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The interaction of xKaiso with xTcf3: a revised model for integration of epigenetic and Wnt signalling pathways

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We demonstrate that a direct interaction between the methyl-CpG-dependent transcription repressor Kaiso and xTcf3, a transducer of the Wnt signalling pathway, results in their mutual disengagement from their respective DNA-binding sites. Thus, the transcription functions of xTcf3 can be inhibited by overexpression of Kaiso in cell lines and *Xenopus* embryos. The interaction of Kaiso with xTcf3 is highly conserved and is dependent on its zinc-finger domains (ZF1-3) and the corresponding HMG DNA-binding domain of TCF3/4 factors. Our data rule out a model suggesting that xKaiso is a direct repressor of Wnt signalling target genes in early *Xenopus* development via binding to promoter-proximal CTGCNA sequences as part of a xTcf3 repressor complex. Instead, we propose that mutual inhibition by Kaiso/TCF3 of their DNA-binding functions may be important in developmental or cancer contexts and acts as a regulatory node that integrates epigenetic and Wnt signalling pathways.

KEY WORDS: Cancer, DNA methylation, Kaiso, Siamois, TCF3, Chromatin

INTRODUCTION

The BTB/POZ transcriptional factor xKaiso is a bimodal DNA-binding protein that is reported to specifically bind methyl-CpGs, or a CTGCNA consensus DNA sequence (Ruzov et al., 2004; Park et al., 2005). Our previous work has established the essential and global role of xKaiso in regulating the timing of zygotic gene activation at the mid-blastula transition (MBT) (Ruzov et al., 2004). Other work proposes a model in which xKaiso specifically binds CTGCNA sequences present in the promoter region of Siamois (and also xWnt11) and interacts with the Wnt effector molecule xTcf3 to promote its stable repression (Kim et al., 2004; Park et al., 2005).

In recent work that is not in keeping with the latter model, we demonstrated that the CTGCNA motifs derived from the promoters of Siamois and xWnt11 are not sequence specific xKaiso-binding sites and these genes are not mis-expressed in xKaiso morphants (Ruzov et al., 2009). Although our loss-of-function experiments did not identify a role for xKaiso in regulating Wnt target genes, two observations suggest a potential role for it in canonical Wnt signalling: the xKaiso protein can be co-immunoprecipitated with xTcf3, and over-expression of xKaiso can suppress axis-duplication that is induced by over-expression of β-catenin in the ventral regions of a four-cell embryo (Park et al., 2005). We wished to determine the molecular basis of the Kaiso/Tcf3 interaction as it has profound implications for the intersection of two important regulatory pathways in amphibian development: regulation of transcriptional silencing in pre-MBT *Xenopus* embryos and in Wnt signalling pathways (Heasman, 2006). We find that the interaction surfaces for both proteins correspond to their previously identified DNA-binding domains. Our data squarely rules out a model for xKaiso repression through stabilisation of xTcf3 binding to DNA. Instead, our analysis suggests that the xKaiso and xTcf3/4 interaction results in their mutual delocalisation from chromatin, a prediction that we demonstrate at the cellular and the DNA levels.

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MATERIALS AND METHODS

Reporter assays and expression constructs

The xKaiso expression constructs (XKaiso/pCS2+MT and HA-tagged) (Kim et al., 2004) were provided by Pierre McCrea. The dKaiso expression construct was from (Ruzov et al., 2004). The Myc-xTcf3, xTcf3dn and β-catenin expression vectors were provided by Randall Moon. The VP16 fusions with the ZF1-3 region of xKaiso (amino acids 447-635) and the HMG domain of xTcf3 were from Ruzov et al. (Ruzov et al., 2009). All reporter assays using SuperTOP/FOP, Tex19 and Siamois luciferase reporters were performed as described previously (Houston et al., 2002; Ruzov et al., 2009).

Embryos and microinjections

Embryos were manipulated as described previously (Houston et al., 2002; Ruzov et al., 2009). At the two-cell stage, the embryos were injected into the animal half with 200-750 pg of sense capped RNA (c-myc-xKaiso mRNA) synthesized in vitro (T3/T7 Cap-Scribe kit, Boehringer).

GST pull-down assays, immunoprecipitation and EMSA

The Kaiso GST fusions were from Ruzov et al. (Ruzov et al., 2009). The TCF4 constructs were provided by Vladimir Korinek. A coupled transcription/translation kit (Promega) was used for in vitro translation/labelling with ^{35}S-Met. GST pull-down assays and immunoprecipitations were performed according to standard protocols in the presence of a 10- to 100-fold excess of recombinant full-length xKaiso or GST where indicated. Samples were visualized by phosphoimaging. EMSA was performed as described previously (Ruzov et al., 2009).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described previously (Ruzov et al., 2009) using myc tagged xTcf3.

Immunostaining

Immunostaining was performed according to standard techniques using *P53*$^{-/-}$ (*Trp53*$^{-/-}$) mouse embryonic fibroblasts. Cells were analysed 24 hours after transfection. Mouse monoclonal anti-T7 tag (Novagen), anti-HA-tag (Sigma), rabbit polyclonal anti-myc (Upstate) and Alexa secondary antibodies were used.

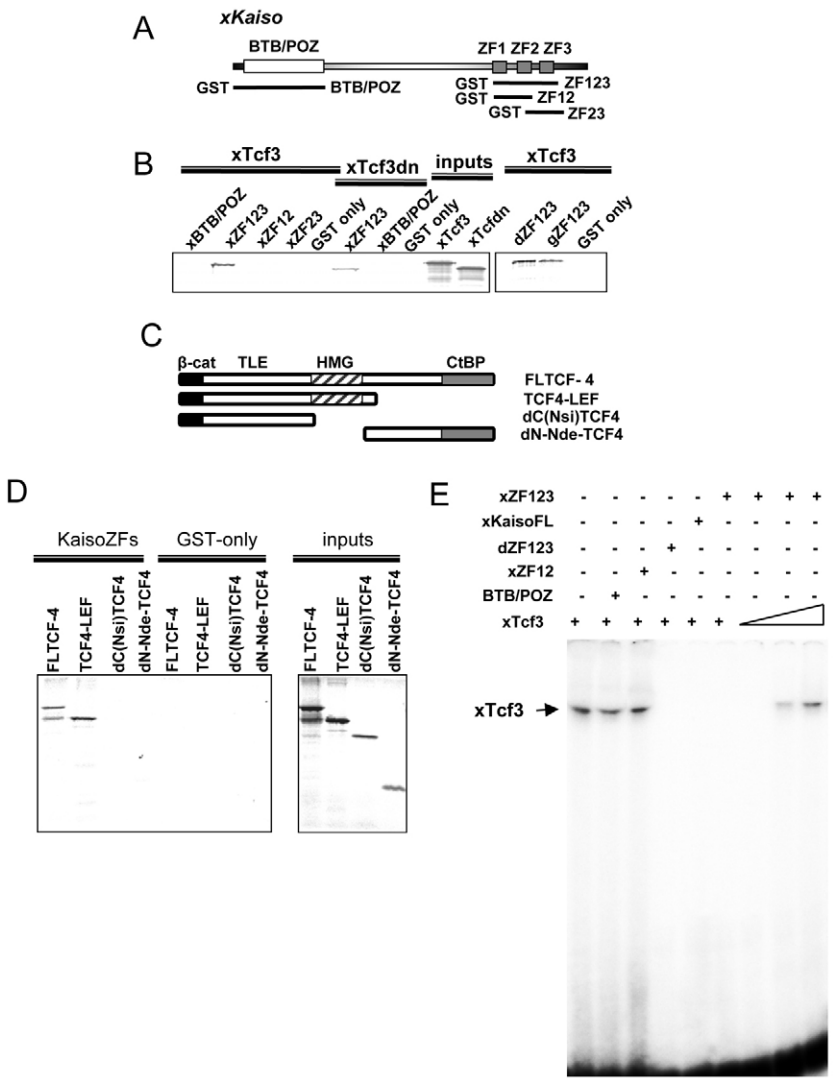

Fig. 1. Kaiso ZF1-3 interacts directly with the HMG domain of TCF factors. (**A**) Kaiso deletion constructs used in pull-down experiments. (**B**) GST pull-down using in vitro transcribed xTcf3 or xTcf3dn with Kaiso recombinant proteins indicated in A. GST was used as a control. (**C**) TCF4 constructs used for in vitro transcription in D. β-cat, β-catenin interaction domain; TLE, TLE/Groucho binding domain; CtBP, CtBP-binding sites; HMG, DNA-binding domain. (**D**) GST pull-down using TCF4 constructs and xKaiso GST-ZF domain (KaisoZFs). GST was used as a control; 1/10 of inputs (xTcf3 and xTcf3dn) are shown. (**E**) EMSA assay using in vitro translated xTcf3 and its DNA-binding site (TCF bs-oligo) in the absence and presence of GST fusions with ZF1-3 of xKaiso, full-length xKaiso or ZF1-3 of dKaiso. xZF2-3 and BTB/POZ fusions, which do not interact with xTCF3 were used as controls. Note absence of the xTcf3/DNA complex (arrow) in the presence of the Kaiso fusions. Increasing the xTcf3 concentration in the presence of xZF1-3 restores the binding of xTcf3 to DNA, supporting the hypothesis that sequestration is the mechanism of inhibition of xTcf3 binding to DNA by Kaiso.

Real-time RT-PCR

Quantitative real-time RT-PCR of Siamois was evaluated as described previously (Houston et al., 2002).

RESULTS AND DISCUSSION

Kaiso directly interacts with the HMG domain of TCF factors via ZF1-3

The TCF family proteins have a multi-domain organisation with a central HMG box DNA-binding region recognizing the sequence A/TA/TCAAA; an N terminus containing the β-catenin-interacting domain adjacent to a Groucho-binding region; and a C-terminal CtBP1 interaction domain (Roose et al., 1998). We demonstrate using recombinant proteins that the DNA-binding, zinc-finger domain of xKaiso (xZF1-3) is sufficient for direct interaction with full-length xTcf3 and dominant-negative xTcf3 (xTcf3dn), which lacks the β-catenin-interacting region (Fig. 1A,B). The ability of Kaiso to bind xTcf3 is conserved, as comparable zinc-finger regions from zebrafish Kaiso (dZF1-3) and chicken Kaiso (gZF1-3) can also interact with xTcf3 (Fig. 1B). The same pattern of interaction is seen between mouse TCF4 and xZF1-3. Deletion analysis suggests that the interaction occurs through the HMG domain of TCF4 (Fig. 1C,D). The experiment was performed in the presence of high concentrations of ethidium bromide to exclude the possibility that the interaction was mediated by non-specific binding to DNA. This suggests that the interaction between Kaiso and TCF3/4 is mutually exclusive of their binding to DNA, and that inhibition of β-catenin activation by Kaiso is not through competitive binding of a shared interaction domain on xTcf3 and TCF4. We tested the first possibility by performing an EMSA with xTcf3 and its target DNA binding site (ATCAAA) in the presence of GST-Kaiso fusions. Binding of xTcf3 to its target sequence was abolished in the presence of the full-length xKaiso, GST-xZF1-3 and GST-dZF1-3. GST alone or GST-xZF1-2 had no effect (Fig. 1E). Increasing the amount of xTcf3 overcomes the inhibitory effect of GST-xZF1-3. Moreover, the interaction between xTcf3 and β-catenin in vitro is not compromised by xZF1-3 (Fig. 2A). We also examined the cellular location of myc-tagged xTcf3 in the absence and presence of Kaiso. xTcf3 localisation in mouse cells is nuclear, with about 40% of cells with a homogenous, as opposed to a speckled, pattern (60%) (Fig. 2B); the latter is reminiscent of the discrete foci observed for endogenous mouse TCF4 (Valenta et al., 2006). xKaiso in mouse fibroblasts exhibits homogenous nuclear staining (Fig. 2C). In the presence of either xKaiso or dKaiso, the pattern of xTcf3 staining was uniformly homogenous, suggesting they alter its nuclear sublocalisation (Fig. 2D).

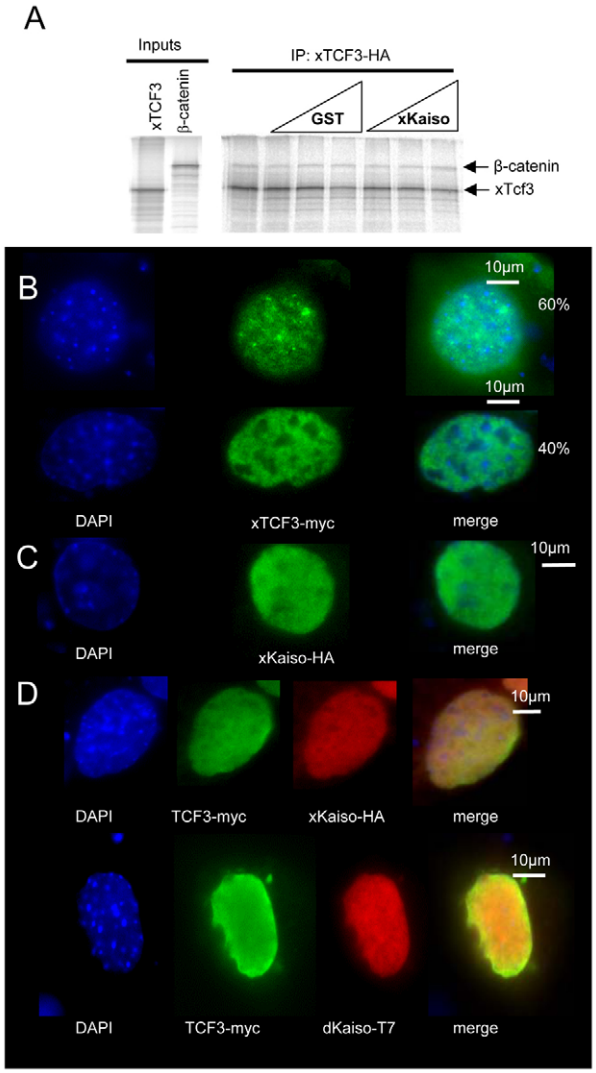

Fig. 2. Kaiso does not affect β-catenin interaction with xTcf3 but can alter the nuclear localisation of TCF3 in mouse fibroblasts. (**A**) In vitro translated ^{35}S-methione labelled β-catenin and HA-tagged xTcf3 were incubated in the presence of an excess of xKaiso full-length (xKaisoFL) protein or GST. xTcf3 was immunoprecipitated with an anti-HA antibody. β-Catenin and xTcf3 proteins are indicated by arrows. 1/10 of inputs are shown. The interaction between β-catenin and xTcf3 is not impaired in the presence of GST-xKaiso or GST. (B,C) Localisation of myc-xTcf3 upon transient transfection into mouse MEFs in the absence and presence of Ha-xKaiso and T7tagged-dKaiso. (**B**) By itself, myc-xTcf3 either exhibits staining at nuclear foci (60% of cells) or homogenous nuclear staining (40%). (**C**) xKaiso by itself exhibits homogenous nuclear staining in 100% of cells. (**D**) In the presence of xKaiso or dKaiso the myc-xTcf3 protein exhibits only homogenous nuclear staining (100% of cells with double staining).

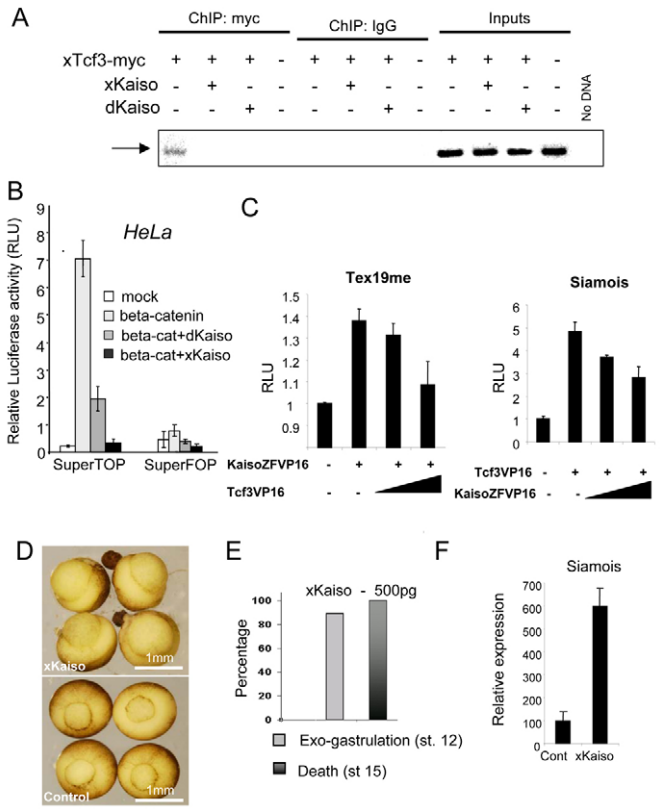

Fig. 3. Kaiso prevents xTcf3 localising to its target promoter and its subsequent activation or repression functions. (**A**) ChIP assay showing that, after transient transfection into A6 cells, myc-xTcf3 can be located at the *Siamois* promoter. Co-expression of xKaiso or dKaiso prevents xTcf3 binding at the promoter. Inputs are shown on the right and the myc-Tcf3 ChIP on the left with a non-specific antibody (Ig) control. (**B**) Overexpression of both dKaiso and xKaiso represses β-catenin-dependent transcription activity in transient transfection assays in HeLa cells. SuperTopflash (SuperTop) was used as a Wnt reporter and SuperFopflash (SuperFop) with mutated TCF3 sites as a control. (**C**) Expression from a methylated luciferase reporter can be enhanced by co-transfection with an xKaisoZFVP16 (KaisoZFVP16) expression plasmid. Targeting to the methylated reporter is provided by the Kaiso-ZFs and transcription activation function by VP16. Increasing amounts of xTcf3HMGVP16 (Tcf3VP16) reduce the activation potential of xKaisoZFVP16. In a reciprocal experiment, activation a Siamois luciferase reporter by xTcf3HMGVP16 is inhibited by the presence of xKaisoZFVP16. (**D**,**E**) Overexpression of myc-xKaiso in *Xenopus* embryos results in the formation of exogastrulae compared with controls at stage 12. Graph shows that by stage 12, over 80% of the embryos (*n*=55) exhibit exogastrulae. (**F**) Real-time RT-PCR analysis of Siamois expression in stage 10 control and embryos injected with myc-xKaiso mRNA (1 ng). Relative to histone, H4 Siamois expression is activated in the myc-xKaiso-injected embryos.

xKaiso displaces xTcf3 from its target promoters

The interaction data suggests that overexpression of xKaiso can displace xTcf3 from its genomic binding sites. Using the chromatin immunoprecipitation (ChIP) technique we could localise myc-xTcf3 to the *Siamois* promoter in A6 cells (Fig. 3A). However, myc-xTcf3 was delocalised in the presence of either Ha-xKaiso or T7tag-dKaiso (Fig. 3A). In contrast to the model of Park et al. (Park et al., 2005), this suggests that Kaiso can disengage xTcf3 from its target genes and alter their expression state. Under the same conditions, we could not localize dKaiso or xKaiso alone to the Siamois promoter; this is not surprising in view of the absence of high-affinity CTGCNA or methyl-CpG binding sites in it (Ruzov et al., 2009).

The SuperTopflash Wnt reporter plasmid contains the luciferase gene under the control of multiple TCF3/4-binding sites, which is activated by the addition of exogenous β-catenin and endogenous TCF3/4 in HeLa cells (Veeman et al., 2003) (Fig. 3B). This

activation was inhibited between four- to sevenfold by co-expression of either xKaiso or dKaiso (Fig. 3B). The transcriptional activity of a control reporter with mutated TCF3-binding sites is unresponsive to β-catenin induction and unaffected by the presence of either Kaiso (Fig. 3B). An obvious explanation of these results is that the Kaiso interaction with the HMG domain of endogenous TCF3/4 blocks its ability to bind DNA and inhibits β-catenin-dependent activation of transcription (Fig. 3B). Conversely, overexpression of xTcf3 interferes with transcriptional activation of a methylated reporter template by an xKaisoZFVP16 fusion (Fig. 3C) in a dose-dependent manner suggesting the Kaiso/TCF3 interaction results in a mutual disengagement of these factors from chromatin-binding sites. This model can account for the inhibition of dorsal axis formation when Kaiso mRNA is co-injected with β-catenin mRNA (Park et al., 2005) as excess xKaiso will displace the xTcf3/β-catenin complex and inhibit axis duplication. Similarly, xKaisoZFVP16 directly inhibits xTcf3HMGVP16 activation of a Siamois reporter in a dose-dependent manner (Fig. 3C).

Loss-of-function studies in *Xenopus* suggest the primary role of xTcf3 is as a repressor of organiser genes, such as Siamois, throughout the early embryo (Houston et al., 2002). This repression is inactivated on the dorsal side by a maternally encoded Wnt signalling pathway. Based on the above results, we predicted that overexpression of xKaiso in developing *Xenopus* embryos would mimic certain aspects of xTcf3 depletion such as ectopic Siamois expression (Houston et al., 2002). To test this, we injected xKaiso mRNA into two-cell embryos and allowed them to develop until gastrulation (stage 10.5-11) (Fig. 3D). In comparison with controls, this results in an exogastrulae phenotype that is distinct from the developmental delay phenotype observed in xKMO morphants, but is similar to the phenotype of embryos that are maternally depleted of xTcf3 (Fig. 3D,E) (Houston et al., 2002; Ruzov et al., 2009). The normalised (relative to histone H4 or GAPDH levels) expression levels of Siamois are increased in the xKaiso mRNA-injected embryos (Fig. 3F). This observation suggests that high levels of xKaiso can interfere with xTcf3 function, resulting in a relative relief of xTcf3/Groucho-mediated repression of Siamois in gastrulating embryos.

These new results call for a revision of the model connecting the xKaiso repressor function and the xTCF3 repression/activation of Wnt-target genes (Kim et al., 2004; Park et al., 2005). The mode of interaction between xKaiso and xTCF3 mutually prevents binding to their cognate DNA sites, which can potentially inhibit the two pathways in which these transcription factors are major participants (Heasman, 2006). However, the recent observation that neither xWnt11 or Siamois expression is altered in xKMO morphants suggests that there is no intersection between these pathways during gastrulation (Ruzov et al., 2009).

Is this model operative in other biological contexts?

The role of Kaiso expressed in adult somatic tissues is not clear as there is no obvious mis-expression of normally silent genes in Kaiso-null mice (Prokhortchouk et al., 2006). In cancer cells, gene expression patterns are highly disturbed and this is mirrored by alterations in the level and genomic distribution of DNA methylation, as well as histone modifications (Ohm et al., 2007). Interestingly, Kaiso levels are increased in colon cancer cells and enhance polyp formation in Min mice ($Apc^{Min/+}$) (Prokhortchouk et al., 2006). This is juxtaposed by positional differences in TCF variant expression in colon cancer (Clevers, 2006). In this case,

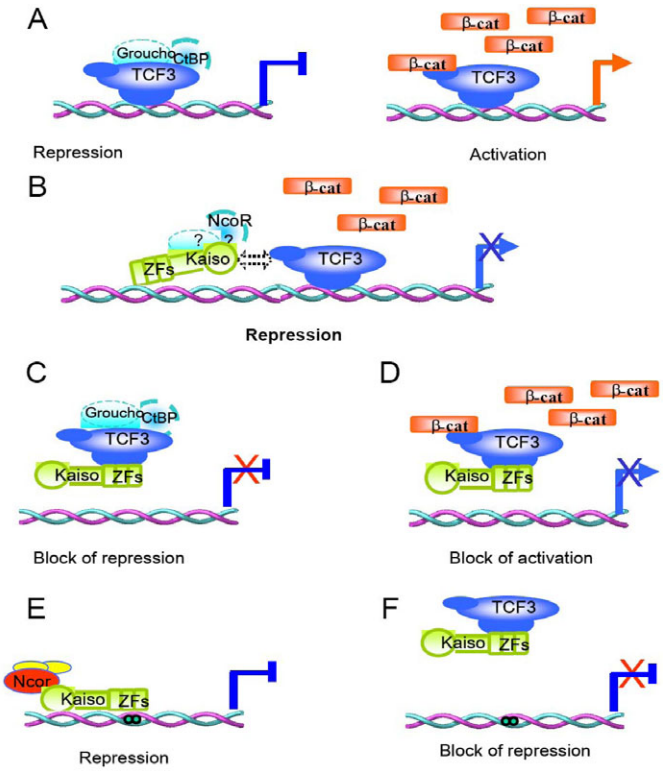

Fig. 4. Models for mutual interference of DNA-binding functions by Kaiso and TCF3. (**A**) A schematic representation of β-catenin-dependent activation of TCF3 target genes. β-Catenin displaces the Groucho/CtBP complex from TCF3 leading to gene activation. (**B**) A model for Kaiso-mediated repression of Wnt target genes that was suggested by Park et al. (Park et al., 2005). Here, it was proposed that repression of the Siamois gene occurs by xKaiso binding to CTGCNA sequences in its promoter and the possible interaction pf Kaiso with xTcf3. Our results suggest this model is not operative (Ruzov et al., 2009). (**C**,**D**) Our model of non-DNA-dependent xKaiso displacement of xTcf3 from target genes proposes that Kaiso overexpression may result in either a block of repression by xTcf3/Groucho or a block of β-catenin/xTcf3-dependent activation. (**E**,**F**) TCF3 can potentially displace Kaiso from its methylated (filed circles) DNA-binding sites and interfere with its repression function.

there is a possible intersection of Kaiso with Wnt signalling pathways, where overexpression of Kaiso could attenuate constitutive Wnt signalling, while at the same time promote cancer progression through silencing of de novo methylated tumour suppressor genes. By the same token, there is a potential for TCF3/4 to displace Kaiso from its cancer target genes and promote their re-expression, which may be coincident with alterations in the tumour environment, such as the transition to metastases phenotype (Fig. 4E). There are two related Kaiso-like proteins, Zbtb4 and Zbtb38, that may also mediate an intersection between epigenetic and cellular signalling pathways in cancer via protein-protein interactions (Filion et al., 2006; Weber et al., 2008). Zbt4 has been shown to inhibit MIZ1 regulation of p21CIP1 expression possibly by displacing this transcription factor from the p21CIP1 promoter (Weber et al., 2008). This suggests that the distributions of these Kaiso-like proteins and their relative abundance within nuclear sub-compartments has the potential to determine their transcriptional output of many signalling pathways.

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