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Kaiso is a key regulator of spleen germinal center formation by repressing Bcl6 expression in splenocytes

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ABSTRACT

Kaiso was previously described as a methylated DNA-binding protein and a transcription repressor interacting with the corepressor protein complex NCoR. In the current study, we show that generation-3 *Kaiso* knockout mice show a phenotype of splenomegaly and large diffused germinal centers (GC). In the spleens of *Kaiso* knockout mice, *Bcl6* (a transcriptional repressor that plays a critical role in GC development in spleen) and *c-Myc* were highly expressed, while the cell cycle arrest genes *p27* (*CDKN1B*), *p21* (*CDKN1A*) and *Gadd45a* were downregulated. Chromatin immunoprecipitation (ChIP) and transcription assays suggested that Kaiso represses Bcl6 expression, and in *Kaiso* knockout mice, derepressed *Bcl6* increased cell proliferation by suppressing *p27* (*CDKN1B*), *p21* (*CDKN1A*) and *Gadd45a*, while upregulating the oncogene *c-Myc*. Further evidence for Kaiso regulation of splenomegaly was provided by B lymphocyte Ramos cells, in which ectopic KAISO repressed *BCL6* and *c-MYC* expression, while concomitantly increasing the expression of the cell cycle arrestors *p21*, *p27* and *Gadd45a*. In summary, derepressed *Bcl6* expression may be responsible for increases in GC cell proliferation and splenomegaly of *Kaiso* knockout mice.

1. Introduction

Kaiso, a member of BTB/POZ protein family, was first isolated as a protein interacting with the armadillo-domain protein p120ctn [1]. Kaiso also binds to methylated CpG dinucleotides within the consensus sequence 5′-CGCG-3′ and the non-methylated consensus sequence 5′-CTGCNA-3′. Kaiso recruits the corepressor NCoR–HDAC complex to promote histone deacetylation, facilitating the formation of repressive chromatin structure within target gene promoters [2].

BCL6, another BTB/POZ family protein, is normally expressed in germinal center B/T cells and other lymphoid tissues. BCL6 also interacts with other regulatory proteins like corepressors BCoR or NCoR via its POZ domain and zinc fingers, regulating the expression of genes involved in B cell activation, differentiation, cell cycle arrest, and apoptosis [3–6]. BCL6 is a key regulator of GC development, and can be regulated both at the transcription and protein levels. For example, NF-kB-mediated transactivation of *IRF4* directly represses *BCL6* transcription [7]. MAP kinase-mediated phosphorylation of the BCL6 protein leads to BCL6 degradation by the ubiquitin–proteasome pathway. Various regulatory mechanisms of BCL6 expression assure precise control of GC reactions. In about 15–40% of DLBCL cases, chromosomal translocations place the *BCL6* gene under the control of heterologous promoters, resulting in elevated BCL6 expression [8–11]. Interestingly, the ONCOMINE database shows a negative correlation between the expression levels of *BCL6* and *KAISO* in several types of lymphoma, including diffuse large B-cell lymphoma (DLBCL, 6 cases), cutaneous follicular lymphoma (6 cases) and marginal zone B-cell lymphoma (5 cases).

In the current study, we show that, in the *Kaiso* knockout mouse, the spleen is enlarged and expresses high levels of Bcl6, as compared to control animals. Our results indicate that Kaiso controls proliferation of splenocytes by activating expression of cell cycle regulatory genes, *p27* (*Cdkn1b*), *p21* (*Cdkn1a*) and *Gadd45a* through transcriptional repression of *Bcl6*.

2. Materials and methods

2.1. Plasmid and antibodies

The pcDNA3.1-KAISO plasmid was prepared by cloning a cDNA fragment encoding the open-reading frame of human KAISO (KIAA0354) into pcDNA3.1 (Invitrogen, CA). The pGL2-Bcl6-Luc fu-

Abbreviations: GC, germinal center; BCL6, B-cell lymphoma 6 protein; DLBCL, Diffuse large B-cell lymphoma.

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sion and pGL2-c-Myc-Luc plasmids were prepared by cloning the Bcl6 promoter (−1 kb to +2.5 kb) and c-Myc (−2.4 kb to +504 bp) into the pGL2-Basic plasmid (Promega, Madison, WI). Antibodies against Bcl6 (sc-858; sc-7388), c-Myc (sc-40) and p21 (sc-397) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The anti-Kaiso (ab12723) antibody was purchased from Abcam (Cambridge, MA, USA), while anti-p27 (Kip1) (06-445) was from Millipore (Billerica, MA, USA), and anti-PCNA (Kip1) (#2586) was from Cell Signaling (Danvers, MA, USA).

2.2. Preparation of splenocytes

Spleens from *Kaiso* WT and knockout C57/6J mice were isolated, rinsed with washing buffer and placed into cell strainers. Spleens were the mashed using the plunger end of a syringe and the homogenate strained into petri dishes. Following centrifugation, supernatants were discarded and pellets resuspended in 1 ml ACK lysis buffer. Splenic cells were then incubated at RT for 5–10 min, followed by the addition of 9 ml of DMEM, centrifugation and resuspension in 3 ml DMEM.

2.3. Genotyping

DNA isolated from the tail biopsies or from the yolk sac was used for genotyping. The presence of the *Kaiso* WT or *Kaiso* KO alleles was verified by PCR using primer sets previously described (Ref. [12] and see below).

2.4. Isolation of total RNA and protein of the spleens of Kaiso WT and Kaiso knockout mice

Total RNA was isolated from spleens using RNeasy mini kits (QIAGEN, Valencia, CA, USA). cDNA was synthesised from 1 μg of total RNA using a QuantiTect reverse transcription kit (QIAGEN, cat. No. 05311), and RT-qPCR performed using SYBR Green Master Mix (Applied Biosystems, CA) The following oligonucleotide primers sets were used for the qPCR assays. qPCR primers used for mouse genes: *Kaiso* Forward, 5′-GCGTGGCCATGGTCTCTTT-3′, Reverse, 5′-GGGCCCGGAATTTTCG-3′; *p21* Forward, 5′-CATTCCCTGCC TGGTTCCTT-3′, Reverse, 5′-CATTCCCTGCCTGGTTCCTT-3′; *Bcl6* Forward, 5′-TCATTTGCGCCAGAAGCA-3′, Reverse, 5′-GACACGCGGTATT GCACCTT-3′; *Gadd45a* Forward, 5′-GGTGAGCCTGAAGAAGGAAGCT-3′, Reverse, 5′-TTCTTGCAGTGCTTTGTAGTTTTTG-3′; *c-Myc* Forward, 5′-CTTCTCTCCTTCCTCGGACTC-3′, Reverse, 5′-GGAGATGAGCCCGAC TCCGACCTC-3′; *18s* Forward, 5′-AGTCCCTGCCCTTTGTACACA-3′, Reverse, 5′-GATCCGAGGGCCTCACTAAAC-3′. qPCR primers used for human genes: *KAISO* Forward, 5′-CCGAGATTCTGCCCACAAA-3′, Reverse, 5′-GGGCGAGTTATTGCTAGCACTAG-3′; *p21* Forward, 5′-A GGGGACAGCAGAGGAAG-3′, Reverse, 5′-GCGTTTGGAGTGGTA-GAAATCTG-3′; *BCL6* Forward, 5′-AATGAGTGTGACTGCCGCTTCT-3′, Reverse, 5′-CACCGGTATGGACGGTCTTG-3′; GADD45A Forward, 5′-CCCCGATAACGTGGTGTTG-3′, Reverse, 5′-GCAGCAAAACGCCTG-GAT-3′; *c-MYC* Forward, 5′-CAGTGGGCTGTGAGGAGGTT-3′, Reverse, 5′-CAGGCTCCTGGCAAAAGGT-3′; *18S* Forward, 5′-AGTCCCT GCCCTTTGTACACA-3′, Reverse, 5′-GATCCGAGGGCCTCACTAAAC-3′.

2.5. Histology and immunohistochemistry

Three *Kaiso* knockout mice and age- and sex-matched *Kaiso* wild type C57/6J mice littermates were sacrificed. Mice tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA). For immunohistochemistry, spleens were fixed overnight in 4% paraformaldehyde at room temperature, as has been described elsewhere [13].

2.6. Western blotting

Ramos cells and splenocytes were harvested and lysed in RIPA buffer (50 mM Tris–HCl (pH 8.0), 1% NP-40, 0.25% sodium deoxycholic acid, 150 mM NaCl, 1 mM EGTA, and complete Mini-Protease cocktail), and total cell-extracted protein (40 μg) was separated on 12% or 10% SDS–PAGE gels. Proteins were then transferred onto Immun-Blot™ PVD membranes (Bio-Rad, Hercusles, CA, USA), blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA) or BSA, and the blotted membranes were incubated with antibodies against Kaiso, Bcl6, p21, p27, and GAPDH and then incubated with anti-mouse or rabbit secondary antibody conjugated with HRP (Vector Laboratories, Burlingame, CA, USA). Protein bands were visualized with ECL solution (PerkinElmer Life Sciences, Santa Clara, California, USA).

2.7. Quantitative chromatin immunoprecipitation (qChIP-reChIP) assays

The molecular interaction between Kaiso and NCoR on the endogenous *Bcl6* promoter in the spleen tissues of the *Kaiso* WT and *Kaiso* knockout mice was analyzed by a standard chromatin immunoprecipitation (ChIP-reChIP) assay protocol, as we have described elsewhere [17]. Quantitative PCR of chromatin-immunoprecipitated DNA was carried out using the following oligonucleotide primer sets, designed to amplify the two *Bcl6* upstream regulatory regions and one regulatory region located in intron 1 of the *Bcl6* gene. (R1, Forward 5′-GTCAATGGTATTAGGTTGAA ATAT-3′ Reverse 5′-TCCTCGAGCTAAATACACAAAAG-3′; R2, Forward 5′-GCCGAGTTTATGGGTAGGAGAGG-3′ Reverse 5′-ACTTCTAA GAACCCCAGTTCTAA-3′; R3, Forward 5′-TCTTTGCGAGGTGTGTGGT GTGAG-3′ Reverse 5′-TCCTTTGAGCTCAAGTTTGCTACC-3′.

2.8. Electroporation

Electroporation was performed using a Neon Transfection System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Ramos and MEF cells were washed with PBS and resuspended in electroporation buffer containing plasmid DNA, and the cells then electroporated (condition: volts 1350 V, pulse width 30 ms, pulse number 1) using a 100 μl tip. After electroporation, cells were suspended in RPMI medium and cultured.

2.9. Cell growth curves

Electrotransfected (with 8 μg KAISO expression vector). Ramos cells were grown in 70 ml T-flasks, cultured overnight, and plated at 5×10^5 cells/well in 24-well dishes. Cell numbers were then counted daily by harvesting cells for 4 days, using an ADAM Automated Cell Counter (Bulldog Bio, New Hampshire, USA).

2.10. Site-directed mutagenesis

Mutations of Kaiso binding sites were introduced by site-directed mutagenesis using the pGL2-Bcl6-Luc WT and pGL2-c-Myc-Luc WT expression plasmids as a template. Conditions of PCR were as follows: denaturation step at 95 °C for 5 min, followed by 18 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min and an elongation step at 68 °C for 10 min. The oligonucleotides used for generation of the mutants were as follows: *KB1* Forward, 5′-CGTCTCTC TAAACTCTCCTCTAACACACTTG-3′, Reverse, 5′-CAAGTGTGTTAGAG GAGAGTTTAGAGAGACG-3′; *KB2* Forward, 5′-ACCAAGTGCCTTTGGT GTGCACTTGAGCAGGACC-3′, Reverse, 5′-GGTCCTGCTCAAGTGCA-CACCAAAGGCACTTGGT-3′; *KB3* Forward, 5′-CACTCTCCCCAACCACT CGACTGACCCAACATCA-3′, Reverse, 5′-TGATGTTGGGTCAGTCGAGT GGTTGGGGAGAGTG-3′.

3. Results and discussion

3.1. Increased splenocytes number and splenomegaly in Kaiso knockout mice

Previously, Prokhortchouk et al. reported that the *Kaiso* knockout mouse lacked any explicit phenotype [12]. Nonetheless, we further husbanded and crossed *Kaiso* knockout mice (Fig. 1A) and in agreement with that report, did indeed observe that the first-(G1) and second-generation (G2) *Kaiso* knockout mice lacked any observable phenotypes. However, generation-3 (G3) *Kaiso* knockout mice showed increase in both bodyweight and spleen size, as compared to their control C57BL/6 strain mouse littermates. The spleens of eight-week-old *Kaiso* knockout mice, age- and sex matched, were larger (86 ± 5 mg) than those of the control mice (56 ± 8 mg) (53% increased spleen mass, see Fig. 1B and C), while the size of the hearts and kidneys were similar in both groups. Increased spleen size also correlated with increased splenocyte numbers, with *Kaiso* knockout mice spleens containing approximately a 4-fold greater number of cells than those of the control littermates (*Kaiso* WT 2.38×10^5 vs. *Kaiso* knockout 8.6×10^5). Thus, these results suggest that *Kaiso* regulates spleen size (and consequently, splenomegaly) by negatively regulating cell proliferation.

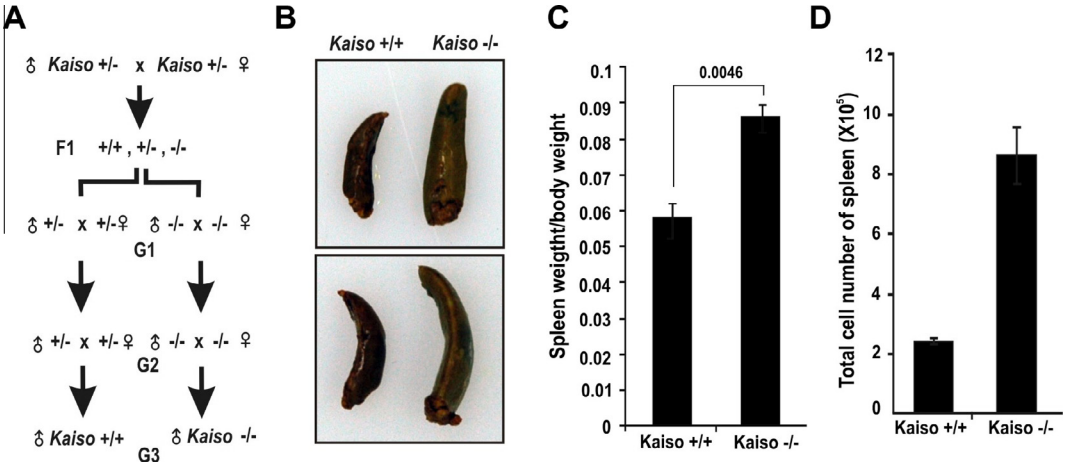

Fig. 1. Increased splenocyte numbers and splenomegaly in *Kaiso* knockout mice. (A) Mouse breeding scheme used to obtain homozygous *Kaiso* knockout (e.g., *Kaiso*$^{-/-}$) generation-3 G3 mice. (B–D) Comparisons of the appearance and total cell numbers in spleens from male *Kaiso* WT ($n = 6$) and *Kaiso* knockout mice ($n = 6$) (shown as means ± s.d.).

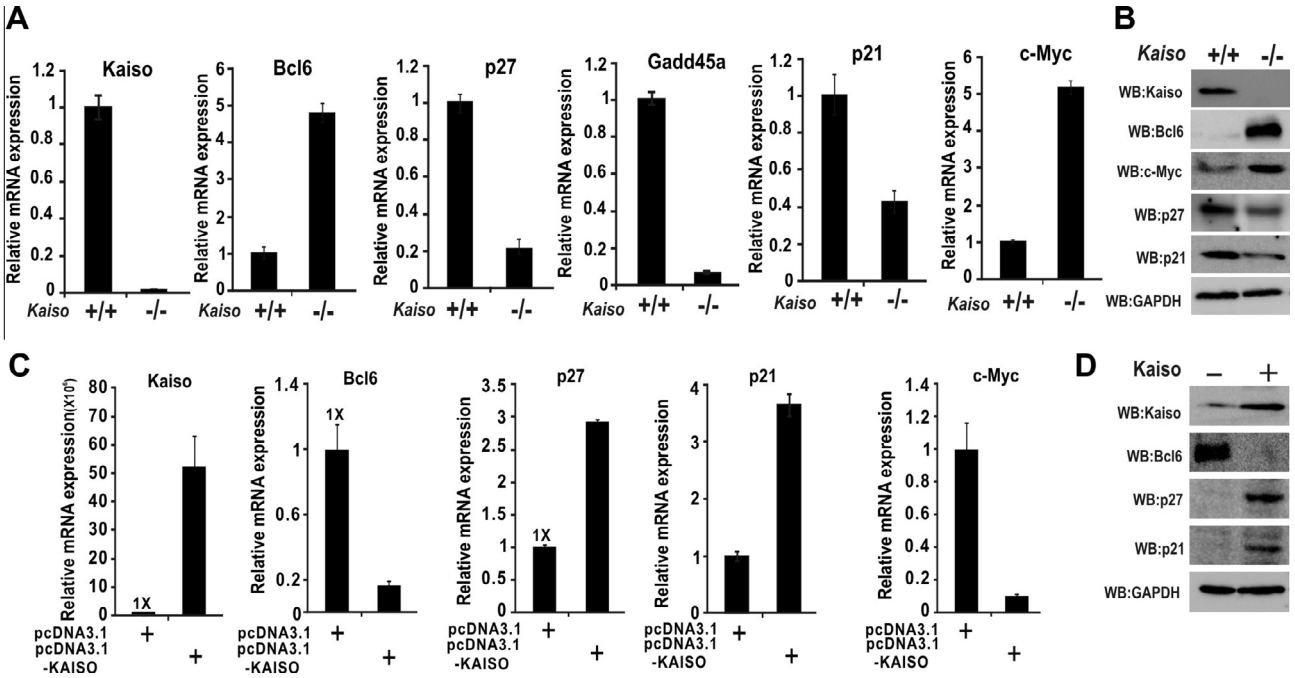

Fig. 2. *Bcl6* expression is high and Bcl6 target genes regulating cell proliferation are decreased in the spleens of *Kaiso* knockout mice. (A) qRT-PCR analyses of mRNA levels of *Kaiso*, *Bcl6* and the *Bcl6* target genes, *p27, Gadd45a*, *p21* and *c-Myc* in spleens from *Kaiso* WT and *Kaiso* knockout mice, with normalization to 18S RNA. (B) Western blots of Kaiso, Bcl6, p27, p21 and c-Myc protein expression in the spleen tissues of the *Kaiso* WT and *Kaiso* knockout mice. GAPDH, control. (C) RT-qPCR analyses of mRNA levels of *Kaiso, BCL6, p27, p21* and c-Myc expression in Ramos B-lymphocyte cells. Ramos cells were transfected with either a control vector or Kaiso expression vector, and then analyzed for expression of endogenous genes, with normalization to 18S RNA. (D) Western blots of Kaiso, Bcl6, p27 and p21 in Ramos cells transfected with Kaiso expression vector and cultured overnight. GAPDH, control.

3.2. Bcl6 expression is high, while Bcl6 target genes regulating cell proliferation are deregulated in the spleens of Kaiso knockout mice

Gene expression microarray analyses spleen mRNA from *Kaiso* WT and knockout mice revealed significantly increased *Bcl6* mRNA expression in the knockout spleens. Bcl6 is a critical gene in germinal center formation, and overexpression of *Bcl6* by chromosomal translocation is causal for diffused large B-cell lymphoma (DLBCL) [3–5]. Based on the enlarged spleen size and increased total number of splenocytes in *Kaiso* knockout mice, we next investigated whether cell proliferation-associated genes were affected by *Kaiso* absence and *Bcl6* overexpression. To validate differential expression of *Bcl6* mRNA obtained from microarray analyses, we isolated total RNA from the spleens of 8 week-old *Kaiso* WT and *Kaiso* knockout mice, with RT-qPCR showing fivefold increased Bcl6 mRNA expression levels in *Kaiso* knockout mice (Fig. 2A). In agreement with previous studies showing Bcl6 regulation of the genes negatively regulating cell proliferation such as *p27* (*CDKN1B*), *p21*

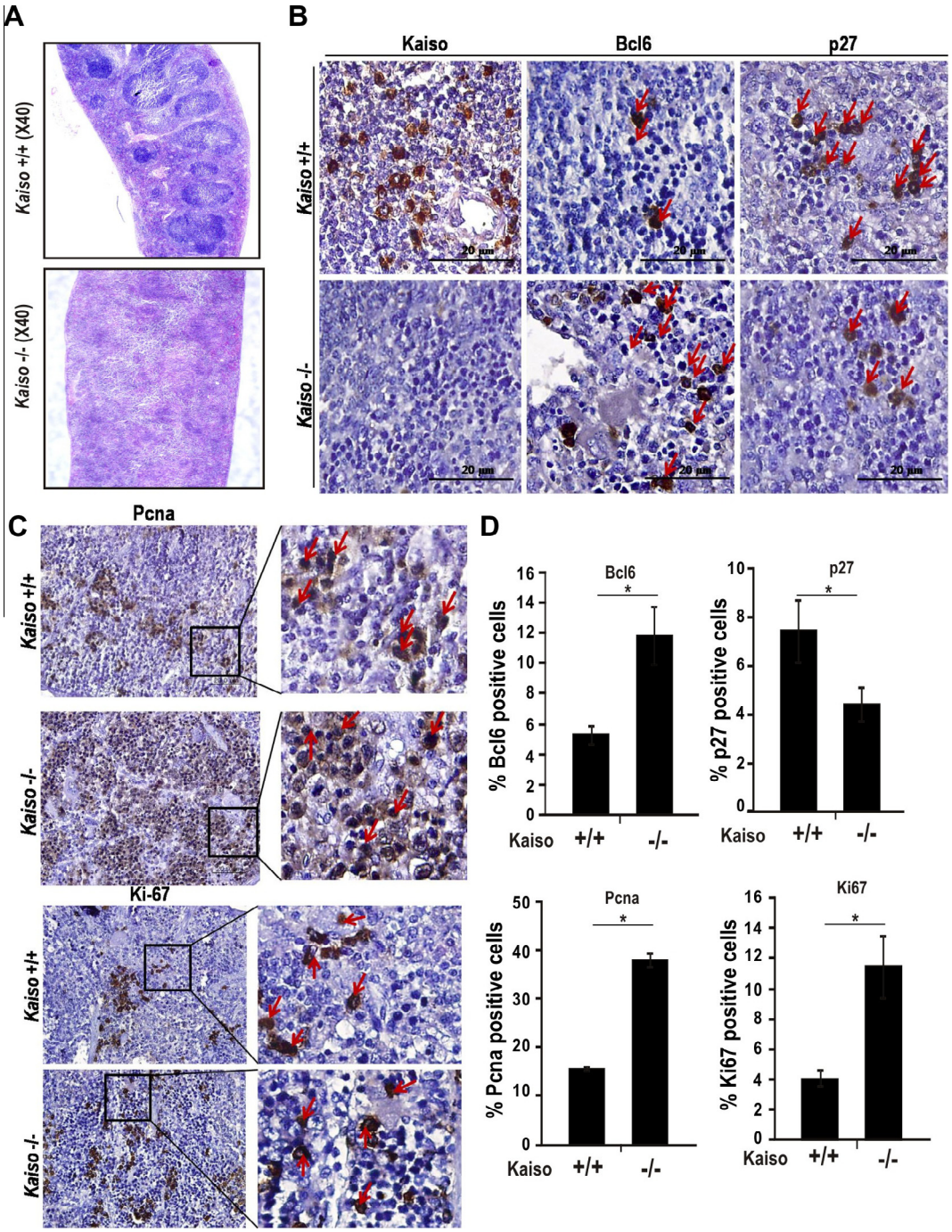

Fig. 3. Spleen morphology and immunohistochemical analysis of expression of Bcl6 and cell cycle regulators in wild-type and *Kaiso* knockout mice. (A) Staining of the spleen tissues sections of 8 week-old *Kaiso* WT and *Kaiso* knockout mice using haematoxylin and eosin (H&E). (B and C) Immunohistochemistry using primary antibodies against Kaiso, Bcl6, p27, Pcna and Ki67 on tissues sections, followed by visualization by DAB staining. (D) Quantification of the number of the cells expressing nuclear Bcl6, p27, Pcan and Ki67, as analyzed by Immunoratio program and depicted in histograms. Data shown is the average of the five randomly selected microscopic areas (400× magnification. $^{*}p = 0.001$. Error bars represent S.D.).

(*CDKN1A*), *Gadd45* and cell proliferation stimulating *c-Myc* [4,14,16], our RT-qPCR and Western blot results showed the cell cycle inhibitors p27 (Cdkn1b), p21 (Cdkn1a) and Gadd45 to be strongly downregulated, while the c-*Myc* was strongly elevated by Bcl6 in spleens of *Kaiso* knockout mice (Fig. 2A and B).

We also examined, a B lymphocyte cell line derived from a Burkitt's patient, Ramos cells, that is known to expresses high levels of *BCL6* and undergo rapid cell proliferation [4]. We thus tested whether ectopic KAISO could repress BCL6 expression and derepress *p21* and *p27* in Ramos cells. In agreement with our above mouse observations, qRT-PCR and Western blot analyses both showed that ectopic KAISO significantly repressed endogenous *BCL6*, while simultaneously inducing endogenous *p27* and *p21* and decreasing c-MYC in Ramos cells (Fig. 2C and D).

Taken together, the reports by others [4,14–16] and out current data show that Kaiso represses *Bcl6* expression, while also relieving transcriptional repression of *p27* (*Cdkn1b*), *p21* (*Cdkn1a*) and *Gadd45*, concurrently decreasing the expression of *c-Myc*.

3.3. Spleen morphology and genes expression analysis of Bcl6 and cell cycle regulators

As described above, the size and total number of splenocytes were increased in *Kaiso* knockout mice. Consequently, we next analyzed spleen tissues of eight weeks-old *Kaiso* WT and *Kaiso* knockout mice by histology and immunohistochemistry. Microscopic analysis of paraffin-embedded, H&E-stained formalin fixed spleen tissue sections showed a large diffused germinal center (GC) and an apparently absent corona (Fig. 3A). *Bcl6* overexpression is primary responsible for DLBCL with diffused GC [15], and we also observed *Kaiso* knockout spleen GC to be highly diffused, concurrent with Bcl6 overexpression.

We also analyzed protein expression of Kaiso, Bcl6, p27, Pcna and Ki67 by immunohistochemistry, showing the number of splenocytes expressing Bcl6 to be significantly increased by 2-fold (Fig. 3B), while p27-expressing cells decreased by 45%. Analogously, the numbers of cells overexpressing Pcna and Ki67, markers of cell proliferation, were increased by approximately threefold in *Kaiso* knockout mice (Fig. 3C and D). The results indicated that derepression of *Bcl6* in the absence of Kaiso stimulates splenocyte proliferation, resulting in a highly diffused GC and splenomegaly. These data further suggest that Kaiso plays an important role in GC formation by repressing expression of *Bcl6*.

3.4. KAISO and the corepressor NCoR bind BCL6 gene promoter, in Ramos cells, thus repressing its transcription

The above results support a role for Kaiso in repression of *Bcl6*. Previously, Kaiso was also shown to repress transcription by interacting with the nuclear corepressor NCoR [2]. Consequently, using chromatin immunoprecipitation analysis (ChIP-reChIP), we examined whether Kaiso and NCoR could bind to the regulatory regions of Bcl6 promoter. Of the three regulatory regions on the Bcl6 promoter (R1, R2 and R3), R1 (bp, −900∼−450) and R3 (bp, +2165∼+2423) region both were immunoprecipitated by the antibodies against KAISO and NCoR ChIP, but only in the spleen of *Kaiso*

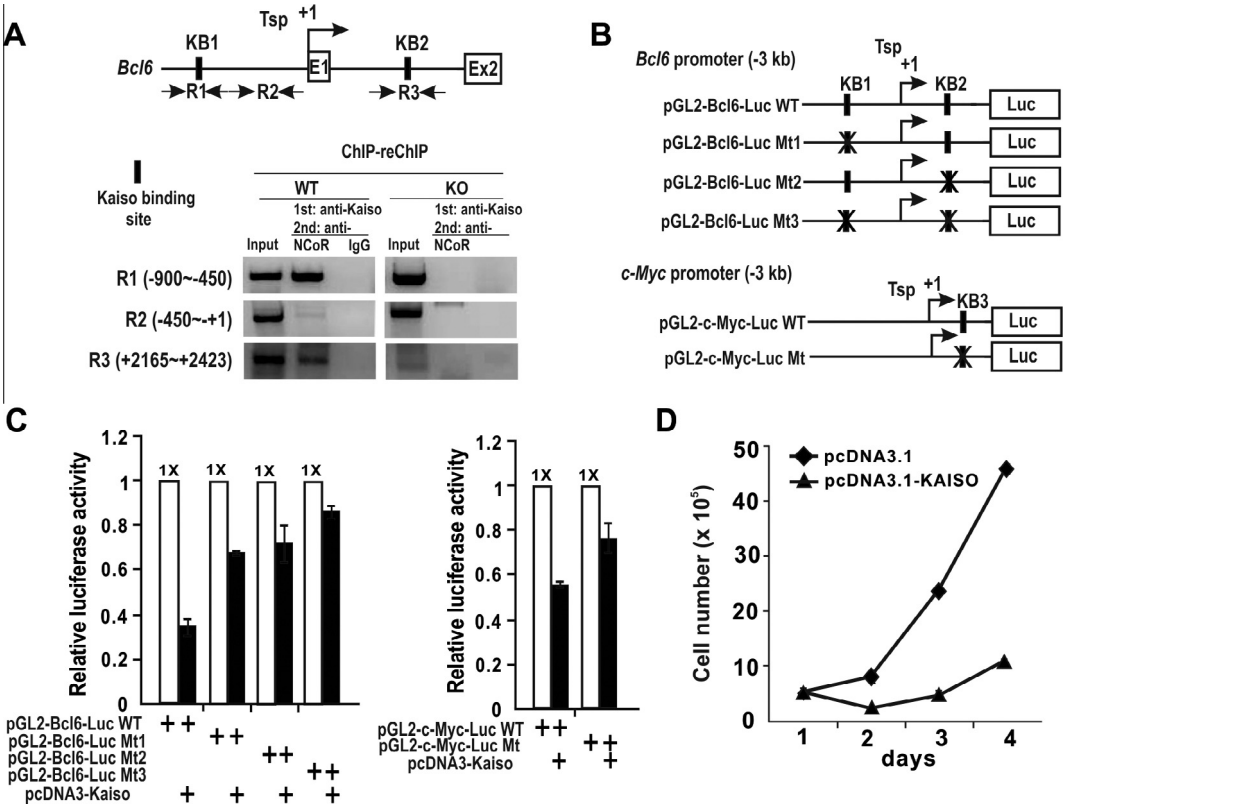

Fig. 4. Kaiso and the corepressor NCoR complex bind the *Bcl6* gene promoter. KAISO inhibits proliferation of Ramos cells by transcriptional repression of *BCL6*. (A) ChIP-reChIP assays. The locations of the R1, R2, R3, of the ChIP PCR primer-binding sites of the *Bcl6* promoter are indicated by arrows. Tsp, transcription start site (+1). DNA binding activity of Kaiso–NCoR complex to the *Bcl6* promoter was detected only in spleens from *Kaiso* WT mice. (B) Structures of the pGL2-Bcl6-Luc WT, pGL2-c-Myc-Luc WT and mutant reporter plasmids tested. Closed rectangular box, Kaiso binding sites; X, mutation introduced, Tsp, transcription start site (+1). (C) Transient transcription assays. Various reporter plasmids and Kaiso expression vector were co-transfected into MEF cells, cultured and analyzed for luciferase activity. Data are presented as the average of three independent assays. Bars indicate standard deviation. (D) *KAISO* inhibits proliferation of Ramos B-lymphocyte cells. Ramos cells were transfected with a KAISO expression vector and cell numbers counted daily using an ADAM Automated Cell Counter.

WT mice. These results, along with Fig. 2, strongly indicate that a KAISO–NCoR complex that binds the promoter of *Bcl6* may directly repress transcription of the gene (Fig. 4A).

We analyzed the *Bcl6* promoter sequence for the potential Kaiso binding sites identical to the Kaiso binding consensus sequence, CTGCNA, and found two potential Kaiso binding sites (KB1, KB2) (Fig. 4A). We tested whether Kaiso can directly repress transcription of the *Bcl6* promoter and also the two potential Kaiso binding sites are important for transcriptional repression, by transient transfection and transcription assays in MEF cells (Fig. 4C). We prepared the Bcl6 promoter-luciferase gene fusion reporter construct derived from the pGL2-Luc Basic. And, to test the functions of the two potential Kaiso binding elements in transcription repression by Kaiso, three other reporter constructs with single or double mutation(s) introduced at the KB1 or KB2 by site-directed mutagenesis. Transcription assays showed that Kaiso represses *Bcl6* promoter potently. The mutation of KB1 or KB2 significantly decreased transcription repression by Kaiso. The mutations of both KB1 and KB2 nearly abolished transcription repression by Kaiso, suggesting that the two elements are important in transcription repression of *Bcl6* by Kaiso (Fig. 4C).

In Xenopus, it was reported that *c-Myc* is the target of Kaiso and Kaiso represses *c-Myc* promoter [18]. Accordingly, we tested whether Kaiso can repress *c-Myc* promoter in mouse. Because mouse *c-Myc* promoter has a potential Kaiso binding site (bp, +356∼+363), we prepared reporter plasmids, a pGL2-c-Myc-Luc WT and pGL2-c-Myc-Luc Mt with mutation at KB3 element (Fig. 4B bottom). Kaiso repressed transcription of *c-Myc* promoter and the mutation of KB3 decreased repression by Kaiso, suggesting the KB3 is probably important in directly transcription repression by Kaiso. Because Kaiso can potentially increase *Blimp1* expression by transcription repression of *Bcl6* gene and increased Blimp1 may repress c-Myc expression [19], Kaiso may also indirectly repress c-Myc expression. These data suggested that Kaiso may repress c-Myc expression by direct transcription repression of *c-Myc* promoter by Kaiso binding or indirectly via transcriptional repression of *Bcl6* promoter by Kaiso.

To further study Kaiso's role in cell proliferation, we examined whether ectopic KAISO can inhibit Ramos cell proliferation by repressing *BCL6* expression. To that end, Ramos cells were transfected with a KAISO expression vector and cell numbers were counted daily. As shown in Fig. 4B, *KAISO* overexpression potently inhibited Ramos cell proliferation by repressing expression of endogenous BCL6. Likewise, Kaiso significantly repressed *Bcl6* transcription by binding to the Bcl6 promoter and recruiting corepressor NCoR (Fig. 4A). These findings strongly suggest that elevated Bcl6 in *Kaiso* knockout mice increases cell proliferation by repressing expression of cell cycle inhibitory genes.

In summary, we show in this report that Kaiso is a negative regulator of splenocyte proliferation by derepressing the cell cycle regulators *p27* (*Cdkn1b*), *p21* (*Cdkn1a*) and *Gadd45a*, through transcription suppression of *Bcl6*. It is intriguing to speculate that deregulation of Kaiso expression may contribute to the pathogenesis of several types of B cell lymphomas, which similarly show GC diffusion and splenomegaly [4,5,15]. Although it is not certain whether elevated *Bcl6* expression in *Kaiso* knockout mice actually contributes to oncogenesis, the spleen is significantly enlarged and germinal centers are highly diffused. To more definitively answer this question, xenotransplantation experiments using *Kaiso* knockout spleen tissues may prove informative in future studies.

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