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

Haoyu Wu1, Peter E. Thijssen1, Eleonora de Klerk1,2, Kelly K. D. Vonk1, Jun Wang1,3, Bianca den Hamer1, Caner Aytekin4, Silvère M. van der Maarel1 and Lucia Daxinger1,*

1Department of Human Genetics, Leiden University Medical Centre, Leiden 2300RC, The Netherlands, 2Department of Microbiology and Immunology, UCSF Diabetes Center, University of California San Francisco, San Francisco, CA 94143-0534, USA, 3Institutes of Biology and Medical Sciences, Soochow University, Suzhou 215123, China and 4Department of Pediatric Immunology, Dr Sami Ulus Maternity and Children’s Research and Educational Hospital, Ankara 06080, Turkey

*To whom correspondence should be addressed at: Tel: +31 0 71 52 69448; Email: l.clemens-daxinger@lumc.nl

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 HILLS. 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 agammaglobulinemia, 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, HILLS/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 Cdca7. 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). Cdca7 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 Cdca7 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 Cdca7 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 Cdca7 mRNA levels in homozygous mutant mESCs. We further demonstrate enrichment of ZBTB24 at the CDCA7 promoter suggesting a direct transcriptional regulation. Finally, we show that CDCA7 levels are affected in ICF2 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<sup>ATBT</sup>, 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<sup>ATBT</sup> mutants 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<sup>ATBT</sup> mice produced no viable homozygous Zbtb24<sup>ATBT</sup> embryos at E9.5 (Fig. 1B). The presence of implantation sites at E9.5 suggests that embryonic death of homozygotes 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<sup>ATBT</sup> deletion allele (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<sup>ATBT</sup> 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 homozygous Zbtb24<sup>ATBT</sup> mutant mESC lines. We decided to continue with three wild-type and three Zbtb24<sup>ATBT</sup> 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 (<i>p</i>-value < 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 data set from mESCs grown on 2i (26) and did not reveal any major differences between wild-type and homozygous Zbtb24<sup>ATBT</sup> 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<sup>ATBT</sup> homozygous mutant mESCs (Fig. 2B and Supplementary Material, Fig. S3A) suggesting that an aberrant Zbtb24<sup>ATBT</sup> 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 Zbtb24<sup>ATTT</sup> mutant mESCs. (A) Genotyping of Zbtb24<sup>ATTT</sup> 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 Zbtb24<sup>ATTT</sup> 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 Zbtb24<sup>ATTT</sup> 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 Zbtb24<sup>ATTT</sup> mESCs. Error bars, SEM from three biological replicates per genotype; NS, non-significant.

Figure 2. Transcriptome analysis of wild-type and homozygous Zbtb24<sup>ATTT</sup> mutant mESCs. (A) MA plot showing RNA-seq analysis results from Zbtb24 wild-type and homozygous Zbtb24<sup>ATTT</sup> 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 Zbtb24<sup>ATTT</sup> mutant mESCs. (B) IGV RNA-seq track showing a putative translation start site at the transgenic Zbtb24 locus in a homozygous Zbtb24<sup>ATTT</sup> 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 Zbtb24<sup>ATTT</sup> 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 Zbtb24<sup>ATTT</sup> 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\textsuperscript{\textemdash\textsc{btb}} homzygous mutant mESCs (Supplementary Material, Fig. S3C). We used polysome profiling to assess whether the aberrant Zbtb24\textsuperscript{\textemdash\textsc{btb}} transcript can be engaged by ribosomes. We found no difference in the ratio of total RNA/polysome-bound RNA between wild-type and Zbtb24\textsuperscript{\textemdash\textsc{btb}} homozygotes suggesting that Zbtb24\textsuperscript{\textemdash\textsc{btb}} 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\textsuperscript{\textemdash\textsc{btb}} homozygous mutant mESCs. Zbtb24 was detected at its predicted size of around 80 kDa in wild-type but severely reduced in the Zbtb24\textsuperscript{\textemdash\textsc{btb}} 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 \textit{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 \textit{Cdca7} protein in Zbtb24\textsuperscript{\textemdash\textsc{btb}} homozygous mutant mESCs (Fig. 3B). The finding that \textit{Cdca7} expression levels are down regulated in Zbtb24\textsuperscript{\textemdash\textsc{btb}} homozygous mutant mESCs was unexpected and to our knowledge, no functional relationship between these two genes has previously been reported. Our observation of \textit{Cdca7} dysregulation in Zbtb24\textsuperscript{\textemdash\textsc{btb}} 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 \textit{Dnmt3b}, Zbtb24, \textit{Cdca7} and \textit{Hells} in wild-type mESCs upon siRNA knock down. Error bars represent the SEM from two independent experiments. t-test $**P < 0.001$. (B) Western blot showing \textit{Dnmt3b} (top, ~110 kDa, multiple variants), \textit{Zbtb24} (middle, ~80 kDa), \textit{Cdca7} (middle) and \textit{Hells} (bottom, ~100 kDa) protein levels after siRNA knock down. Tubulin is shown as a loading control.
Zbtb24<sup>ABTT</sup> mutant mESCs, we found significantly reduced Cdc7 mRNA levels (t-test P < 0.05) upon Zbtb24 depletion (Fig. 3C and D). Reversely, Cdc7 knock down did not affect Zbtb24 mRNA levels, suggesting that Zbtb24 acts upstream of Cdc7. 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 Cdc7 in mESCs, suggesting convergence of the ICF2 and ICF3 genes.

Consistent with our data from mESCs, we found downregulation 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 CDC7 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 Cdc7, Cdc40 and Ostc (Fig. 5A).

Next, we cloned the predicted mouse Cdc7, Cdc40 and Ostc promoters into a Luciferase reporter vector and transfected wild-type and homozygous Zbtb24<sup>ABTT</sup> mutant mESCs. Luciferase activity was detected in wild-type but not homozygous Zbtb24<sup>ABTT</sup> mutant mESCs for Ccd7 and Ostc, whereas we could not detect luciferase activity for Cdc40 (Fig. 5B). The Cdc40 gene is located adjacent to the Wasf1 gene and it could be that Cdc40 expression is regulated by elements distal to the Cdc40 gene (Supplementary Material, Fig. S5B). We then cotransfected homozygous Zbtb24<sup>ABTT</sup> mutant mESCs with the reporter constructs and a human GFP-ZBTB24 overexpression construct. Luciferase activity was detected for Cdc7 and Ostc, 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 Zbtb24<sup>ABTT</sup> mutant mESCs. First, we measured expression levels of Cdc7, 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 Cdc7, Cdc40 and Ostc (Fig. 5A).

**Figure 4.** CDCA7 is down regulated in siZBTB24 human cell lines. (top) qRT-PCR and (bottom) a representative Western-blot analysis showing down regulation of both ZBTB24 and CDCA7 levels in siZBTB24 U2OS (left) and WI38 (right) cells. Error bars – SEM from three independent knock down experiments. t-test *P < 0.05, **P < 0.01, ***P < 0.001. TUBULIN (50 kDa) is shown as a loading control. CDCA7 (40 kDa).
demonstrating that the GFP-ZBTB24 overexpression construct was able to rescue the Zbtb24
DBT 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 we found ZBTB24 enrichment at the CDCA7 and OSTC promoter regions when compared to 28S control region in U2OS cells transfected with a 3xTy1_ZBTB24 construct. Error bars – SEM from two independent experiments. t-test *P < 0.05, **P < 0.01.

Figure 5. Zbtb24 regulates Cdca7 expression level by modulating the Cdca7 promoter. (A) qRT-PCR showing Cdca7, Cdc40 and Ostc expression levels in wild-type and Zbtb24DBT homozygous mutant mESCs. mRNA levels of all three genes could be rescued by overexpression of GFP- or 3xTy1 tagged ZBTB24. Error bars – SEM from three biological replicates. t-test *P < 0.05, **P < 0.01. (B) Luciferase activity assay showing the interaction between endogenous Zbtb24 and overexpressed full-length ZBTB24 with the Cdca7 and Ostc promoters in wild-type and homozygous Zbtb24DBT mutant mESCs. Error bars – SEM from three biological replicates. t-test *P < 0.05, **P < 0.01, ***P < 0.001. (C) (top) TSS regions of CDCA7 and OSTC. Primers (black arrows) for ChIP-qPCR were designed in the predicted promoter regions (~1 kb upstream of TSS) of CDCA7 and OSTC. (bottom) ChIP-qPCR analysis showing an enrichment of ZBTB24 at the CDCA7 promoter region when compared to a R28S control region in U2OS cells transfected with a 3xTy1_ZBTB24 construct. Error bars – SEM from two independent experiments. t-test *P < 0.05, **P < 0.01.
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 Zbb24<sup>ABTB</sup> 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 Zbtb24 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 Zbb24<sup>ABTB</sup> transcript contributes to the early embryonic lethality observed in the homozygotes. However, the observation that an independent allele produced by the IMPC 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 Zbtb24<sup>ABTB</sup> 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 Ethics Committee of the Leiden University Medical Center and patient material was obtained after obtaining informed consent.

Zbtb24 BTB deletion mouse

Zbtb24<sup>ABT</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 back-crossed 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 (31966-021; Gibco), Sodium Pyruvate (11360; Gibco), 2-Mercaptoethanol (31350; Gibco) and Leukemia Inhibitory Factor (ESG1107; Millipore) plus MEK inhibitor PD0325901 (1 μM) (Axon Medchem) on 0.1% gelatine. U2OS cells were maintained in DMEM (11360; Gibco) supplemented with 10% FCS, 1% Pen-Strep, 1% sodium pyruvate and 1% HEPES (all Invitrogen Life Technologies, Bleiswijk, The Netherlands). T-cells were obtained from fresh blood samples as described previously (1).

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 (13778; Life Technologies) reagent were used. About 1 mg/ml (pH 7.4) PEI (23966; Polysciences) was used in the transfection mixture. Total RNA was extracted using QIAzol (5346994; Qiagen). 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.

RNA isolation and qRT-PCR

Total RNA was extracted using QIAzol (5346994; Qiagen). 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 into WIG 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>ABT</sup> mutant mESCs (30).

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 washed 4 times with IP Buffer and resuspended in 20 μl IP Buffer. After heating for 5 min at 95 °C, 2 μl of 2× SDS-PAGE loading buffer was added, followed by heating for 5 min at 95 °C. Western blots were performed using anti-GFP antibody (ab6557; Abcam) and detected using an enhanced chemiluminescence (ECL). Densitometric analysis was done with ImageJ software.

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>ABT</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).
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 μM) was used to quench cross-linking for 5 min. Cells were washed twice with cold PBS and lysed with NP Buffer [150 μM NaCl, 50 μM Tris–HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, Protease inhibitor cocktail (05065489001; 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 μM NaCl], high-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 500 μM NaCl), LiCl washing buffer [0.25 M 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-isoamylol (15593-049; Life Technologies) and then incubated with antibodies 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 μM NaCl], high-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 500 μM NaCl), LiCl washing buffer [0.25 M 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-isoamylol (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 (05065489001; Roche) and Phosphatase inhibitor cocktail (04906837001; 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), Cdc7a (15249-1-AP; Proteintech, 1:500), Dnm1B (Ab16049; Abcam, 1:1000), Hells (11955-1-AP; Proteintech, 1:1000), Ty1 (SA84800032; Sigma, 1:1000) and Tubulin (T6199; Sigma, 1:5000). Goat anti-Rabbit 800CW (926-32211; Westburg, 1:5000), Donkey anti-mouse 680RD (926-68072; Li-Cor, 1:5000), Goat anti-guinea pig AS94 (A-11076; Thermofisher, 1:5000) and Donkey anti-Goat 800CW (926-32214; Li-Cor, 1:5000) were used as secondary antibodies. Membranes were analyzed on Odyssey (Wesburg).

Luciferase reporter assay
Suspended Cells were transfected with Lipofectamine 2000 (11668-027; Life Technologies). Cells were harvested 2 days after transfection and treated with Dual-Luciferase Reporter Assay System kit (E1910; Promega). A Perkin Elmer precisely 1420 Multilabel counter victor 3 was used to measure Luciferase intensity.

Southern blot
Southern blotting was used to determine integration of the PGK-NEO cassette. Primers used to generate the probe can be found in Supplementary Material, Table S1. A buffer containing 0.125 M Na2HPO4 (pH 7.2), 10% PEG6000, 0.25 M NaCl, 1 mM EDTA and 7% SDS was used for hybridization for 16–24 h at 65 °C. Final washing conditions were 2× SSC-0.1% SDS (p13E-11). The blot was exposed for 16–24 h to Phosphorimager screens and analyzed with the Image Quant software program (Molecular Dynamics).

Cloning
Full length human ZBTB24 was PCR amplified from a cDNA library obtained from HCT116 cells using Phusion high fidelity DNA polymerase (NEB) with the following primers: ZBTB24_F and ZBTB24_R. The obtained fragment was subcloned into the Zero Blunt Topo cloning vector. To introduce restriction sites (BglII and SalI), we amplified the coding sequence using Phusion with the following primers: ZBTB24_F2 and ZBTB24_R2. Subsequently, the ZBTB24 ORF and the pEGFP-c1 expression vector (Promega) were digested with BglII and SalI and ligated. To generate the 3xTy1_ZBTB24 construct, 3xTy1 tag was amplified from pTALYM9 vector (47876; Addgene) with flanking restriction sites (Nhel/BglII (3xTy1-Nhel_I, 3xTy1-BglII_R), and GFP was replaced by 3xTy1 in pEGFP-c1 vector containing ZBTB24. For Luciferase assay experiments around 1000 bp upstream of the Cdc7a and Cdc40 transcription start sites were PCR amplified (Cdc7a-pro-MluI_F, Cdc7a-pro-HindIII_R, Cdc40-pro-MluI_F, Cdc40-pro-HindIII_R) with flanking restriction sites for cloning (MluI/HindIII), and the PCR product was inserted into the pGL3-Basic vector. Around 1000 bp upstream of the Ostc transcription start site was obtained by PCR (Ostc-pro-MluI_F, Ostc-pro-BglII_R) with flanking restriction sites for cloning (MluI/BglII), the PCR product was inserted into the pGL3-Basic Vector. All constructs were verified using Sanger sequencing. Primers used can be found in Supplementary Material, Table S1.

Polysome profiling
Polysome RNA was extracted from mESCs cultured in 10-cm dishes. Translation elongation was blocked and cytoplasmic RNA was recovered as described previously (33). Lysate was layered on a sucrose gradient (7–46% sucrose) and separated by ultracentrifugation at 35 000 rpm in a SW 41 Ti rotor (210 000 g) for 3 h at 4 °C. About 15 fractions (750 μl each) were collected from the top and digested with proteinase K (0.15 mg/750 μl) for 30 min at 42 °C in the presence of 1% sodium dodecyl sulphate. RNA was extracted by acid phenol (Ambion) purification followed by ethanol precipitation. For each sucrose gradient separation, the polysome profile was determined on a Bioanalyzer (Agilent) with the RNA 6000 Nano kit. Fractions containing polysomes (corresponding to fractions 10–15) were combined.

Total RNA was extracted from mESCs using NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of polysome or total RNA using BioScript MMLV Reverse Transcriptase (Bioline) with 40 ng of random hexamer and oligo(dT)18 primers. qRT-PCR was performed with Zbtb24-RT-PCR primers (Supplementary Material, Table S1) on a LightCycler 480 (Roche) using SensiMix reagent (Bioline). PCR efficiency was determined using the LinRegPCR program (34,35). Ratios between Zbtb24 relative expression levels in total RNA and polysome RNA were calculated and significance was tested performing a t-test.

Supplementary Material
Supplementary Material is available at HMG online.
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References


