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14. ABSTRACT: Kaiso is a new member of the POZ-ZF transcription factor family, which was first identified as a binding partner for the cell-adhesion co-factor p120ctn. Preliminary work from our lab indicated that Kaiso is misexpressed in ~40% of human breast tumors and identified <i>cyclin D1</i> as a putative Kaiso target gene. The purpose of this project is to elucidate the mechanisms by which Kaiso's transcriptional regulation of <i>cyclin D1</i> affects breast cell proliferation and contributes to breast tumorigenesis. Using such techniques as artificial promoter assays and semi-quantitative RT-PCR, we sought to determine how Kaiso regulates <i>cyclin D1</i> promoter binding and expression. Our work to date demonstrates that Kaiso is a transcriptional repressor of the <i>cyclin D1</i> gene and implicates Kaiso and p120ctn activity in modulating canonical Wnt signaling.						
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Introduction

Most invasive and fatal human tumors originate from epithelial cells with reduced intercellular adhesion, and increased cellular mobility to distinct vital organs [1]. The E-cadherin-catenin complex is the major cell-cell adhesion system in epithelial cells and its malfunction is implicated as a contributing factor to ~50% of metastatic human carcinomas [1, 2]. Cadherins are tissue-specific transmembrane cell adhesion molecules that are vital for the maintenance of normal cell-cell contacts and tissue architecture [1, 2]. The catenins, α -, β -, γ -, and p120^{ctn}, are a family of cytoplasmic proteins that bind and regulate cadherin function via direct and indirect interactions [1, 3]. Although E-cadherin reduction and catenin malfunction strongly correlate with the invasive and metastatic phenotype of tumor cells, some tumors retain normal function of E-cadherin and the classical catenins (β - and γ -catenin). This implicates other catenins, e.g. the atypical catenin p120^{ctn}, as key factors in tumor progression.

During the characterization of p120^{ctn} function, my supervisor, Dr. Daniel, then a post-doctoral fellow, cloned and identified a novel POZ-ZF transcription factor, Kaiso, as a p120^{ctn} binding partner [4]. The POZ-ZF family of transcription factors are strongly implicated in cancer as oncoproteins or tumor suppressors [4-6]. Consistent with this idea, we observed that Kaiso is misexpressed in ~40% of human breast tumors and is a potential transcriptional regulator of the cell cycle regulatory gene *cyclin D1*, which is aberrantly expressed in many human breast cancers [7-9]. These early findings raise the possibility that Kaiso and p120^{ctn} are components of an epithelial cell-specific signalling pathway, where Kaiso regulates genes involved in breast cell proliferation and its binding partner p120^{ctn} (hereafter, p120) serves to modulate its function. This possibility led to our hypothesis that **Kaiso plays a role in breast cell proliferation and tumorigenesis via its regulation of *cyclin D1* expression.**

Body

Specific Aim 1: To examine Kaiso expression and function in human breast tumours by analysing human breast tissue samples, and assess the effects of Kaiso misexpression on breast cell proliferation and transformation. Due to a few setbacks obtaining access to human breast tumor tissue banks at Yale University, and through the Cooperative Breast Cancer Tissue Resource (CBCTR), I was unable to make significant progress with this aim. However we have

initiated a formal collaboration with a pathologist (Dr. L. Elavathil) at McMaster University and we are currently in the process of appropriating human breast tumor tissue for the analyses of Kaiso and Cyclin D1 expression.

Another goal of specific Aim 1 was to generate a Kaiso overexpression system using lentiviral delivery methods. However we encountered several problems during the drug-selection step. Various other methods were employed to circumvent this problem, however they were unsuccessful. Another member of our laboratory group has now successfully generated stable virally transduced Kaiso over-expression and depletion systems and these are now available for general use by all lab members. Our plan is to now to use these retroviral systems to determine whether Kaiso inhibits or promotes breast tumor cell proliferation and/or colony formation. If Kaiso inhibits cell growth, it would be implicated as a tumor suppressor, if it promotes proliferation, it would be implicated as an oncoprotein.

Specific Aim 2: Determine if Kaiso regulates cyclin D1 expression. Using a pGL3-Basic 1748CD1 artificial promoter construct, I performed luciferase-reporter assays to determine the effects of Kaiso overexpression on luciferase expression under the control of a partial *cyclin D1* promoter construct. These experiments clearly demonstrated that human Kaiso is a dose-dependent transcriptional repressor of the *cyclin D1* promoter (Figure 1, Appendix). The maximum repression level attained was 2-fold. This was our first experimental evidence that Kaiso may indeed regulate *cyclin D1* transcript expression. I next utilized site-directed mutagenesis to create point-mutations in the two sequence-specific Kaiso binding sites (KBS) located within the *cyclin D1* promoter fragment of the 1748CD1 construct. Unexpectedly, Kaiso was still able to repress luciferase expression under the control of the mutant KBS *cyclin D1* promoter constructs (Figure 2). Visual analysis of the *cyclin D1* promoter revealed the presence of several potential methylatable CpG islands. Since Kaiso is a bi-modal transcription repressor that also recognizes and binds methyl-CpG, we postulated that Kaiso's effects on the *cyclin D1* promoter may be due to its association with methylated DNA [10], in addition to the sequence-specific KBS.

To investigate this possibility I analyzed Kaiso effects on the 962CD1 luciferase reporter construct, which lacks one KBS and contains all putative CpG islands. Kaiso was able to repress luciferase expression from the 962CD1 partial *cyclin D1* promoter construct 2-fold (Figure 2),

suggesting that indeed its methylated DNA-binding capabilities are involved in its transcriptional repression of *cyclin D1*. In light of this new finding I decided to carry out electrophoretic mobility shift assays (EMSA) to characterize Kaiso binding to *cyclin D1* promoter-derived probes *in vitro*. One KBS within the *cyclin D1* promoter is located 24 nucleotides downstream of the transcriptional start site (CD1+24), is contained within a putative CpG island of the promoter region, and is a part of the 962CD1 construct described above, which Kaiso was able to repress. 30-mer probes derived from the CD1+24 KBS and surrounding region were methylated *in vitro* and utilized in EMSA experiments along with a panel of GST-Kaiso fusion proteins. These experiments demonstrate that Kaiso binds to the methylated CD1+24 probe efficiently (Figure 3). Furthermore, mutation of the Kaiso binding site within this methylated probe diminished but did not abolish Kaiso binding to the CD1+24 probe (Figure 3). This is the first experimental evidence that Kaiso binds specifically to methylated sequences within the *cyclin D1* promoter, and raises the exciting possibility that Kaiso binding to the *cyclin D1* promoter and resulting transcriptional activity may be dually directed by its sequence-specific and methylated DNA binding capabilities.

We have also begun to analyze the effects of Kaiso misexpression on *cyclin D1* transcript levels through preliminary methods while our viral expression system is being optimized. To begin these analyses we decided to use transient transfection methodology in the MCF-7 breast tumor cell line because it is readily transfected by conventional methods. Our preliminary experiments have indicated that Kaiso depletion by plasmid-based RNA interference (RNAi) results in a three-fold increase in *cyclin D1* mRNA (Figure 4). This finding, although preliminary, corroborates data obtained through luciferase assay methodology and provides a solid foundation for further analysis of Kaiso misexpression effects on *cyclin D1* expression and their contribution to breast tumorigenesis.

Specific Aim 3: To analyse catenin function in the transcriptional regulation of *cyclin D1*. Since the p120/Kaiso interaction is reminiscent of the β -catenin/TCF interaction, and β -catenin/TCF are well-documented transcriptional regulators of *cyclin D1*, I investigated whether Kaiso and p120 synergize or antagonize with β -catenin/TCF regulation of *cyclin D1* using luciferase assays. Kaiso inhibited β -catenin-mediated activation of the *cyclin D1* promoter while p120 enhanced this activation (Figure 5). This exciting finding corroborates those made by us and our

collaborator (Dr. Pierre McCrea) that Kaiso and p120 are indirect regulators of canonical Wnt signalling [12, 13]. I next analysed the effects of p120 overexpression alone on the *cyclin D1* promoter and found that p120 activated *cyclin D1* expression (Figure 6), however repeated experiments could not definitely conclude that p120 relieves Kaiso-mediated repression of the promoter. Together these results suggest that p120 activation of the *cyclin D1* promoter may be independent of its interaction with Kaiso, and that the p120 transcription activation effects may be due to other intracellular signalling pathways that p120 is involved in, e.g. p120 regulation of the RhoA family of GTPases [11].

Key Research Accomplishments

1. Determined that Kaiso is a transcriptional repressor of the *cyclin D1* gene, a function that may not be regulated by p120^{ctn}.
2. Determined that Kaiso antagonizes the β -catenin-mediated activation of *cyclin D1*-luciferase reporter whereas p120^{ctn} alone enhances it.
3. Discovered a potential methylation-specific mechanism of *cyclin D1* repression by Kaiso.

Reportable Outcomes

1. **Abstract presentation***. Abena A. Otchere and Juliet M. Daniel. *The cell cycle regulator cyclin D1 is a target gene of the POZ-ZF transcription factor Kaiso*. New England Biolabs Annual Symposium. Toronto, Ontario. May 2005. (*Winner, Best Poster).
2. **Conference Delegate**. Edward A. Smuckler Memorial Workshop: Pathobiology of Cancer. Presented by the American Association for Cancer Research. Snowmass Village, Colorado. July 2005.
3. **Manuscript in preparation**. Abena A. Otchere, Kevin F. Kelly, Monica Graham, and Juliet M. Daniel. *Kaiso is a transcriptional repressor of the cyclin D1 gene*.

Conclusions

Our first year of this funding grant analysing *cyclin D1* as a putative Kaiso target gene in the context of breast tumorigenesis has been very productive. Our initial findings through this grant have indicated that Kaiso is a transcriptional repressor of the *cyclin D1* gene and hint that this transcriptional repression of *cyclinD1* may differ mechanistically from its other target genes such as *matrilysin* or *Wnt11* [13, 14]. Only a few aspects of these aims remain to be completed, namely analysis and comparison of Kaiso and Cyclin D1 protein levels in breast tumour tissue samples (Aim 1), assessment of Kaiso misexpression effects on cell proliferation and transformation (Aim 1), and completion of our analysis of Kaiso misexpression effects on *cyclin D1* transcript and protein levels (Aim 2). Although we could not initially execute Specific Aim 1 due to reagent unavailability and lack of expertise, we are now in a position to perform tissue microarray staining for Kaiso and Cyclin D1 in collaboration with Dr. L Elavathil, pathologist at McMaster University. Our future investigations will analyse the role that Kaiso plays directly in the cell biology of breast tumorigenesis. Our findings will hopefully clarify our understanding of how Kaiso contributes to this process for the future development of novel diagnostic, prognostic, and therapeutic tools in the fight against breast cancer.

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Appendix

Figure 1: Kaiso represses transcription from the *cyclin D1* promoter in a dose-dependent manner. The 1748CD1 minimal *cyclin D1* promoter construct was co-transfected with increasing amounts of human Kaiso expression vector, up to 1.0 μg , in HeLa cells. Kaiso overexpression in this system results in the dose-dependent repression of luciferase expression, to reach a saturation point of just over 2-fold repression (A). Immunoprecipitation and Western Blot analyses demonstrated that Kaiso is ectopically expressed at a high level in HeLa cells (B).

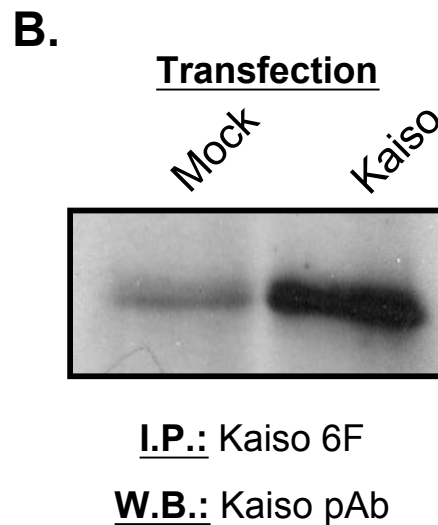
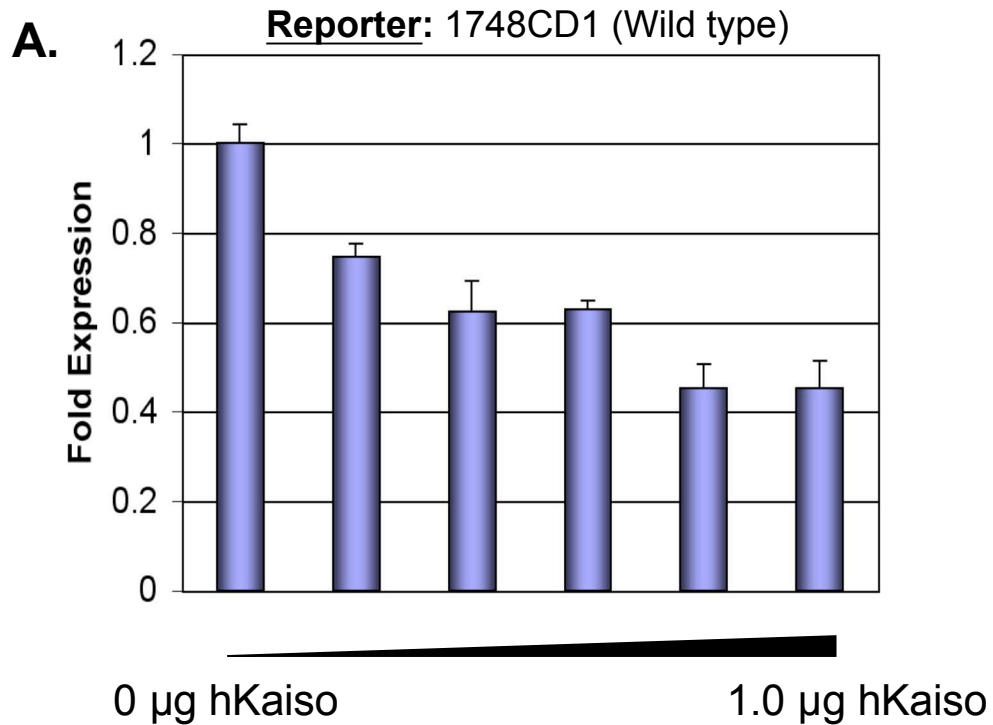


Figure 2: Kaiso represses luciferase expression from KBS point mutant *cyclin D1* promoter constructs. Kaiso was co-transfected along with the 1MK2MK-CD1 KBS mutant partial *cyclin D1* promoter construct which contains a single base pair point mutation in each of the two KBS located within the 1748CD1 construct. Kaiso was unexpectedly able to repress luciferase expression 2-fold under the control of the KBS mutant *cyclin D1* promoter (A). Luciferase assays using the 962CD1 partial *cyclin D1* promoter construct, which lacks a KBS but contains putative CpG islands demonstrated a dose-dependent repression of luciferase expression by Kaiso to a maximum repression of 2-fold (B).

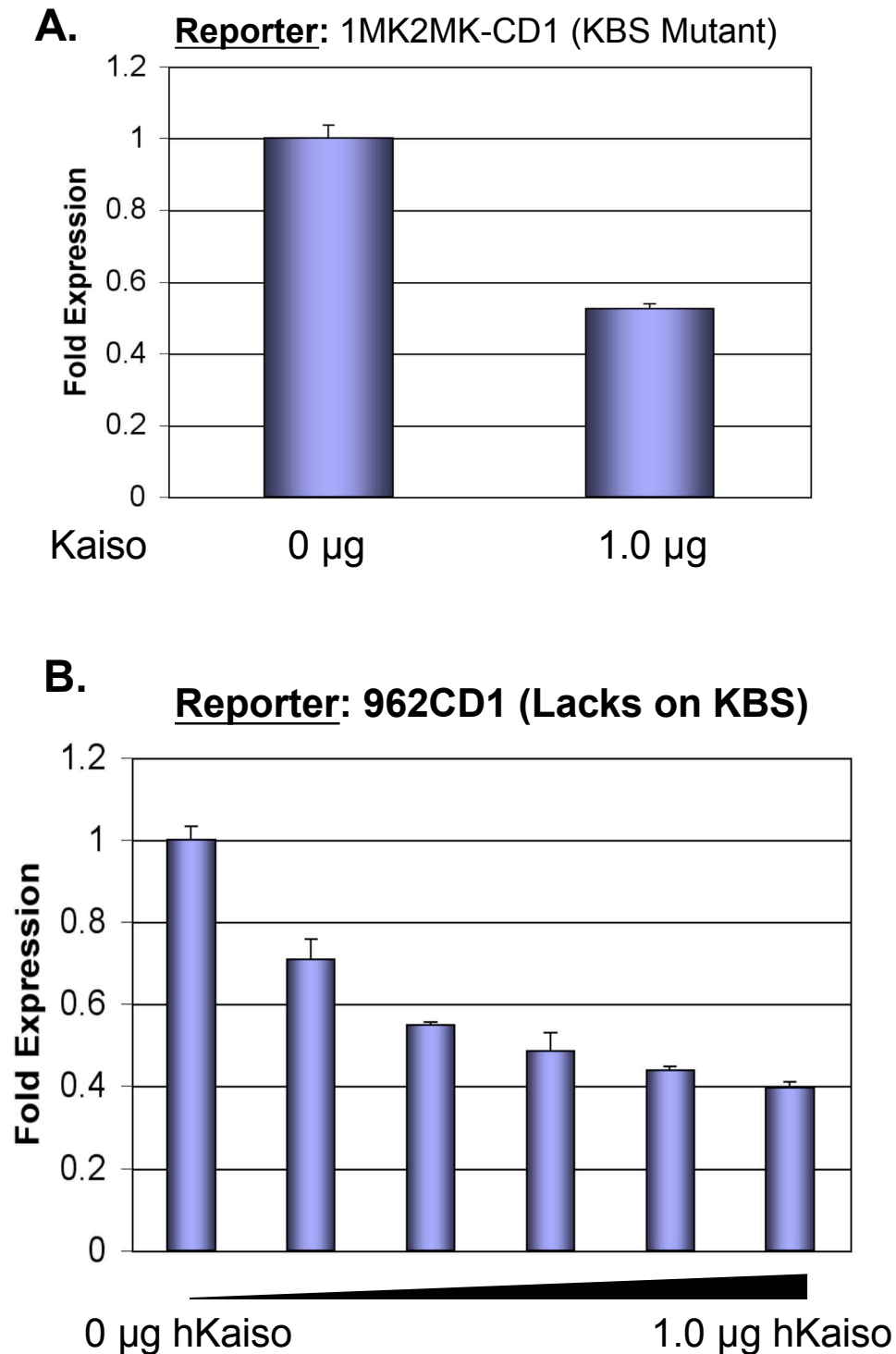


Figure 3: Kaiso binds methylated *cyclin D1* promoter-derived probes *in vitro*. CD1+24 oligos were methylated *in vitro*, radiolabelled and incubated with GST-hKaiso fusion proteins. The Kaiso proteins bind efficiently to the methylated wild type CD1+24 probe, with hZF and hΔAR1 showing the strongest DNA-binding affinity. Methylated KBS mutant CD1+24 probes (ATTTTA) were also incubated with Kaiso proteins, which were able to bind the oligos, albeit at a lesser affinity than their binding to methylated wild type probe.

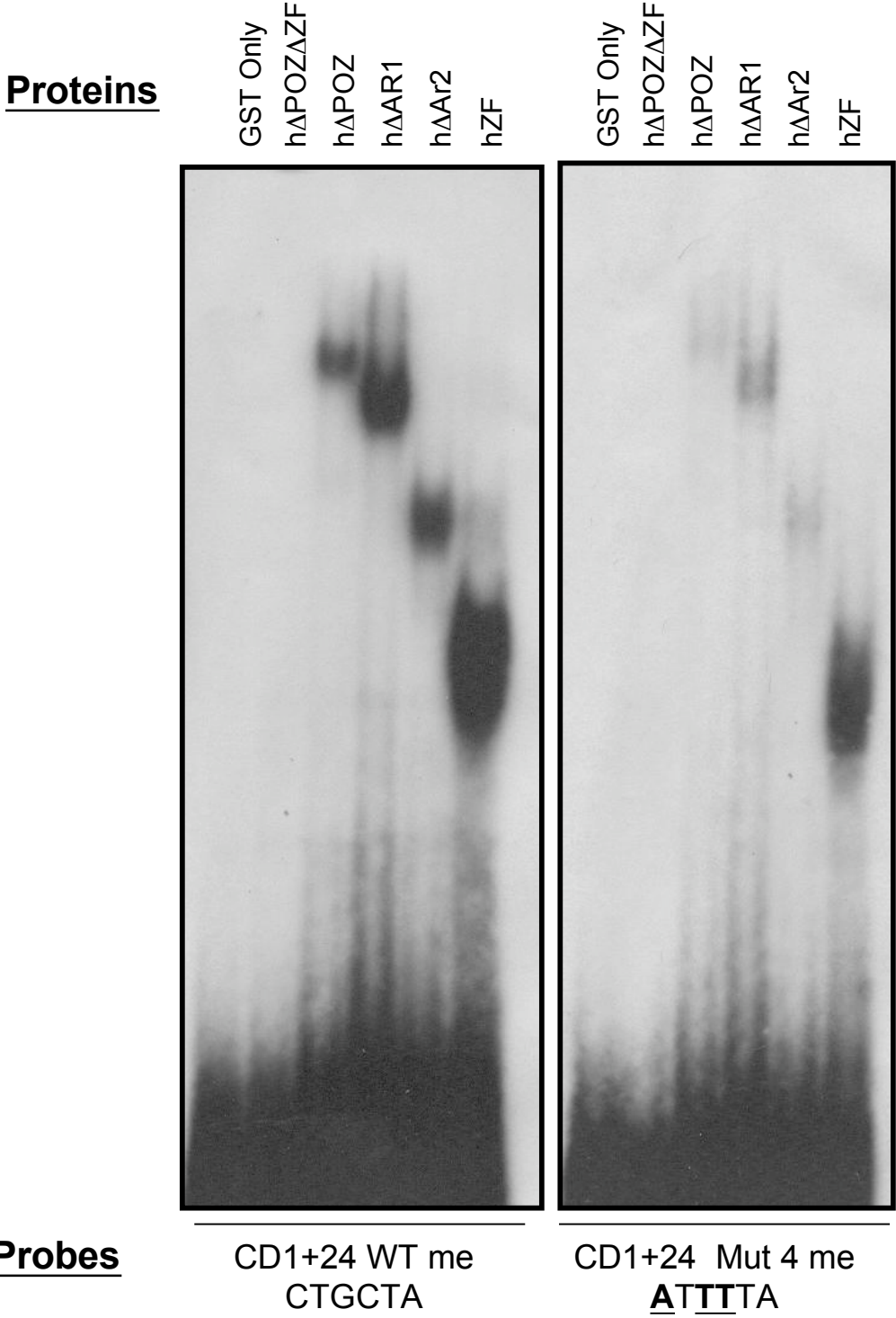


Figure 4: Kaiso depletion enhances *cyclin D1* expression. MCF-7 cells were transfected at a low confluency with a Kaiso-specific siRNA plasmid vector, and harvested 24 hours post-transfection for total RNA. RNA samples were subjected to semi-quantitative RT-PCR using Kaiso-, cyclin D1-, and GAPDH-specific primers and PCR products were separated by agarose gel electrophoresis. Kaiso mRNA was efficiently depleted by this method and resulted in a 3-fold increase in cyclin D1 mRNA levels (A). Immunoprecipitation and Western Blot experiments demonstrated that Kaiso depletion by RNAi could also be detected at the protein level (B).

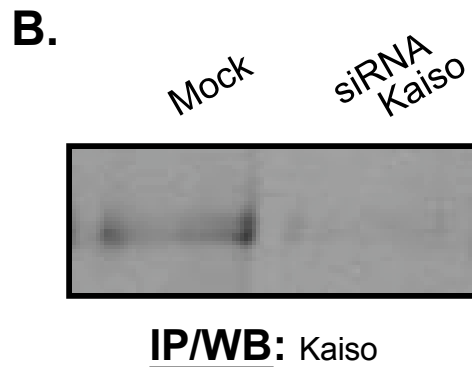
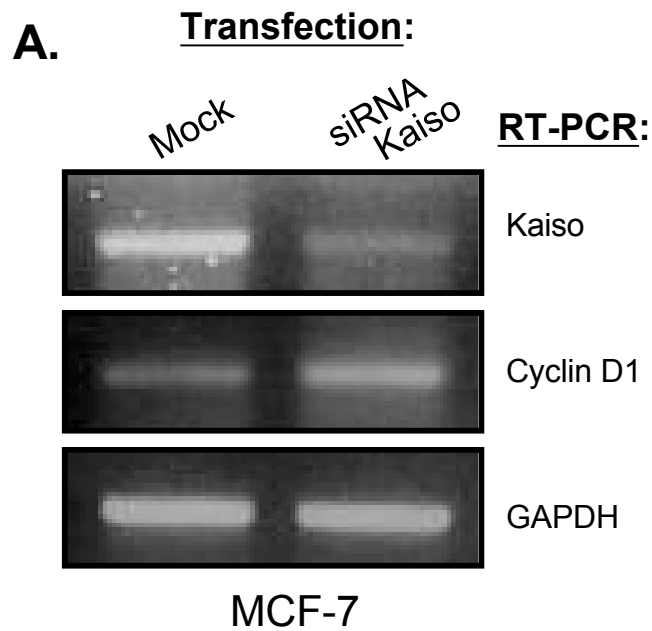
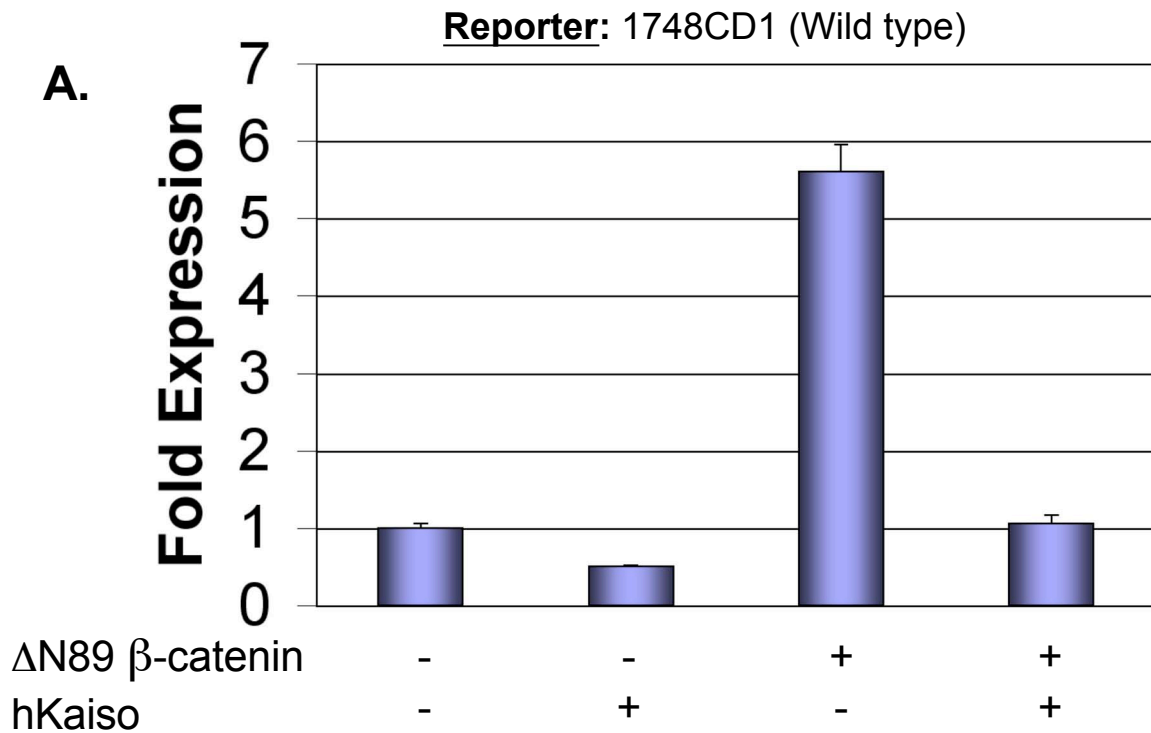
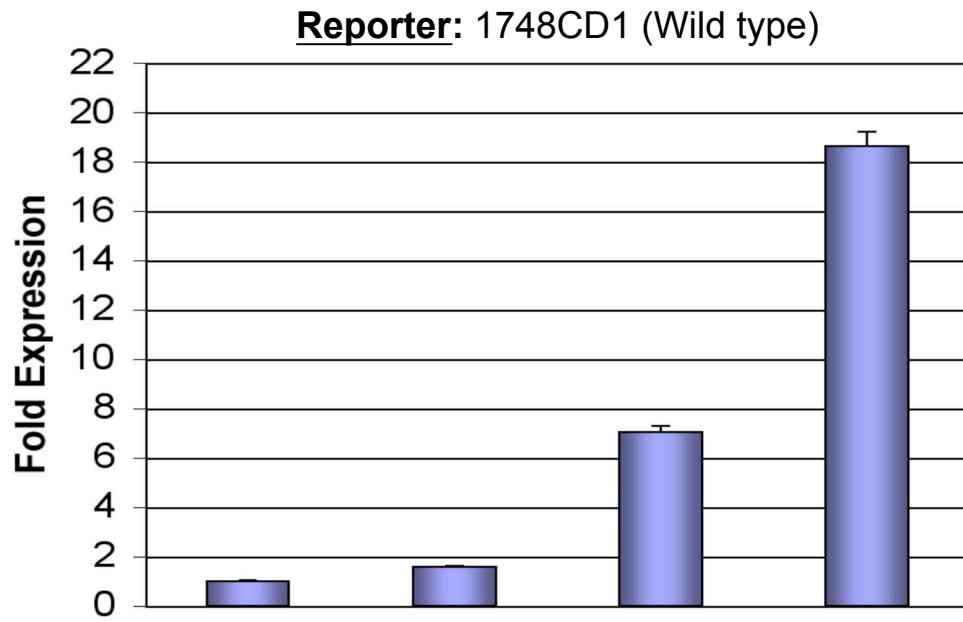


Figure 5: Kaiso and p120 modulate β -catenin mediated activation of the *cyclin D1* promoter. p120/Kaiso are reminiscent of β -catenin/TCF, well-known regulators of *cyclin D1*, leading us to believe that they may synergize or antagonize β -catenin/TCF regulation of *cyclin D1*. To investigate this possibility the wild type 1748CD1 partial *cyclin D1* promoter reporter construct was co-transfected with Kaiso, p120, and an N-terminally truncated and constitutively active β -catenin expression construct, Δ N89. β -catenin activated the *cyclin D1* promoter 5-6-fold, however Kaiso inhibited this activation and brought luciferase expression back to basal levels (A). Conversely, p120 enhanced β -catenin activation of the *cyclin D1* promoter to double its normal level (B). Both p120 and β -catenin are efficiently expressed through transient transfection methods (C).



B.



p120	-	+	-	+
Δ N89 β -catenin	-	-	+	+

C.

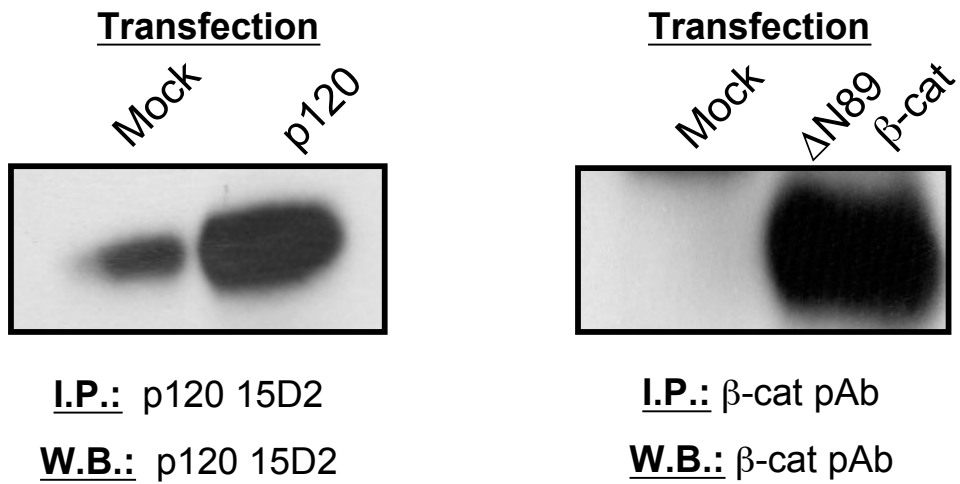


Figure 6: p120 activates the *cyclin D1* promoter. To assess whether p120 could regulate the *cyclin D1* promoter, p120 was co-transfected with the 1748CD1 luciferase reporter construct and cells were subsequently assayed for luciferase expression. Repeated experiments demonstrated that p120 activates the *cyclin D1* promoter 1.5-fold.

