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Heterozygous deletion of *Atbf1* by the *Cre-loxP* system in mice causes preweaning mortality

Xiaodong Sun¹, Xiaoying Fu¹, Jie Li¹, Changsheng Xing¹, David W. Martin², Helen Heju Zhang², Zhengjia Chen³, and Jin-Tang Dong^{1,*}

¹Department of Hematology and Medical Oncology, Emory University School of Medicine, Winship Cancer Institute, Atlanta, GA

²Transgenic Mouse & Gene Targeting Core, Emory University School of Medicine, Atlanta, GA

³Department of Biostatistics and Bioinformatics, Emory University, Atlanta, GA

Summary

ATBF1 is a large nuclear protein that contains multiple zinc-finger motifs and four homeodomains. In mammals, ATBF1 regulates differentiation, and its mutation and/or downregulation is involved in tumorigenesis in several organs. To gain more insight into the physiological functions of ATBF1, we generated and validated a conditional allele of mouse *Atbf1* in which exons 7 and 8 were flanked by *loxP* sites (*Atbf1^{fllox}*). Germline deletion of a single *Atbf1* allele was achieved by breeding to *Elia-cre* transgenic mice, and *Atbf1* heterozygous mice displayed reduced body weight, preweaning mortality, increased cell proliferation, and attenuated cytokeratin 18 (CK18) expression, indicating haploinsufficiency of *Atbf1*. Floxed *Atbf1* mice will help us understand such biological processes as neuronal differentiation and tumorigenesis.

Keywords

Atbf1; *Cre-loxP*; conditional knockout; heterozygous; preweaning mortality

ATBF1 (AT-motif binding factor 1), also named ZFX3 for zinc finger homeobox 3, belongs to a small family that is structurally characterized by multiple zinc-finger motifs and three or four homeodomains. In human and mouse, the *ATBF1* gene contains 11 exons, and exon 10 is the largest (5454 bps). Using distinct promoters, *ATBF1* produces two splicing variants, *ATBF1-A* and *-B*, which share exons 4 to 11 but differ in 5' exons. Both isoforms encode large proteins. *ATBF1-A* uses a start codon in exon 3 to generate a protein of 404-kD. *ATBF1-B* uses a start codon in exon 4 to produce a protein of 300-kD (Miura *et al.*, 1995a; Morinaga *et al.*, 1991). Although both isoforms were reported, *ATBF1-A* is the predominant one with an expression level much higher than *ATBF1-B*. In mouse, only *Atbf1-A* was detectable at the mRNA and protein levels (Ishii *et al.*, 2003; Miura *et al.*, 1995b).

ATBF1 functions in multiple biological processes including cell differentiation and tumorigenesis. It could regulate region-specific central nervous system differentiation (Ishii *et al.*, 2003); its expression decreases at postnatal stage but remains detectable in mature neurons (Jung *et al.*, 2005; Watanabe *et al.*, 1996); and its expression is up-regulated in retinoic acid-treated p19 cells, an in vitro model of neuronal differentiation (Ido *et al.*, 1994;

*Correspondence to: Jin-Tang Dong, Emory University Winship Cancer Institute, 1365C Clifton Rd. Room C4080, Atlanta, GA 30322. Telephone: 404-712-2568. Fax: 404-712-2571. j.dong@emory.edu.

Jung *et al.*, 2005; Miura *et al.*, 1995a). ATBF1 may also function in myogenic differentiation (Berry *et al.*, 2001). ATBF1 regulates several differentiation genes including alpha-fetoprotein (AFP), aminopeptidase N and neurod1 (Jung *et al.*, 2005; Kataoka *et al.*, 2000; Morinaga *et al.*, 1991; Yasuda *et al.*, 1994). ATBF1 may affect atrial fibrillation, since genetic variations in introns of *ATBF1* are associated with atrial fibrillation (Benjamin *et al.*, 2009; Gudbjartsson *et al.*, 2009; Li *et al.*, 2011).

ATBF1 has also been suggested to be a tumor suppressor in cancers of the breast, prostate, stomach and liver. In humans, *ATBF1* is located at 16q22, frequently deleted in prostate and breast cancers (Dong, 2001). *ATBF1* undergoes frequent somatic mutations in human prostate cancer, some of which are protein-truncating (Sun *et al.*, 2005). A germline variant of *ATBF1* was linked to prostate cancer risk (Xu *et al.*, 2006), and *ATBF1* mutation was detected in gastric cancers (Cho *et al.*, 2007). In highly malignant AFP-producing gastric cancers, *ATBF1* expression is absent, and its role in transcriptional repression of the *AFP* oncogene has been demonstrated (Kataoka *et al.*, 2001; Yasuda *et al.*, 1994). Re-establishing the expression of *ATBF1* in human prostate and breast cancer cell lines inhibits cell growth (Dong *et al.*, 2010; Sun *et al.*, 2005; Sun *et al.*, 2007), and changes in subcellular localization alter its function in cancer gene regulation (Mabuchi *et al.*, 2010; Mori *et al.*, 2007) (our unpublished data). ATBF1 may therefore be a tumor suppressor in human tissues, though this has not been examined *in vivo*.

For functional studies of ATBF1 *in vivo*, we developed a mouse strain in which exons 7 and 8 of *Atbf1* were flanked by *loxP* sites so they can be deleted upon spatiotemporally controlled expression of Cre recombinase. Removal of exons 7 and 8 leads to a frame shift in *Atbf1* translation, resulting in the removal of approximately 70% of the *Atbf1*-A protein sequence including most of the zinc finger motifs and all four homeodomains. The targeting vector *pFlexible* was used, containing the positive/negative selectable marker *PGK-puroΔtk* and *loxP* and *Frt* sequences (van der Weyden *et al.*, 2005).

Three *Atbf1* genomic fragments were amplified by PCR from genomic DNA of TL-1 embryonic stem cells (derived from 129S6/SvEvTac mice) and subcloned into the *pFlexible* vector (Fig. 1a). The linearized targeting construct was then electroporated into TL1 embryonic stem cells. After selection in puromycin-containing medium, ES cell clones were screened for homologous recombination by Southern blot analysis. Two clones showed the expected 10.9-kb wildtype and 8.4-kb targeted fragments using the 5' probe (P1) (Fig. 1b). One had the expected 5.1-kb targeted fragment for the 3' probe (P2), indicating that this clone contained a correctly targeted allele in which exons 7 and 8 are flanked by *loxP* sites and the *PGK-puroΔtk* selection marker cassette by *Frt* sites (Fig. 1b). The mutant allele was termed *Atbf1^{PGK}*.

The ES cell clone with correct recombinations was injected into blastocysts and implanted into recipient female mice. Nine chimeric male mice were obtained. Of the six male mice bred with C57BL/6J female mice, three had germline transmission of the targeted allele. Genotypes of the *Atbf1^{PGK}* mice were confirmed by Southern blot analysis using the same strategy as for ES cells. All subsequent genotyping was performed by a PCR strategy (Fig. 1a, 2a).

The *Atbf1^{PGK}* mice were then bred with *ACT-FLPe* transgenic mice, which universally express the FLP enzyme, and the *PGK-puroΔtk* cassette was removed from the genome (Fig. 1a). Mice were then bred to C57BL/6J mice to obtain floxed *Atbf1* allele without *Flp*, which was termed *Atbf1^{fllox/wt}*. Mice with single (*Atbf1^{fllox/wt}*) and double (*Atbf1^{fllox/fllox}*) floxed alleles were viable and fertile, and showed no gross abnormality. The allele with the deletion of exons 7 and 8 was termed *Atbf1^{Δ7&8}*.

Using a PCR-based genotyping strategy (Fig. 2a), three *Atbfl* genotypes could be detected with one pair of primers: *Atbfl^{flox}*, *Atbfl^{wt}* and *Atbfl^{Δ7&8}*. To verify the excision of exons 7 and 8 by Cre, *Atbfl^{flox/wt}* mice were inbred and mouse embryonic fibroblasts (MEFs) were prepared from embryos at day 13.5 of gestation. Cultured MEFs were then infected with adenoviruses bearing either green fluorescent protein (Ad-GFP) or Cre (Ad-Cre). After Ad-Cre infection, the *Atbfl^{flox}* allele was abolished and the *Atbfl^{Δ7&8}* allele appeared, indicating the excision of exons 7 and 8 by Cre (Fig. 2b). Deletion of exons 7 and 8 generated a truncated mRNA, which was also detected by a PCR approach (Fig. 2c). The *Atbfl* protein was significantly reduced in *Atbfl^{flox/wt}* MEFs and was not detectable in *Atbfl^{flox/flox}* MEFs after the infection of Ad-Cre (Fig. 2d). Because the antibody was produced by a peptide sequence within the deleted region, it is unknown whether the deleted *Atbfl* allele is expressed as a truncated protein.

To examine the effect of whole-body deletion of *Atbfl*, we crossed *Atbfl^{flox/wt}* mice with heterozygous *Elia-cre* transgenic mice, which express Cre in early stage of embryogenesis under the control of the adenoviral *Elia* promoter and thus cause the deletion of *loxP*-flanked sequences in most if not all tissues including germ cells (Lakso *et al.*, 1996). Some F1 mice were further bred with C57BL/6J mice to achieve germline transmission of the deletion. All F1 and F2 offspring were genotyped with tail DNA and the PCR method (Fig. 3a), which detected different status of *Atbfl* deletion in the *Cre⁺/Atbfl^{flox/wt}* bigenic mice: no band for the *Atbfl^{Δ7&8}* allele (no deletion, mice 2 and 5 in Fig. 3a); complete excision of *Atbfl* in all cells (mouse 6); and incomplete excision (3 bands, mice 1 and 8), reflecting that the *Atbfl* deletion only occurred in some but not all cells due to heterogeneous expression of Cre post the zygote stage. In individual mice, a mosaic genotype was present at a similar extent in all major organs tested (Fig. 3b). Unexpectedly, only 55% (51 of 92) of the *Cre⁺/Atbfl^{flox/wt}* bigenic mice survived weaning, suggesting a preweaning mortality phenotype in these mice (Table 1). Most of the dead mice had complete excision of *Atbfl*. Breeding *Cre⁺/Atbfl^{flox/wt}* mice with C57BL/6J mice demonstrated germline transmission of the *Atbfl^{Δ7&8}* allele and more severe preweaning mortality in the heterozygous mice. Among a total of 70 mice with heterozygous *Atbfl^{wt/Δ7&8}*, 29 (41%) died within the first 48 hours after birth, additional and 20 (29%) died within 4 weeks after birth (Table 1). Some of the remaining *Atbfl^{wt/Δ7&8}* mice died after weaning. The mortality was sex-independent.

Except for the early postnatal mortality, *Atbfl^{wt/Δ7&8}* mice were not distinguishable from their wildtype littermates at birth. However, whereas *Atbfl^{wt/Δ7&8}* mice kept growing and gaining weight until they died, it became obvious at day 10 after birth that they were much smaller, and their body weights were significantly lighter ($p < 0.0001$) than the wildtype littermates (Fig. 3c, 3d). These mice were also hypoactive and usually required extended weaning.

Mating mice with heterozygous deletion (*Atbfl^{wt/Δ7&8}*) with wildtype C57BL/6J mice (*Atbfl^{wt/wt}*) produced 83 *Atbfl^{wt/wt}* and 33 *Atbfl^{wt/Δ7&8}* pups, which was different from the expected ratio of 1:1 wildtype to heterozygous pups according to Mendelian distribution. A reduction of heterozygous pups by 60.2% suggests that deletion of one *Atbfl* allele also causes partial prenatal lethality in mice. When *Atbfl^{wt/Δ7&8}* mice were inbred, conception time was prolonged and only two pregnancies were observed so far. No pups were delivered, further suggesting that defects in embryonic development had occurred. Due to the limited number of sexually mature *Atbfl^{wt/Δ7&8}* mice and a low pregnancy rate in these mice, we were unable to fully characterize *Atbfl* null embryos or mice. In addition, preweaning mortality and growth retardation were not observed when *Atbfl* was knocked out in a tissue-specific manner (e.g., the prostate and mammary gland, unpublished data).

Several mechanisms could be responsible for the postnatal mortality in heterozygous mice, including haploinsufficiency, monoallelic expression, imprinting and dominant negative property of the mutant allele. As the first step in addressing this question, we measured *Atbf1* expression at mRNA and protein levels in 8 major organs including the brain, liver, lung, heart, intestine, kidney, spleen and stomach. Tissues were collected from both the wildtype (*Atbf1*^{wt/wt}) and heterozygous (*Atbf1*^{wt/ Δ 7&8}) mice at postnatal day 1 and day 21. Real-time RT-PCR with primers from exon 6 and exon 8 of mouse *Atbf1* was performed to measure the *Atbf1* mRNA expression. At postnatal day 1, *Atbf1* mRNA level was the highest in the brain, whereas at postnatal day 21, the expression level was relatively higher in the liver, lung, heart and kidney and lower in the brain, intestine, spleen and stomach (Fig. 4a). At both time points, *Atbf1* mRNA level in the heterozygous (*Atbf1*^{wt/ Δ 7&8}) mice was about half of that in the wildtype mice in most organs examined (Fig. 4a). Immunohistochemistry (IHC) for Atbf1 on tissue sections confirmed that deletion of one *Atbf1* allele reduced Atbf1 protein expression (Fig. 4c). These results excluded the possibility of monoallelic expression or imprinting, while suggesting that haploinsufficiency of *Atbf1* causes preweaning mortality in the *Atbf1* heterozygous mice. RT-PCR with a pair of primers from exons 4 and 5 of *Atbf1*, which amplifies both the wildtype (wt) and the expected truncated (mt) *Atbf1* mRNA showed similar mRNA expression in the majority of the tissues at both postnatal day 1 and day 21 (Fig. 4b). Taken together with the results from the primers from exons 6 and 8, these results indicate the existence of a truncated *Atbf1* mRNA in these organs of the *Atbf1* heterozygous mice. Therefore, we can not exclude the possibility that the *Atbf1* ^{Δ 7&8} allele may produce a truncated protein that interferes with the wildtype Atbf1 function.

Histologically, we did not detect any abnormalities in the organs by comparative pathologic analysis and blood cell counting (Fig. 4c and data not shown). The preweaning mortality and growth retardation were not due to milk sucking or lactation defects, because milk was visible in the stomach of the *Atbf1* heterozygous pups. Human ATBF1 could modulate cell proliferation and differentiation, so we measured the expression of Ki-67, a proliferation marker, and cytokeratin 18 (CK18), a differentiation marker, in the *Atbf1*^{wt/ Δ 7&8} mice. In the kidney, loss of one *Atbf1* allele caused significant increase in Ki-67 expression while attenuating CK18 expression (Fig. 4c). However, it is unknown whether these changes caused preweaning mortality.

In summary, we developed a mouse strain in which the *Atbf1* gene is flanked by *loxP* sequences (*Atbf1*^{fllox/wt}) so the gene can be conditionally deleted by controlled expression of Cre. *Atbf1* is haploinsufficient, and knockout of one allele induced prenatal and preweaning mortality in mice. Availability of mice with a floxed *Atbf1* allele could facilitate functional examination of Atbf1 under biological conditions. For example, we specifically knocked out prostate *Atbf1* and observed prostatic intraepithelial neoplasia, supporting a tumor suppressor role of Atbf1 (manuscript in preparation).

Methods

Generation of the targeting construct

Genomic DNA of TL-1 mouse embryonic stem cells was used as the template for generating mouse *Atbf1* genomic fragments. The 5' homologous arm was a 6.2-kb fragment from intron 6 of *Atbf1*, and the 3' homologous arm was 4.7-kb from intron 8. The targeting sequence was a 0.9-kb fragment spanning part of intron 6, exon 7, intron 7, exon 8 and part of intron 8. All three fragments were amplified by PCR using the LA Taq polymerase (TaKaRa, Berkeley, CA) and were cloned into the T-vector (Promega, Madison, WI). After confirmation by DNA sequencing, the 5' homologous arm, the targeting sequence, and the 3' homologous arm were sequentially inserted into the EcoR I, Hind III, and Not I sites of

the *pFlexible* vector (van der Weyden *et al.*, 2005). The gene-targeting construct was then confirmed by DNA sequencing.

Generation of *Atbfl^{flox}* and *Atbfl^{Δ7&8}* mice

After linearization by digestion with the *Sfi* I restriction enzyme, the gene-targeting construct was electroporated into TL1 embryonic stem cells, which were then cultured in puromycin-containing medium to kill cells without gene integration. Puromycin-resistant clones were picked, expanded sequentially in 96-well and 24-well plates, and screened for homologous recombination by Southern blotting. PCR primers for generating hybridization probes were: 5'-TGGCTGGAGCGGACTGAGA-3' and 5'-ATGTTGTGGAGCCTCTGAAAATGG-3' for the P1 probe and 5'-CATATGCTGGGGTCCTCCTGTGA-3' and 5'-TGAGGCATGGCAGCACAGTCT-3' for the P2 probe. The ES clone with correct recombination was then expanded, injected into blastocysts, and implanted into pseudopregnant recipient female mice. Chimeric mice were bred with C57BL/6J mice to obtain mice with germline transmission. The mutant allele was termed *Atbfl^{PGK}*. Mice with an *Atbfl^{PGK}* allele were bred with *ACT-FLPe* transgenic mice (Jackson Laboratory, Bar Harbor, ME) to remove the *Frt*-flanked *PGK-puroΔtk* cassette, resulting in the floxed *Atbfl* allele *Atbfl^{flox}*. Heterozygous *Atbfl^{flox/wt}* mice were further bred to *Elia-Cre* transgenic mice (kindly provided by Dr. Tongzhong Ju, Emory University) to delete exons 7 and 8 to produce mice with the deleted *Atbfl* allele (*Atbfl^{Δ7&8}*). All alleles were maintained in the 129S6/C57BL/6J background. The generation and usage of the mice were approved by the Emory University Institutional Animal Care and Use Committee.

Genotyping by PCR

PCR was applied to genomic DNA from tail biopsies and MEFs to genotype various alleles of *Atbfl* and other genes. PCR products of the *Atbfl^{PGK}* allele (528 bp) were distinguished from that of the *Atbfl^{wt}* allele (457 bp) when using primers F1 (5'-GGCCCTTGACTGCATTTCTTTCCTGT-3') and R1 (5'-CCCAAGACCCAGTGCCCCAAAA-3'). Similarly, alleles of *Atbfl^{flox}* (1248 bp), *Atbfl^{wt}* (1071 bp) and *Atbfl^{Δ7&8}* (289 bp) were detected in a single PCR by using primers F1 and R2 (5'-ATTCGTTAATGGGAAGGTGTCAGA-3'). Primers used for genotyping other genes included: 5'-CGGTCGATGCAACGAGTGAT-3' and 5'-CCACCGTCAGTACGTGAGAT-3' for *Cre* (529 bp), 5'-CTAGGCCACAGAATTGAAAGATCT-3' and 5'-GTAGGTGGAAATCTAGCATCATCC-3' for *Il2* (324 bp), and 5'-CACTGATATTGTAAGTAGTTTGC-3' and 5'-CTAGTGCGAAGTAGTGATCAGG-3' for *Flp* (725 bp).

RT-PCR and real-time RT-PCR

Total RNA was extracted from MEFs or mouse tissues by Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1 μg of total RNA using the iScript reverse transcription kit (Bio-Rad, Hercules, CA). PCR primers used for amplifying fragments transcribed from all three *Atbfl* alleles (*Atbfl^{flox}*, 532 bp; *Atbfl^{wt}*, 532 bp; and *Atbfl^{Δ7&8}*, 197 bp) were 5'-GGCCAGATCTTACCATCC-3' and 5'-CAGGGAGGAACATGCTACTAGG-3', with the former from exon 5 and the latter from exon 9 of mouse *Atbfl*. *Atbfl* mRNA levels were measured in mouse organs by real-time RT-PCR using 1) primers 5'-AGAGCAAGAGGGCAGCGTCATC-3' and 5'-CGGTTACGTCAGCGTTGCTATAC-3', which span exons 6 to 8 and only amplify the wildtype *Atbfl* cDNA; 2) primers 5'-AAGCTGCGGCTGCACACAGTCAAC-3' and 5'-TCACTGCGCTGGTGCTTCATGG-3', which span exons 4 and 5 and amplify both the wildtype and the truncated *Atbfl* cDNA. Mouse *β-actin* mRNA, which was used as the internal control, was detected with primers 5'-GATCTGGCACCACCTTCT-3' and 5'-GGGGTGTGAAGGTCTCAAA-3'.

Immunoblotting and immunohistochemistry (IHC)

Atbf1 protein in MEFs was analyzed by immunoblotting. Briefly, MEFs were infected with Cre-expressing or control adenoviruses at an MOI of 200. Cell lysates were then prepared in the RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 10% glycerol, 20 mM β -glycerophosphate) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). A total of 50 μ g protein lysates were separated by 4% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membranes. After incubation with an anti-Atbf1 antibody (kindly provide by Dr. Masakiyo Sasahara of Toyama Medical and Pharmaceutical University, Toyama, Japan) (Ishii *et al.*, 2003), blots were then incubated with horseradish peroxidase-conjugated anti-IgG secondary antibodies (Sigma-Aldrich, St. Louis, MO) and visualized with an enhanced chemiluminescence system (Thermo Scientific Pierce Protein Research Products, Rockford, IL).

Atbf1 protein was analyzed by immunohistochemistry (IHC) in mouse tissues according to standard procedures. Briefly, tissue sections were deparaffinized, rehydrated, and washed in PBS. Antigen retrieval was done by heating slides in a microwave oven for 15 min in a citrated buffer (pH 6.0, 10 mM trisodium citrate). After blocking with 5% normal goat serum in Tris-buffered saline with 0.1% Tween-20 (TBST), tissue sections were incubated with primary antibodies at 4°C overnight, followed by an incubation with EnVision Polymer-HRP secondary antibodies (Dako, Glostrup, Denmark) at room temperature for 40 min. After the application of DAB-chromogen, tissue sections were mounted and visualized under microscopes. Primary antibodies used in this study included anti-Atbf1 (Ishii *et al.*, 2003), anti-Ki-67 (Lab Vision of Thermo Scientific, Kalamazoo, MI), and anti-CK18 (GeneTex, San Antonio, TX) antibodies.

Body weight measurement

Body weights were measured for the *Atbf1* heterozygous mice and their wildtype littermates aged at 13 to 29 days. Only a few litters were followed at multiple time points because of the frequent mortality. Body weights of the two groups (*Atbf1*^{wt/wt} vs. *Atbf1*^{wt/ Δ 7&8}) at different ages were graphically plotted against ages, and a general linear model (GLM) was used to test whether there was a significant difference in mouse body weight between the two groups. A *P* value of 0.05 was considered significant. The SAS statistical package V9.2 (SAS Institute Inc., Cary, NC) was used for data management and analysis.

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Figure 1. Generation of the floxed *Atbf1* allele (*Atbf1*^{lox}) in mice

a. Design of cloning vectors and targeting strategies. Part of the *Atbf1* gene (from exon 6 to exon 10) is shown at top (wildtype allele). *LoxP* and *Frt* sequences are marked by triangle and diamond-shaped boxes respectively. The targeting construct was generated by inserting three *Atbf1* fragments (6.2 kb, 0.9 kb and 4.7 kb) into *pFlexible*. Two hybridization probes (P1 and P2) used in Southern blot analyses are indicated in the mutant allele. The *PGK-puroΔtk* selection cassette was deleted by mating *Atbf1*^{PGK} mice with *ACT-FLPe* mice to generate the *Atbf1*^{lox} allele. Digestion of targeted genomic DNA by *Xba* I yields a 10.9 kb fragment for the wildtype allele and an 8.4 kb fragment for the mutant allele when the 5' probe (P1) is used, while digestion by *Xho* I yields 7.4 kb wildtype and 5.1 kb mutant bands when the 3' probe (P2) is used. b. Southern blot analyses of wildtype (wt) and targeted (PGK) alleles in ES cells, showing expected mutant fragments for both 5' and 3' probes.

Figure 2. Excision of exons 7 and 8 of *Atbf1* by the Cre recombinase

a. Schematic representation of Cre-mediated deletion of exons 7 and 8. *LoxP* and *Frt* sequences are marked by triangle and diamond-shaped boxes respectively. Primers F1 and R2 are indicated by arrow heads. The *Atbf1*^{Δ7&8} deletion allele was induced by Cre from adenoviruses or *Ella*-cre mice. b. Five MEF clones incubated with adenoviruses expressing GFP or Cre were genotyped by a PCR using primers F1 and R2, identifying three *Atbf1* alleles: *Atbf1*^{flox} (1248 bp), *Atbf1*^{wt} (1071 bp) and *Atbf1*^{Δ7&8} (289 bp). c. Detection of the wildtype (532 bp) and truncated (197 bp) *Atbf1* mRNA by RT-PCR in MEFs with different status of *Atbf1* deletion. d. Detection of Atbf1 protein in MEFs with different *Atbf1* deletion status by immunoblotting. Molecular masses of protein standards are shown to the left of the gel.

Figure 3. Reduced body size and weight in mice with deletion of one *Atbf1* allele

a. Representative genotyping PCR. Eight mice were examined for *Atbf1*, *Cre*, and *Il2* (an internal control) genotypes. Three *Atbf1* alleles (*Atbf1^{flox}*, *Atbf1^{wt}* and *Atbf1^{Δ7&8}*) were detected in a single PCR. Two mice with the *Cre⁺/Atbf1^{flox/wt}* genotype (#1 and #8) showed a mosaic genotype. M, 1 Kb plus DNA standard (Invitrogen). b. Mosaic genotypes in different organs. Nine organs from mouse #1 in panel 3a were examined for the *Atbf1* genotype. B, brain; Lv, liver; Lg, lung; H, heart; I, intestine; K, kidney; Sp, spleen; St, stomach; T, tail. c. Representative images of 21-day old mice with the wildtype (wt/wt) or heterozygous deletion (wt/Δ7&8) of *Atbf1*. d. Weights of wildtype mice (wt/wt, filled circles) and heterozygous mice (wt/Δ7&8, open circles) aged at 13 to 29 days. *Atbf1* heterozygous mice were significantly lighter than their wildtype littermates.

Figure 4. Reduced *Atbf1* expression in mice with *Atbf1* deletion

a. Knockout of *Atbf1* reduced the expression of the wildtype *Atbf1* mRNA in most organs of mice at postnatal day 1 (left) and day 21 (right), as detected by real-time RT-PCR. The ratio of *Atbf1* to β -actin mRNA levels for wildtype mouse brain was set to 1, and that for other organs were adjusted accordingly. The reduction by about half is obvious in the brain (B), liver (Lv), lung (Lg), heart (H), kidney (K) and stomach (St) but not in the intestine (I) and spleen (Sp) in mice at 21-day-old (right). b. Detection of the truncated *Atbf1* mRNA by real-time RT-PCR in the brain, liver, heart, intestine, kidney and spleen but not in the lung and stomach of the *Atbf1* heterozygous mice. c. Molecular and histological analyses of mouse kidneys from both *Atbf1* wildtype (wt/wt) and heterozygous (wt/ Δ 7&8) mice. HE, representative images from Hematoxylin and Eosin stained sections. IHC staining confirmed reduced *Atbf1* expression, increased Ki-67 expression and attenuated CK18 expression in the *Atbf1* heterozygous mice (wt/ Δ 7&8) when compared to wildtype mice (wt/wt). All scale bars are 100 μ m.

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Table 1

Numbers of live mice obtained for different genotypes.

Atbfl genotypes	Cre ^{lox} - wt/wt	Cre ⁺			Cre ⁻ wt/Δ7&8
		flox/wt	flox/wt/Δ7&8*	wt/Δ7&8 [§]	
total					
At birth	442	7	25	60	92
48 hrs after birth	434	7	23	41	71
Weaning (4 weeks)	431	6	21	24	51

* , deletion of exons 7 and 8 occurred only in some but not all cells in Cre⁺/Atbfl^{flox/wt} bigenic mice, showing a mosaic genotype.

§ excision of exons 7 and 8 was complete in all cells in Cre⁺/Atbfl^{flox/wt} mice.

¶ P values for comparisons between Cre⁻/Atbfl^{wt/Δ7&8} and Atbfl^{wt/wt} at different time points were all smaller than 0.0001.