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The POZ-ZF transcription factor Znf131 is implicated as a regulator of Kaiso-mediated biological processes

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ABSTRACT

Znf131 belongs to the family of POZ-ZF transcription factors, but, in contrast to most other characterized POZ-ZF proteins that function as transcriptional repressors, Znf131 acts as a transcriptional activator. Znf131 heterodimerizes with the POZ-ZF protein Kaiso, which itself represses a subset of canonical Wnt target genes, including the cell cycle regulator Cyclin D1. Herein, we report a possible role for Znf131 in Kaiso-mediated processes. Notably, we found that Znf131 associates with several Kaiso target gene promoters, including that of *CCND1*. ChIP analysis revealed that Znf131 indirectly associates with the *CCND1* promoter in HCT116 and MCF7 cells via a region that encompasses the previously characterized +69 Kaiso Binding Site, hinting that the Znf131/Kaiso heterodimer may co-regulate Cyclin D1 expression. We also demonstrate that Kaiso inhibits Znf131 expression, raising the possibility that Kaiso and Znf131 act to fine-tune target gene expression. Together, our findings implicate Znf131 as a co-regulator of Kaiso-mediated biological processes.

1. Introduction

The **Po**x virus and **z**inc finger (POZ)-**z**inc **f**inger (ZF) family of transcription factors is a rapidly growing family of proteins that are characterized by an N-terminal POZ domain, which mediates protein-protein interactions, and a C-terminal DNA-binding domain comprised of multiple zinc fingers [1]. While best known for their roles in vertebrate development, mounting evidence demonstrates that POZ-ZF proteins also play crucial roles in tumorigenesis [1]. For example, the well-characterized POZ-ZF proteins PLZF and BCL-6 are implicated in promyelocytic leukemia and B-cell lymphoma, respectively [2,3], while the more recently characterized POZ-ZF protein Kaiso is linked to several cancers including pancreatic [4], colon [5,6], prostate [7] and breast [8,9]. Kaiso was initially identified as a binding partner of the Armadillo protein and Src kinase substrate $p120^{ctn}$ [10,11], and like most POZ-ZF proteins, Kaiso acts primarily as a transcriptional repressor [12–18]. During our characterization of Kaiso, we identified the minimally characterized POZ-ZF transcription factor Znf131 as a Kaiso interaction partner [19,20].

In vitro, Znf131 recognizes and binds the **Z**nf131 **B**inding **E**lement (ZBE), a 12-bp palindromic sequence that is separated by a variable number of intervening nucleotides (GTCGCR-N_X-YGCGAC; R-purine, Y-pyrimidine, N-any nucleotide). In contrast to most characterized POZ-ZF transcription factors, artificial promoter-reporter assays revealed that Znf131 functions as a transcriptional activator. Notably, we have previously shown that Znf131's transcriptional activity can be attenuated by Kaiso co-expression [19]. To-date, no *bona fide* Znf131 target genes have been identified. However, since Znf131 and Kaiso interact via their POZ domains [19], we hypothesized that Znf131 may co-regulate Kaiso target genes, and thus play a role in Kaiso-mediated biological processes. Herein, we report that Znf131 associates with a subset of Kaiso target genes at the **K**aiso **B**inding **S**ite (KBS). We also found that ectopic Znf131 expression activated a minimal Cyclin D1 (*CCND1*) promoter that we previously showed was repressed by Kaiso [13]. Interestingly, Znf131-mediated activation of the *CCND1* promoter was attenuated upon Kaiso co-expression in a dose-dependent

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manner. Additionally we report an inverse correlation between Znf131 and Kaiso expression in intestinal cells, but a direct correlation in colorectal cancer (CRC) tumors. Together, our findings are the first to implicate Znf131 in the co-regulation of Kaiso target genes, suggesting that Znf131 imposes upon Kaiso-mediated homeostasis.

2. Materials & methods

2.1. Animal ethics statement

All mice were housed and handled in accordance with the McMaster University Animal Research Ethics Board, under the utilization protocol 14-08-29. Mice were euthanized by CO_2 asphyxiation and cervical dislocation.

2.2. Cell culture

Human colon carcinoma (HT29, HCT116) and human breast carcinoma (MCF7) cells were purchased from the American Type Culture Collection and cultured as previously described [9,13,21]. For time course experiments, MCF7 cells were transiently transfected with the empty pCS2+ vector or with pCS2+MT-mZnf131 for 24 h. Cells were synchronized at G_0 for 24 h post-transfection and released from G_0 upon addition of 10% FBS. Cells were lysed at the indicated time points for western blot analysis.

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA analysis was performed as previously described, and using the same oligonucleotides as listed in Ref. [13].

2.4. Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described [21] using the following antibodies: anti-Kaiso mAb 6F [22], anti-Znf131 [19], Histone H3 (Abcam), or Non-specific IgG (Active Motif). The primers used to amplify the selected Kaiso target genes are listed in Table 1.

2.5. Promoter-reporter assay

Promoter-reporter assays were performed as described in Ref. [21] with the following changes: 1×10^5 cells were seeded into 6-well dishes and incubated for at least 12 h until the cells were ~50–60% confluent. Cells were co-transfected with 5 μg total plasmid DNA. Co-transfection with β-galactosidase was used to determine transfection efficiency. Statistical significance was determined by one-way ANOVA with Tukey's post-hoc test.

2.6. Western blot

Isolation of both intestinal epithelial cells (IECs) and cultured cells were performed as previously described [21]. The following antibodies were used as follows: Znf131 rabbit pAb at 1:1000 [19]; Cyclin D1 rabbit pAb at 1:5000 (U.S. Biologicals); β-tubulin mouse mAb at 1:50,000 (Sigma Aldrich); and β-actin mouse mAb at 1:50,000 (Sigma Aldrich).

2.7. Quantitative real time-PCR

RNA isolation and cDNA synthesis were performed as previously described [21]. Gene expression levels were normalized to β-actin for cultured cells, or hydroxymethylbilane synthase (HMBS) for murine IECs. Changes in gene expression were quantified using the standard curve method, and Student's *t*-test (unpaired, two-tailed) was used for statistical calculations. A p-value ≤ 0.05 was considered statistically significant. Primers used are listed in Table 2.

2.8. Immunohistochemistry (IHC)

Intestinal tissue processing and IHC were performed as described previously [23]. Briefly, de-paraffinized and rehydrated intestinal tissues were permeabilized with 0.05% TBS-Tween 20 for 15 min. Following heat-mediated antigen retrieval with 10 mM trisodium citrate, pH 6.0 for 15 min, and quenching of endogenous peroxidase activity with 3% H_2O_2 in TBS for 10 min, Znf131 rabbit polyclonal antibody [19] was incubated overnight at 4°C at 1:300. Following incubation with biotinylated secondary antibody, tissues were incubated with Vectastain Elite ABC reagent and Vectastain DAB substrate (Vector Labs). Tissues were then counterstained with hematoxylin, differentiated with acid alcohol, blued with Scott's tap water substitute and dehydrated in an increasing ethanol series. Slides were mounted using Polymount (Polysciences Inc.) and imaged using the AperioScope slide scanner.

2.9. Gene expression analyses

The publically available colon cancer dataset GSE39582 was obtained from the Gene Expression Omnibus [24], and was pre-processed as described by Marisa et al. [24]. Differential expression of *ZNF131* and *ZBTB33* between tumor and normal samples was examined using *t*-tests with Welch correction (stats package in R). Pearson correlation analysis was performed using GraphPad software.

3. Results

3.1. Znf131 associates with several Kaiso target genes, including CCND1

The finding that Znf131 and Kaiso interact via their POZ domains [19] prompted us to examine whether Znf131 might also associate with, and co-regulate, Kaiso target genes. To this end, ChIP of HCT116 cell lysates was performed using Kaiso- and Znf131-specific antibodies. We found that both Kaiso and Znf131 co-occupied the *MTA2*, *ID2*, *MMP7*, *WNT11* and *CCND1* promoters, all of which have been implicated in tumorigenesis and/or Wnt signaling [25–30] (Fig. 1 A). These results suggest that Znf131 might synergize or antagonize Kaiso-mediated regulation of these target genes.

Table 1
List of primers used for ChIP-PCR.

Target Gene	Fwd Primer 5' – 3'	Rev Primer 5' – 3'
CCND1	CACACGGACTACAGGGGAGTT	CTCGGCTCTCGCTTCTGCTG
MTA2	GCTAAGGCGCGCGAGTCTTTG	GAGAACAAGGCCCACTGCTCGGC
ID2	CAGCCCCGCACTTACTGTACTGTA	CATTGGCGGAAGGTGGCACGT
MMP7	AAGGAGACCCAAAGAAGGGA	GAACATCACCAAAATCCTGTGG
WNT11	TGTCTGTTTGTTTGAGACCGA	CGTCTCTACTACGAATACAA
ZNF131	TTCCCCCATGGTTTCATCTA	TTGCTGGATAGACGCACAAG

Table 2
List of primers used for qRT-PCR.

Target	Fwd Primer 5' – 3'	Rev Primer 5' – 3'
hβ-actin	CTCTTCCAGCCTTCCTTCCT	AGCACTGTGTTGGCGTACAG
mHMBS	GATGGGCAACTGTACCTGACTG	CTGGGCTCCTCTTGGAATG
Znf131[a]	GATTCTGCTCTAGCACTGTTGGC	GCTTCTTACTGACTCCACCTTCTT

[a] NB – Znf131 primers were used to amplify murine and human *Znf131* transcripts.

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