Converging disease genes in ICF syndrome: ZBTB24 controls expression of CDCA7 in mammals

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Abstract

For genetically heterogeneous diseases a better understanding of how the underlying gene defects are functionally interconnected will be important for dissecting disease etiology. The Immunodeficiency, Centromeric instability, Facial anomalies (ICF) syndrome is a chromatin disorder characterized by mutations in DNMT3B, ZBTB24, CDCA7 or HELLs. Here, we generated a Zbtb24 BTB domain deletion mouse and found that loss of functional Zbtb24 leads to early embryonic lethality. Transcriptome analysis identified Cdca7 as the top down-regulated gene in Zbtb24 homozygous mutant mESCs, which can be restored by ectopic ZBTB24 expression. We further demonstrate enrichment of ZBTB24 at the CDCA7 promoter suggesting that ZBTB24 can function as a transcription factor directly controlling Cdca7 expression. Finally, we show that this regulation is conserved between species and that CDCA7 levels are reduced in patients carrying ZBTB24 nonsense mutations. Together, our findings demonstrate convergence of the two ICF genes ZBTB24 and CDCA7 at the level of transcription.

Introduction

The Immunodeficiency, Centromeric instability and Facial anomalies (ICF; OMIM 242860/614069) syndrome is an autosomal recessive disorder characterized by immunodeficiency, developmental delay and facial anomalies. Patients present with hypo- or a-gammaglobulinemia, in the presence of circulating B cells, causing recurrent and often fatal respiratory and gastrointestinal infections. All patients show DNA hypomethylation of the centromeric satellite II and III repeats, which together with chromosomal abnormalities in phytohemagglutinin-stimulated cells are the molecular hallmarks for the ICF syndrome (1,2). Based on the underlying genetic defects, ICF syndrome can be divided into at least five different clinically indistinguishable subgroups (ICF1, ICF2, ICF3, ICF4 and ICFX) (3). About half of the ICF patients carry mutations in the de novo DNA methyltransferase 3B gene (DNMT3B), which is referred to as ICF1 (1,4). ICF2 patients carry mutations in the zinc-finger- and BTB domain containing 24 gene (ZBTB24) and account for ~30% of the ICF cases (1,5,6). Recently, mutations in cell division cycle associated 7, CDCA7 and helicase, lymphoid-specific, HELLS/LSH, have been shown to be causative for the ICF3 and ICF4 subtypes respectively, and together with few unexplained cases (ICFX) account for the remaining ICF cases (3). Explaining the molecular mechanisms underlying ICF syndrome has remained difficult. The majority of molecular studies...
have focused on the analysis of gene expression and DNA methylation patterns in cell lines of normal and ICF1 patients. DNA hypomethylation and aberrant expression of germ line genes and genes important for immune function have been reported (7–11). ICF1 mouse models carrying hypomorphic Dnmt3b mutations have been described and shown to recapitulate some of the disease aspects such as hypomethylation of repeat sequences, aberrant expression of germ line genes and craniofacial abnormalities (12–14). The most prominent difference between mouse and human is the absence of B cell defects in an ICF1 mouse model. In contrast, apoptosis of T-cells was reported after birth (12). Hells loss of function mice display genome-wide DNA hypomethylation and perinatal lethality (15,16). In addition, B and T cell abnormalities have been reported in Rag2−/− mice that were injected with Hells−/− fetal liver cells (17). In mouse embryonic stem cells (mESCs), Hells has been proposed to facilitate Dnmt3b recruitment to its targets (18). This highlights a functional convergence between Dnmt3b and Hells and provides a possible explanation for the involvement of both genes in the ICF syndrome.

Little is known about the molecular function of Zbtb24 and Cdc7. Zbtb24 is a member of the BTB domain family of proteins, of which many are important for hematopoietic differentiation (19). Zbtb24 contains eight C2H2 zinc fingers and a function as a transcription factor has been suggested (20). Cdc7 has previously been shown to interact with Myc and is involved in neoplastic transformation and hematopoietic stem cell emergence (21,22). Mouse Zbtb24 can localize to heterochromatin (5) and it has been shown that knock down of Zbtb24 and Cdc7 in mouse embryonic fibroblasts leads to hypomethylation of minor satellite repeats, suggesting a role for the two factors in the maintenance of DNA methylation (3).

In this study, we show that mice homozygous for a Zbtb24 BTB-domain deletion are early embryonic lethal and that Cdc7 expression is lost in ES cells from these mice. We find that this regulation is conserved between species and that overexpression of human ZBTB24 can restore Cdc7 mRNA levels in homoyzous mutant mESCs. We further demonstrate enrichment of ZBTB24 at the CDC7 promoter suggesting a direct transcriptional regulation. Finally, we show that CDC7 levels are affected in ICF patients carrying ZBTB24 nonsense mutations.

Results
Generation of Zbtb24 BTB-domain deletion mouse and derivation of mESC lines
To study the molecular function of Zbtb24, the Zbtb24 gene was mutated by partial replacement of the BTB-domain region with a PGK-Neomycin (PGK-NEO) cassette, in antisense orientation, using standard gene targeting techniques (Fig. 1A and Supplementary Material, Fig. S1A). This results in the deletion of the last 15 bps of the non-coding exon 1 and the first 430 bps of exon 2, which encodes the BTB and AT-hook domains, and the first zinc finger motif. Southern blot and long range PCR were used to show integration of the PGK-NEO cassette and no random insertion was found (Supplementary Material, Fig. S1). Based on previous biochemical studies on BTB-domain containing proteins, this deletion is predicted to disrupt protein-protein interactions and regulation of gene expression through altered chromatin conformation (23). Mice heterozygous for the Zbtb24 BTB-domain deletion, Zbtb24+/−, appeared to be normal, fertile and indistinguishable from their wild-type littermates at weaning (~3 weeks of age) and throughout adulthood. Following heterozygous intercrosses, no viable homozygous Zbtb24−/− mice were recovered from a total of 129 offspring. The proportions of wild-type (n = 45) and heterozygous (n = 84) offspring were as expected for homozygous lethality (Fig. 1B). Timed matings between heterozygous Zbtb24−/− mice produced no viable homozygous Zbtb24−/− embryos at E9.5 (Fig. 1B). The presence of implantation sites at E9.5 suggests that embryonic death of homoyzogotes occurs sometime between E4.5–9.5.

To date, no knock out mouse model for Zbtb24 has been described but recently, mousephenotype.org reported complete homozygous lethality pre-weaning for the Zbtb24−/− (https://www.mousephenotype.org/, date last accessed July 26, 2016). This is in agreement with our observations.

The early embryonic lethality prompted us to establish Zbtb24−/− mutant mESC lines. In total, 24 blastocysts from two heterozygous intercrosses were put into culture, which resulted in the establishment of 19 independent mESC lines. Genotyping identified six wild-type, eight heterozygous and five homoyzgous Zbtb24−/− mutant mESC lines. We decided to continue with three wild-type and three Zbtb24−/− homozygous mutant mESC lines and maintained the mESCs in the presence of the two small-molecule inhibitors PD0325901 and CHIR99021, together known as 2i. mESCs grown in 2i resemble cells in the pre-implantation embryo (24) and have reduced DNA methylation levels genome wide (25). Wild-type and homozygous Zbtb24−/− mutant mESCs were phenotypically normal, showed a homogeneous morphology characteristic for 2i and were positive for the expression of pluripotency marker genes (Fig. 1C and D).

Transcriptome-wide differences between wild-type and homozygous Zbtb24−/− mutant mESCs
To characterize the effect of the Zbtb24 BTB-domain deletion mutation on the mESC transcriptome we performed RNA sequencing (RNA-seq) on total RNA isolated from independent wild-type and Zbtb24−/− homozygous mutant mESC lines, established and cultured feeder free in 2i (n = 3 per genotype). Genome wide we found a total of 139 significantly differentially expressed genes, using a cutoff that defined significant changes as at least 0.5 log fold difference in gene expression and an adjusted P-value of <0.05 (Fig. 2A and Supplementary Material, File S1). A similar number of up- and down-regulated genes were observed relative to wild-type controls. Functional annotation clustering of differentially expressed genes using KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis did not show any enrichment for specific terms and chromosome graph showed homogeneous distribution of differentially expressed genes over the mouse genome (Supplementary Material, Fig. S2A). Analysis of gene expression levels for known lineage specification genes showed that there was a good correlation with a published RNA-seq dataset from mESCs grown on 2i (26) and did not reveal any major differences between wild-type and homozygous Zbtb24−/− mutant mESCs (Supplementary Material, Fig. S2B).

By disrupting exon 1 and 2 of the Zbtb24 gene we intended to create a knockout mouse model. Unexpectedly, our RNA-seq analysis revealed that Zbtb24 mRNA levels were not affected in Zbtb24−/− homozygous mutant mESCs (Fig. 2B and Supplementary Material, Fig. S3A) suggesting that an aberrant Zbtb24−/− transcript can be produced in the mutants. To enable characterization of the transgenic Zbtb24 locus we mapped to an artificial mouse genome reference containing the sequence of the Zbtb24 locus with the inserted reverse PGK-NEO cassette, resembling our transgene insertion. This analysis suggested that an aberrant Zbtb24 transcript initiated in the PGK promoter
Figure 1. Characterization of homozygous Zbtb24ΔBTB mutant mESCs. (A) Genotyping of Zbtb24ΔBTB heterozygous mice. The wild-type allele was detected as a 363-bp band (P2 + P1) and the mutant allele as a 321-bp band (P3 + P1). Black arrows indicate primers. (B) Timed matings and intercrosses of Zbtb24ΔBTB mice. Data shows the number of mice observed (and in brackets the percentage) at E9.5–11.5 and 3 weeks. (C) Cellular morphology of wild-type and homozygous Zbtb24ΔBTB mutant mESCs grown feeder free on 2i. Scale bar, 250 μm. (D) qRT-PCR analysis of pluripotent gene expression levels in wild-type and homozygous Zbtb24ΔBTB mESCs. Error bars, SEM from three biological replicates per genotype; NS, non-significant.

Figure 2. Transcriptome analysis of wild-type and homozygous Zbtb24ΔBTB mutant mESCs. (A) MA plot showing RNA-seq analysis results from Zbtb24 wild-type and homozygous Zbtb24ΔBTB mutant mESCs. Three independent biological replicates were analyzed per genotype. Significantly differentially expressed genes are shown in red, adj P < 0.05. Black data points represent genes whose expression were not significantly altered in Zbtb24ΔBTB mutant mESCs. (B) IGV RNA-seq track showing a putative translation start site at the transgenic Zbtb24 locus in a homozygous Zbtb24ΔBTB mutant mESC line. Bidirectional transcription of the PGK promoter was detected. An ATG is present near the TSS region of the mutant Zbtb24 transcript that does not affect the reading frame of the protein suggesting that a truncated Zbtb24 protein can be produced. The green and blue lines represent a nucleotide mismatch in sequencing reads. (C) Polysome profiling in wild-type and homozygous Zbtb24ΔBTB mutant mESCs. Error bars – SEM, two biological replicates for each genotype. (D) Western blot showing full-length Zbtb24 (~80 kDa) in wild-type and severely reduced levels in homozygous Zbtb24ΔBTB mutant mESCs. Tubulin is shown as a loading control.
flanking the NEO cassette (Supplementary Material, Fig. S3A and B). No aberrant transcription from genes neighboring the Zbtb24 locus was observed in the Zbtb24 homozygous mutant mESCs (Supplementary Material, Fig. S3C). We used polysome profiling to assess whether the aberrant Zbtb24 transcript can be engaged by ribosomes. We found no difference in the ratio of total RNA/polysome-bound RNA between wild-type and Zbtb24 homozygotes suggesting that Zbtb24 mRNA could be translated into an amino-terminal truncated Zbtb24 protein that lacks the BTB and AT hook domains (Fig. 2C). Finally, we used a C-terminal Zbtb24 antibody and Western-blot analysis to determine whether Zbtb24 protein could be detected in Zbtb24 homozygous mutant mESCs. Zbtb24 was detected at its predicted size of around 80 kDa in wild-type but severely reduced in the Zbtb24 homozygous mutant mESCs (Fig. 2D). Furthermore, we could not detect an additional band in the mutant mESCs that could be indicative of an amino-terminal truncated Zbtb24 protein. Together, our results suggest that the amino-terminal truncated Zbtb24 protein lacking the BTB and AT hook domains is likely to be unstable.

The top significantly down-regulated gene (2.57 log2 fold change and adjusted P-value 4.58E-131) in our RNA-seq dataset was Cdca7 (Fig. 3A), in which mutations have recently been shown to be causative for ICF3 (3). Western-blot analysis revealed that the decrease in mRNA levels correlates with reduced levels of Cdca7 protein in Zbtb24 homozygous mutant mESCs (Fig. 3B). The finding that Cdca7 expression levels are down regulated in Zbtb24 homozygous mutant mESCs was unexpected and to our knowledge, no functional relationship between these two genes has previously been reported.

Our observation of Cdca7 dysregulation in Zbtb24 homozygous mutant mESCs prompted us to examine whether additional relationships between the four ICF genes exist. We conducted knock down experiments on wild-type mESCs, using two independent siRNAs against each of the four ICF genes, Dnmt3b, Zbtb24, Cdca7 and Hells. Consistent with the results from our
Zbtb24DBT mutant mESCs, we found significantly reduced Cda7 mRNA levels (t-test P < 0.05) upon Zbtb24 depletion (Fig. 3C and D). Reversely, Cda7 knock down did not affect Zbtb24 mRNA levels, suggesting that Zbtb24 acts upstream of Cda7. No additional links between the four ICF genes at transcript levels were observed (Fig. 3C and D). Together our results show that Zbtb24 can directly or indirectly regulate transcription of Cda7 in mESCs, suggesting convergence of the ICF2 and ICF3 genes.

Consistent with our data from mESCs, we found down regulation of CDCA7 mRNA (t-test P < 0.05) upon siRNA-mediated ZBTB24 knock down in human fetal lung fibroblasts (WI38) and a human osteosarcoma cell line (U2OS) (Fig. 4). When we knocked down CDCA7 using three siRNAs in U2OS cells, we did not detect down regulation of ZBTB24, which is consistent with our result in mESCs (Supplementary Material, File S1). We found that overexpression of ZBTB24 could restore transcription levels of Cdca7, Cdc40 and Ostc (Fig. 5A).

Next, we cloned the predicted mouse Cdca7, Cdc40 and Ostc promoters into a Luciferase reporter vector and transfected wild-type and homozygous Zbtb24DBT mutant mESCs. Luciferase activity was detected in wild-type but not homozygous Zbtb24DBT mutant mESCs. First, we measured expression levels of Cdca7, Cdc40 and Ostc. These three genes were most significantly down regulated in our RNA-seq experiment and therefore considered to be putative direct targets of Zbtb24 (Supplementary Material, File S1). We found that overexpression of ZBTB24 could restore transcription levels of Cdca7, Cdc40 and Ostc (Fig. 5A).

Zbtb24 regulates Cda7 expression level by activating the promoter region of Cda7

ZBTB24 protein is highly conserved between mouse and human and contains a BTB domain, an AT hook domain and eight zinc fingers of the classical Cys2-His2 type (Supplementary Material, Fig. S5A). Based on its zinc finger structure it has been suggested to function as a transcription factor (20). To investigate whether ZBTB24 has transcriptional capacity we overexpressed full-length human GFP-ZBTB24 and 3xTy1-ZBTB24 in homozygous Zbtb24DBT mutant mESCs. First, we measured expression levels of Cdca7, Cdc40 and Ostc. These three genes were most significantly down regulated in our RNA-seq experiment and therefore considered to be putative direct targets of Zbtb24 (Supplementary Material, File S1). We found that overexpression of ZBTB24 could restore transcription levels of Cdca7, Cdc40 and Ostc (Fig. 5A).

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demonstrating that the GFP-ZBTB24 overexpression construct was able to rescue the Zbtb24 BTB phenotype (Fig. 5B). These results suggest that functional Zbtb24 is needed at the Cdca7 and Ostc promoters to activate transcription.

To test whether ZBTB24 directly or indirectly affects Cdca7 expression, we overexpressed both 3xTy1 and 3xTy1_ZBTB24 in U2OS cells separately and performed ChIP followed by qRT-PCR. We first performed immunoprecipitation to test specificity of the Ty1 antibodies (Supplementary Material, Fig. S5C). For the ChIP-qPCR we decided to focus on Cdca7 and Ostc since we failed to detect Luciferase activity in the predicted promoter region of Cdc40. Using primers in the predicted promoter regions of Cdca7 and Ostc and 28S ribosomal RNA as a control region we found ZBTB24 enrichment at the Cdca7 and Ostc promoter regions when compared to 28S ribosomal RNA (Fig. 5C).

Together, these experiments suggest that ZBTB24 modulates Cdca7 expression levels by directly binding to the Cdca7 promoter.
CDCA7 deregulation upon ZBTB24 loss is reflected in ICF2 patient-derived cell lines

Next, we were interested to determine if CDCA7 levels were also affected in ICF2 patients carrying ZBTB24 mutations. To address this, we used qRT-PCR to measure ZBTB24 and CDCA7 mRNA levels in two ICF2 families. One previously unpublished patient was found to be homozygous for a 1 bp deletion in exon 2 that encodes the Zinc finger domain of ZBTB24 (c.917delA; p.[N306IfsX4]) by Sanger candidate gene sequencing (Supplementary Material, Fig. S6). This mutation results in a frameshift and a premature stop codon three amino acids downstream and has previously been described in a different family (6). We found reduced levels of CDCA7 mRNA in the newly identified ICF2 patient when compared to the heterozygous unaffected parents in fibroblasts (Fig. 6). A less apparent result was obtained using RNA isolated from expanded T-cells from a different patient (patient 55 in Weemaes et al. (1)) carrying a homozygous nonsense ZBTB24 mutation (c.958C>T; p.[R320X]). One explanation for the observed discrepancy between fibroblasts and T-cells is that the regulation is not conserved between all tissues or cell types. Alternatively, T-cell stimulation could mask the ZBTB24-dependent regulation of CDCA7 since in zebrafish, cdca7 was shown to participate in a regulatory network controlling thymus development (22).

Discussion

In this study we have revealed that the two ICF genes ZBTB24 and CDCA7 converge at the level of transcriptional regulation. Absence or disruption of ZBTB24 protein results in the down regulation of CDCA7, most likely because of a direct interaction of the ZBTB24 transcription factor with the CDCA7 promoter, and this is found in mESCs and in somatic cells.

Mice homozygous for a BTB-domain deletion are early embryonic lethal suggesting an important function for Zbtb24 in development. The early embryonic lethality phenotype of the Zbb24ABT homozygotes was surprising given that ICF is a recessive disorder and most ICF2 patients carry nonsense mutations in the ZBTB24 gene. One possibility for the more severe phenotype in the mouse is that Zbb24 has acquired a different function during mouse early development. Alternatively, using a different inbred mouse strain or a mixed background could result in improved viability of the homozygous embryos. A third possibility could be that the aberrant PGK/NEO-driven Zbb24ABT transcript contributes to the early embryonic lethality observed in the homozygotes. However, the observation that an independent allele produced by the IPMC shows a similar homozygous lethality phenotype makes this less likely.

So far, no clear phenotypic differences between the ICF subtypes have been identified. However, it appears that ICF2 patients show a higher incidence of intellectual disability (1). At a molecular level, up-regulation of germ-line genes including Mael and Syce1 has been reported in ICF1 mouse models and in whole blood from ICF1 patients (10,13). In contrast, ICF2 patients did not show this molecular signature (10). Consistent with this, expression levels of germ line genes were not affected in our homozygous Zbtb24ABT mutant mESCs. Our findings in mouse and human cell lines indicate that disruption of ZBTB24 leads to down regulation of CDCA7, the gene causative for ICF3. If and to what extent this contributes to the ICF syndrome phenotype requires further investigation. Highly overlapping phenotypic features suggest convergence of all ICF genes in pathways involved in immunity, chromatin regulation and development. HELLS can interact with DNMT3B to mediate DNA methylation establishment (17,18,27,28). It is possible that ZBTB24 and CDCA7 act in common at the level of DNA methylation maintenance.

In conclusion, we have identified ZBTB24 as a direct transcriptional activator of CDCA7 and established a functional link between the two ICF genes. Our observations suggest that investigations into functional relationships between disease causing genes are likely to shed light on the mechanisms underlying other genetically heterogeneous diseases.
Materials and Methods

Ethics statement

All procedures involving animals were approved by the Animal Ethics Committee of the Leiden University Medical Centre and by the Commission Biotechnology in animals of the Dutch Ministry of Agriculture. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center and patient material was obtained after obtaining informed consent.

Zbtb24 BTB deletion mouse

Zbtb24<sup>ABTB</sup> mice were generated by homologous recombination using standard gene targeting techniques in a C57BL/6j background. Transgenic mice were genotyped by PCR and the wild-type allele was detected as a 363-bp band and the mutant allele as a 321-bp band (genotyping primers are provided in Supplementary Material, Table S1). For timed matings, the day of a vaginal plug was designated as E0.5. All experimental procedures and crosses were carried out on mice that were backcrossed to C57BL/6j for at least 10 generations.

Patient Material

Patient derived skin fibroblasts were generated by expanding primary cultures from skin biopsies and cultured in DMEM F12 (31331) supplemented with 20% FCS, 1% Pen-Strep, 1% sodium pyruvate and 1% HEPES (all Invitrogen Life Technologies, Bleiswijk, The Netherlands). T-cells were obtained from fresh primary cultures from skin biopsies and cultured in DMEM F12 ([Knockout DMEM (10829-018; Gibco), 10% FBS (DE14-801F; BioWhittaker), NEAA (11140; Gibco), l-Glutamine (25030-123; Gibco), Sodium Pyruvate (11360; Gibco), 2-Mercaptoethanol (31350; Gibco) and Leukemia Inhibitory Factor (ESG1107; Millipore)] plus MEK inhibitor PD0325901 (1 mM) and Leukemia Inhibitory Factor (ESG1107; Millipore) plus MEK inhibitor PD0325901 (1 μM) and GSK3 inhibitor CHIR99021 (3 μM) (Axon Medchem) on 0.1% gelatine. U2OS cells were maintained in DMEM (31966-021; Gibco) supplemented with 10% FCS (10270-106; Gibco) and 1% Pen-Strep (15140-122; Gibco). WI38 cells were maintained in the same medium used for patient derived skin fibroblasts. For transfections, siRNAs with a final concentration of 40 nM and RNAiMAX (Thermo) were used. About 1 mg/ml (pH 7.4) PEI (23966; Polysciences) was used in the transfection reagent were used. About 1 mg/ml (pH 7.4) PEI (23966; Polysciences) was used in the transfection

Cell culture and siRNA transfection

mESCs were generated from E3.5 blastocysts after natural mating and were cultured without feeders in mESC medium [Knickout DMEM (10829-018; Gibco), 10% FBS (DE14-801F; BioWhittaker), NEAA (11140; Gibco), l-Glutamine (25030-123; Gibco), Sodium Pyruvate (11360; Gibco), 2-Mercaptoethanol (31350; Gibco) and Leukemia Inhibitory Factor (ESG1107; Millipore)] plus MEK inhibitor PD0325901 (1 μM) and GSK3 inhibitor CHIR99021 (3 μM) (Axon Medchem) on 0.1% gelatine. U2OS cells were maintained in DMEM (31966-021; Gibco) supplemented with 10% FCS (10270-106; Gibco) and 1% Pen-Strep (15140-122; Gibco). WI38 cells were maintained in the same medium used for patient-derived skin fibroblasts. For transfections, siRNAs with a final concentration of 40 nM and RNAiMAX (Thermo) reagent were used. About 1 mg/ml (pH 7.4) PEI (23966; Polysciences) was used in the transfection assay for overexpression. Cell cultures tested negative for mycoplasma on a regular basis.

RNA isolation and qRT-PCR

Total RNA was extracted using QIAzol (5346994; Qiajen). About 1 μg of total RNA was used for reverse transcription with the RevertAid H Minus First Strand cDNA Synthesis Kit (K1632; Thermo). qRT-PCR was performed in triplicate on a C1000TM Thermal cycler (Bio-Rad) with SYBR Green (170-8887; Bio-Rad). Data was normalized to β-actin for mouse and GUSB for human. Primer sequences are provided in Supplementary Material, Table S1.

Pre-processing, alignment of RNA-seq reads and differential expression analysis

After sequencing, adapter sequences were removed, and raw reads were filtered for low quality reads (Q20 > 97%). An average of 20 million paired-end reads were aligned to the mouse genome (mm9) using GSNAP (version 2013-11-27), with the following parameters: -N 1 -n 1. BAM files were converted into mpileup files using SAMTools, and subsequently converted intowig format for visualization purposes, using a custom python script. The number of mapped reads was quantified at gene level using HTSeq (version 0.6.1p1) with default parameters (union mode), based on Ensembl annotation (version 64) (ftp://ftp.ensembl.org/pub/release-64/gtf/mus_musculus/Mus_musculus.NCBIM37.64.gtf.gz) (29). The R Bioconductor package DESeq2 (version 1.6.3 on R version 3.1.1) was used for analysis of differential gene expression analysis between wild-type mice and homozygous Zbtb24<sup>ABTB</sup> mutant mESCs (30).

Mapping of RNA-seq reads to the artificially engineered Zbtb24 locus

The mouse mm9 genome reference sequence was modified by inserting a reverse PGK-Neomycin cassette between exon1 and exon2 of the Zbtb24 locus to create an artificial genome reference resembling our transgenic mouse. Gmap_build.data (31) was used to build a reference genome for GSNAP and subsequently reads from one wild-type and one homozygous Zbtb24<sup>ABTB</sup> mutant mESC samples were aligned to this artificial reference genome. The Sashimi-plot option in IGV was used to visualize the alignment (32).

Statistical Analysis

A Student’s t test and Standard error of mean (SEM) were used for all the statistical analysis. Unless otherwise stated statistical analysis was done either on at least two biological replicates or at least two independent experiments. Values of P < 0.05 were considered significant.

Immunoprecipitation

Cells were washed twice with cold PBS and lysed in IP Buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA) with Protease inhibitor cocktail (05056489001; Roche) and Phosphatase inhibitor cocktail (04906837001; Roche) on ice. A BCA kit (23225; Thermo) was used to determine protein concentration. Protein A/G plus-Agarose Beads (sc-2003; Santa Cruz) were first incubated with 1 μg Ty1 antibodies (SAB4800032; Sigma; C15200054; Diagenode) at 4°C for at least 1 h. Total cell extracts were pre-cleared with beads and equal amounts of total protein were added into beads-antibody mix and incubated at 4°C overnight. After immunoprecipitation, beads were

RNA sequencing libraries

RNA-seq libraries were generated by BGI from total RNA using the TruSeq RNA-Seq library prep kit (Illumina), with an insert size of ~160 bp. Three independent biological replicates for each group (wild-type or homozygous Zbtb24<sup>ABTB</sup> mutant ESCs) were sequenced paired-end (2 x 91 bp) on the Illumina HiSeq2000 platform (Accession number for RNA-seq data: European Nucleotide Archive ERP010761 http://www.ebi.ac.uk/ena/data/view/ERP010761, date last accessed July 26, 2016).

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washed with IP buffer 4 times and then boiled for 10 min to collect samples. IP samples were analyzed by Western blotting.

Chromatin immunoprecipitation

Cells were cross linked with 1% formaldehyde (344198; Calbiochem) for 10 min at RT and glycine (125 μl) was used to quench cross-linking for 5 min. Cells were washed twice with cold PBS and lysed with NP Buffer ([150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 2 mM EDTA, 1% Triton X-100] with Protease inhibitor cocktail (05056489001; Roche)). Nuclei were sheared by sonication (Diagenode Bioruptor Pico). Protein A and G Beads (10002D, 10003D; Life Technologies) were first blocked with BSA (A7906; Sigma) and then incubated with antibodies at 4°C for at least 4 h. About 2 μg Ty1 antibody (C15200054; Diagenode) coupled with beads were incubated with sheared chromatin at 4°C overnight. After immunoprecipitation, beads were washed with low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 150 mM NaCl), high-salt washing buffer (0.1% SDS, 1% Triton X-100, 2% EDTA, 20% Tris–HCl (pH 8.1), 500 mM NaCl), LiCl washing buffer (0.25 μg LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl (pH 8.1)) and TE buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA). DNA was extracted using phenol-chloroform-isooamylol (15593-049; Life Technologies) and used for analysis. Primers can be found in Supplementary Material, Table S1.

Western blotting

Cells were lysed in Cell Lysis buffer (20 mM triethanolamine (T1377; Sigma), 0.14 M NaCl, 0.1% Sodium deoxycholate (D6750; Sigma), 0.1% SDS, 0.1% Triton X-100) with Protease inhibitor cocktail (05056489001; Roche) and Phosphatase inhibitor cocktail (04096837001; Roche) on ice. BCA kit was used to determine protein concentration. Equal amounts of total cell extracts were separated on a NuPAGE gel (4-12%; NP0321), and transferred to a Nitrocellulose Blotting Membrane (10600016; Life Sciences). The following primary antibodies were used: Zbtb24 (PM085; MBL Life Science, 1:1000), Cdca7 (15249-1-AP; Proteintech, 1:500), Dnmt3b (Ab16049; Abcam, 1:1000), Hells (11955-1-AP; Proteintech, 1:1000), Ty1 (SAB4800032; Sigma, 1:1000), Goat anti-Rabbit 800CW (926-32214; Li-Cor, 1:5000), and Donkey anti-Goat A594 (A-11076; Thermofisher, 1:5000) were used as secondary antibodies. Membranes were separated on a NuPAGE gel (4–12%; NP0321), and transferred to an a Nitrocellulose Blotting Membrane (10600016; Life Sciences). The following primary antibodies were used: Zbtb24 (PM085; MBL Life Science, 1:1000), Cdca7 (15249-1-AP; Proteintech, 1:500), Dnmt3b (Ab16049; Abcam, 1:1000), Hells (11955-1-AP; Proteintech, 1:1000), Ty1 (SAB4800032; Sigma, 1:1000), Goat anti-Rabbit 800CW (926-32214; Li-Cor, 1:5000) were used as secondary antibodies. Membranes were analyzed on Odyssey (Westbur).
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References


