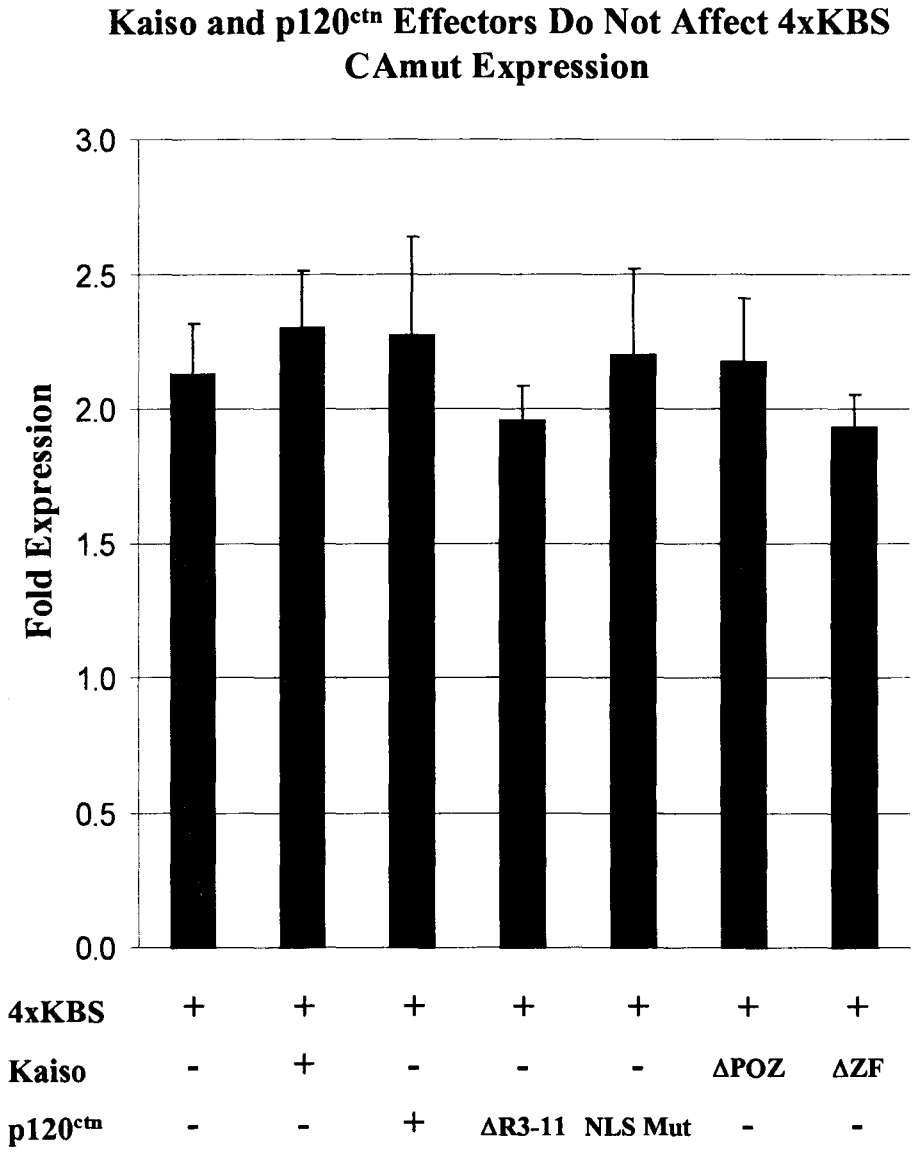
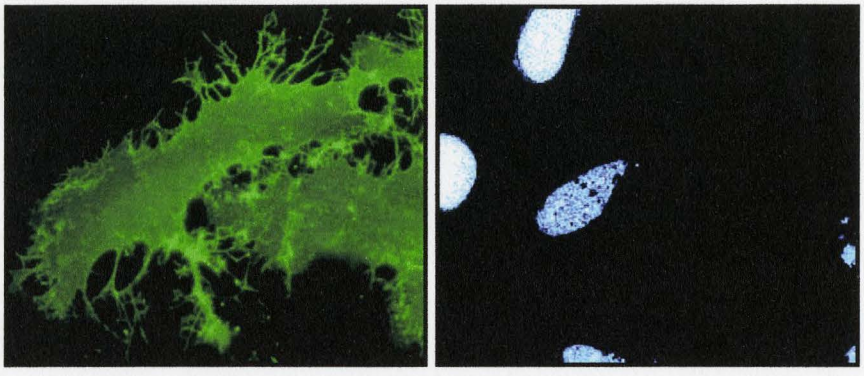


Figure B-3:*Kaiso and p120^{ctn} constructs act specifically on the 4xKBS element.*. Co-transfection of Kaiso and p120^{ctn} effectors with the 4xKBS CAmut reporter did not significantly affect luciferase expression levels. This confirms that the trends observed are specific to the 4xKBS *cis*-element.

Nuclear Localization of GFP-p120^{ctn}

GFP-p120 WT



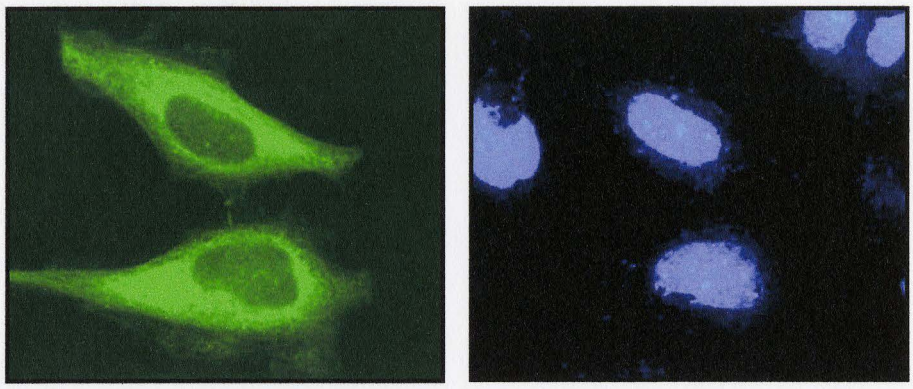
GFP **Hoechst**

Cell Line: HeLa

Figure B-4: *p120^{ctn} translocates to the nucleus of HeLa cells.* **G**reen **f**luorescent **p**rotein (GFP)-fused p120^{ctn} was transfected into HeLa cells and visualized via immunofluorescent microscopy. GFP-p120^{ctn} partially co-localizes with Hoechst stained nuclei.

Nuclear Localization of the GFP-p120^{ctn} NLS Mutant

GFP-p120^{ctn} NLS mut



GFP **Hoechst**

Cell Line: HeLa

Figure B-5: *p120^{ctn} NLS mut does not translocate to the nucleus of HeLa cells.* GFP-fused NLS mut p120^{ctn} was transfected into HeLa cells and visualized via immunofluorescent microscopy. NLS mut p120^{ctn} does not co-localize with Hoechst stained nuclei.

Kaiso Binds to the *CyclinD1* Probe

Probe: ***cyclinD1***

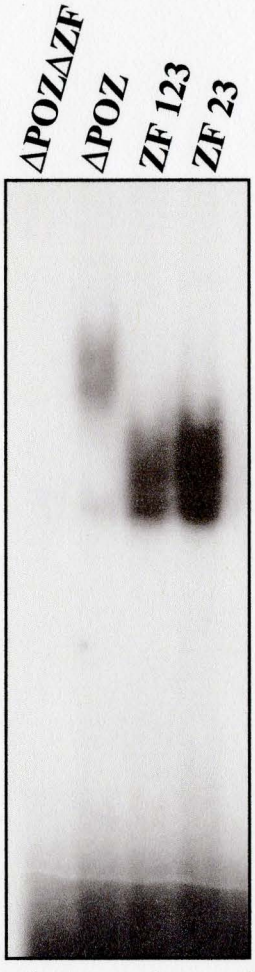


Figure B-6: *Kaiso binds to an oligonucleotide probe derived from the cyclinD1 promoter.* Kaiso-GST fusion proteins ΔPOZ, ZF 123, and ZF 23 bind to a *cyclinD1* probe derived from a region in the *cyclinD1* promoter containing the KBD site, while ΔPOZ ΔZF does not.

Human *E-cadherin* Promoter

GGCCTGCTAG CTCAGTGCTC CATGGCTCAC ACCTGAAATC
CTAGCACTTT GGGAGGCCAA GGCAGGAGGA TCGCTTCAGC
CCAGGAGTTC GAGACCAGGC TGGGCAATAC AGGGAGACAG
CGCCCCCACT GCCCCTGTCC GCCCCGACTT GTCTCTCTAC
AAAAAGGCAA AAGAAAAAAA AAATTAGCCT GGCGTGGTGG
TGTGCACCTG TACTCCCAGC TACTAGAGAG GCTGGGGCCA
GAGGACCGCTT GAGCCCAGGA GTTCGAGGCT GCAGTGGCTG
TGAATCGCAC CACTGCACTC CAGCTTGGGT GAAAGAGTGA
GCCCCATCTC CAAAACGAAC AAACAAAAAT CCCAAAAAAC
AGAACTCAGC CAAGTGTAAA AGCCCTTTCT GATCCCAGGT
CTTAGTGAGC CACCGGCGGG GATGGGATTC GAACCCAGTG
GAATCAGAAC CGTCGAGGTC CCAATAACCC ACCTAGGACC
CTAGCAACTC AGGTAGAGGG TCACCGCCGT CTATGCGAGG
CGGGGTGGGC GGGCCGTCAG CTCCGCCCTG GGGAGGGGTC
CGCGCTGCTG GGCTGTGGCC GGCAGGTGAA CCCTCAGCCA
ATCAGCGGTA CGGGGGGGCG GTGCACCGGG GGTCACCTGG
CTGCAGCCAC GCACCCCCTC TCAGTGGCGT CGGACTGCAA
AAGCACCTGT GAGCTTGCGG AAGTCAGTTC AGACTCCAGC
CCGCTCCAGC CCGGCCCGAC CCGACCGCAC CCGGCGCCTG
CCCTGCTCGG CGTCCCCGGCC AGCATGGGCC

Minimal KBS site: CTGCNA
Minimal CpG site: $MGN_{(0\text{-}3)}MGN_{(3\text{-}8)}MG$ } can delete 1st or 3rd **MG**

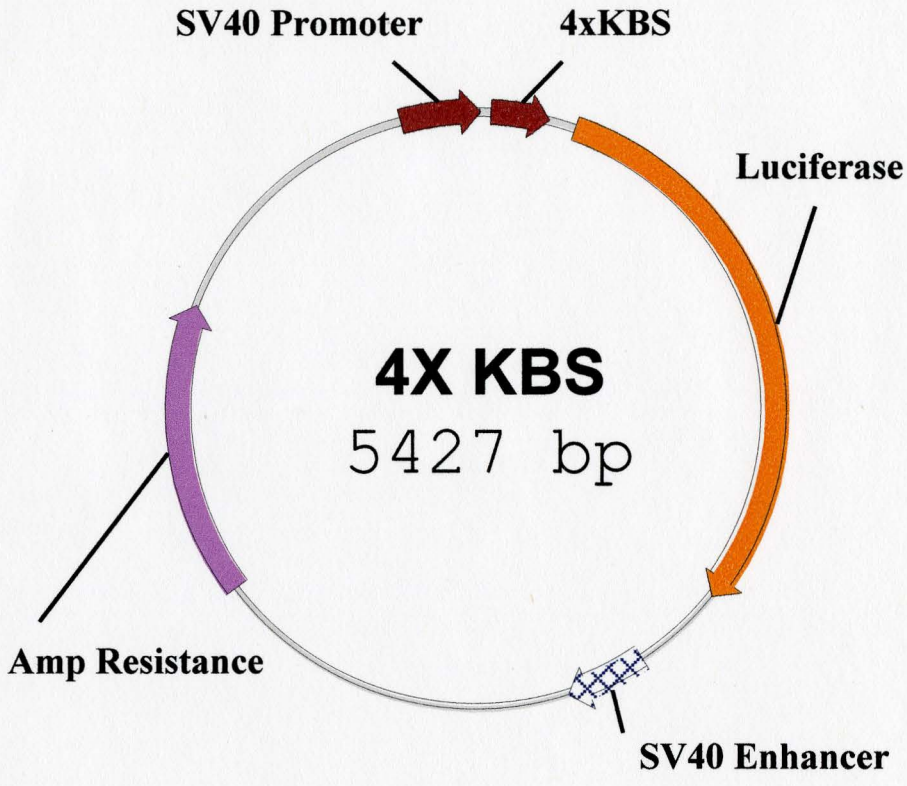
Figure B-7: *Schematic representation of the human E-cadherin promoter.* The consensus KBS site and properly spaced methyl-CpG sites are shown in the human *E-cadherin* promoter.

Appendix C: Vector Information

Table C-1: Promoters for Luciferase Reporter and Effector Plasmids

Effector/Reporter	Plasmid	Promoter
Kaiso	pCDNA3	CMV
Antisense Kaiso	pLXSN	MMLV/MMSV
pSilencer Kaiso	pSilencer	U6 (RNAPIII)
ΔPOZ Kaiso	pCDNA3	CMV
ΔPZF Kaiso	pCDNA3	CMV
p120^{ctn}	pRcRSV	RSV
ΔR3-11	pRcRSV	RSV
p120^{ctn} NLS Mut	pRcRSV	RSV
pGL3	pGL3 Control	SV40

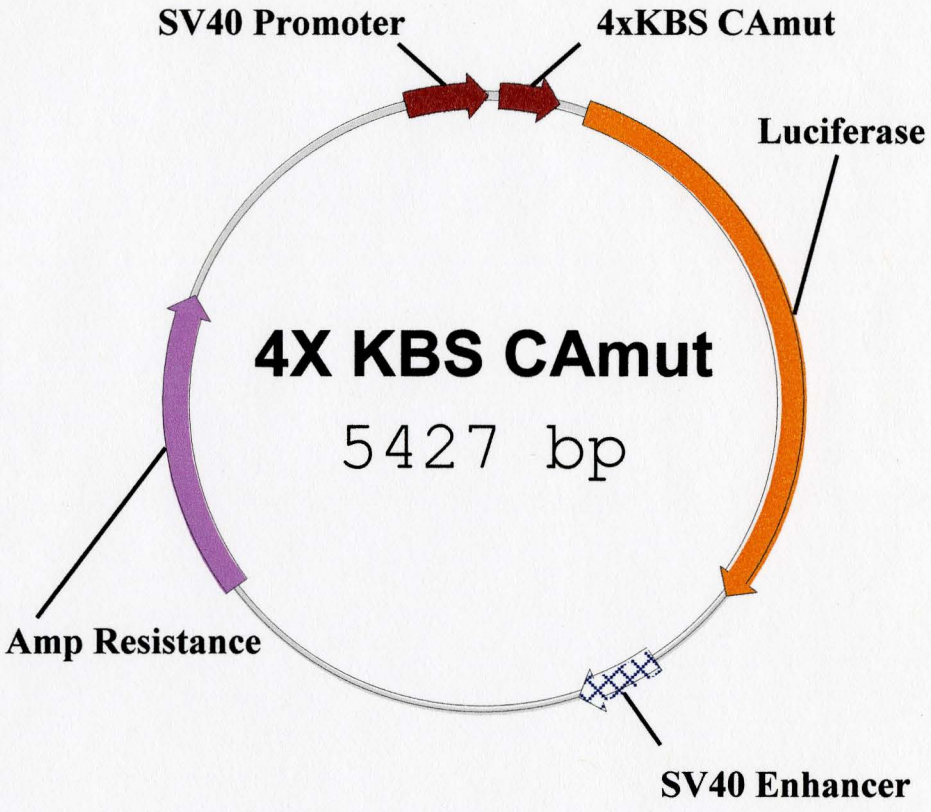
Schematic Representation of the 4xKBS Construct



Consensus KBS: TCCTGCNA

Figure C-2: *Schematic representation of the 4x KBS plasmid.* Four copies of the KBS (TCCTGCNA) sequence were cloned into the pGL3 Control vector upstream of the firefly *luciferase* gene. The plasmid also carries an SV40 promoter, SV40 enhancer, and ampicillin resistance gene.

Schematic Representation of the 4x KBS CAmut Construct



Consensus KBS Mutation: TCATGCNA

Figure C-3: *Schematic representation of the 4x KBS CAmut plasmid.* Four copies of the mutated KBS (TCATGCNA) sequence were cloned into the pGL3 Control vector upstream of the firefly *luciferase* gene. The plasmid is identical to the 4x KBS plasmid except that the mutation harboured in the KBS abrogates Kaiso-DNA binding.

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