Zfp423 Is Required for Normal Cerebellar Development†

Søren Warming, Rivka A. Rachel, Nancy A. Jenkins, and Neal G. Copeland*

Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute, Frederick, Maryland 21702

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Zinc finger protein 423 (also known as Ebf-associated zinc finger protein, Ebfa3) binds to and negatively regulates Ebf1, a basic helix-loop-helix transcription factor required for B-cell lineage commitment and olfactory epithelium development. *Zfp423* also binds to Smad1/Smad4 in response to Bmp2 signaling. *Zfp423* contains 30 Krüppel-like zinc fingers that are organized into discrete clusters; some zinc fingers are used to bind DNA, while others mediate *Zfp423*'s interaction with other signaling proteins such as Ebf1 and Smad1/Smad4. Previously, we showed that *Zfp423* is an oncogene whose upregulation following retroviral integration in murine B cells leads to an arrest in B-cell differentiation and the subsequent development of B-cell lymphomas. To study the biological functions of *Zfp423* in vivo, we used recombineering and gene targeting to generate mice that carry conditional as well as null alleles of *Zfp423*. Homozygous *Zfp423* null mice are runted and ataxic, the cerebellum is underdeveloped, and the vermis is severely reduced. In the remaining cerebellar structures, the Purkinje cells are poorly developed and mislocalized. In mice carrying a hypomorphic *Zfp423* gene trap allele, *lacZ* expression in the cerebellum correlates with the Purkinje cell layer, suggesting that these phenotypes are a result of a Purkinje cell-intrinsic defect.

Zfp423 is one of two members of a novel transcription factor family of zinc finger proteins with 30 Krüppel-like zinc finger (ZF) domains. The other family member is *Zfp521* (also known as ecotropic viral integration site 3, *Evi3*) (10, 26). *Zfp423* was initially identified as an interaction partner of Ebf1 in a yeast two-hybrid screen by using a cDNA library from rat olfactory epithelium (19), where it modulates the transcriptional activation mediated by Ebf1. *Zfp423*/Ebf1 heterodimerization involves the most C-terminal zinc fingers of *Zfp423*, ZFs 28 to 30, whereas *Zfp423* homodimerization occurs independently of these ZFs (20), and DNA binding of a palindromic target sequence occurs via the seven (8) most N-terminal ZF domains.

The olfactory/early B-cell factor (Ebf) transcription factor family consists of four members, all with a characteristic helix-loop-helix domain (22, 24). Expression of *Ebf* genes during development is restricted mostly to neuronal tissues (24); all four members are expressed in olfactory tissue and also show overlapping expression patterns in other areas of the developing central nervous system. In cerebellum, *Ebf1* and *Ebf2* expression is found in Purkinje cells, with *Ebf1* expression being higher than that of *Ebf2*. In adult mice, *Ebf1* is expressed in several tissues, whereas the expression of the other family members is restricted mostly to olfactory tissue. Within olfactory tissue, *Ebf1*, -2, -3, and -4 expression is restricted to the neuronal and basal layers. The overlapping pattern of *Ebf* expression in olfactory tissue suggests functional redundancy, and whereas mice without *Ebf1* lack immunoglobulin-expressing B cells, demonstrating that *Ebf1* is required for normal B-cell development, olfactory tissue in these mice develops

normally (13). A comprehensive analysis of chick *Ebf1* showed that in the hindbrain region, *Ebf1* is required for neuronal differentiation and migration from the ventricular zone to the mantle layer (4), suggesting that Ebf1 might coordinate the coupling of neuronal differentiation with migration after cell cycle exit.

Interestingly, *Zfp423* has also been shown to associate with activated Smad (contraction of Sma and Mad) transcription factors in response to bone morphogenetic protein 2 (Bmp2) signaling (7). In *Xenopus laevis*, the *Zfp423* orthologue, *Xoaz*, activates the homeobox regulator of mesoderm and neural development, *Xvent-2*. The ZF domains of *Zfp423* involved in Bmp2 signaling are separate and independent from the Ebf1-interacting ZFs; binding to activated Smad1 and Smad4 occurs via ZFs 14 to 19, and binding to the Bmp2 response element occurs via ZFs 9 to 13.

We have previously reported that *Zfp423* is a frequent retroviral integration site in murine B-cell lymphomas (27). *Zfp423* is not normally expressed in B cells, and retroviral integration results in ectopic activation and overexpression of this gene. *Zfp521* on the other hand is expressed throughout B-cell development, albeit at very low levels (26). Murine B-cell tumors with retroviral integration in either *Zfp423* or *Zfp521* show high-level expression of *Zfp423* or *Zfp521*, respectively, as well as the surrogate B-cell receptor light chain gene *lambda5* (27). Ebf1, together with E47, activates *lambda5* expression in early B-cell development (18), and based on the high homology with *Zfp521*, the result of *Zfp423* activation is most likely the same as that of overexpression of *Zfp521*: B-cell lymphoma due to inhibition of B-cell differentiation through modulation of Ebf1 activity (9, 26, 27).

The ability of *Zfp423* to both act in Bmp2 signaling, via Smad interaction, and modulate Ebf activity opens up several possibilities for the function of this gene in vivo. However, besides the effect of *Zfp521* and *Zfp423* overexpression in the development of B-cell lymphoma, little is known about the biological functions of this multi-zinc finger gene family.

* Corresponding author. Mailing address: Mouse Cancer Genetics Program, 1050 Boyles Street, Bldg. 539, Room 229, National Cancer Institute, Frederick, MD 21702. Phone: (301) 846-1260. Fax: (301) 846-6666. E-mail: copeland@ncifcrf.gov.

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In the studies described here, we used “recombineering” (recombination-mediated genetic engineering in *Escherichia coli* [14]) and gene knockout to create null as well as conditional alleles of *Zfp423* and, in the process, uncovered for the first time an essential role for *Zfp423* in normal cerebellar development.

MATERIALS AND METHODS

Mice with a conditional *Zfp423* allele. The conditional gene-targeting vector was constructed using a novel recombineering approach developed in our laboratory, essentially as described previously (14). Detailed recombineering protocols and information on how to receive the recombineering reagents can be found on our website at <http://recombineering.ncicrf.gov>. A CJ7/129 BAC library (CT7; Invitrogen) was screened with a *Zfp423* genomic probe to identify a bacterial artificial chromosome (BAC) containing the genomic region of interest (clone no. 398E19, catalog no. 96022; Invitrogen). BAC DNA was purified using the Nucleobond BAC maxi kit (Clontech, BD Biosciences). The BAC was transferred into the modified *E. coli* strain DY380 by electroporation (11). To create a new backbone for targeting vectors with no homology to the BAC backbone, we PCR amplified 2 kb of pBluescript SK(−). The forward primer contains an *NheI* recognition site. The reverse primer contains recognition sites for the following enzymes: *BamHI*, *XhoI*, *Sall*, *ClaI*, *HindIII*, *EcoRI*, *SpeI*, *XbaI*, and *NotI*. *NheI*, *BamHI*, and *NotI* sites are underlined in the primer sequences given below. The base pairs recognizing pBluescript are in italics. The following primers were used: pBlight F, 5′AAATAAGCTAGCGAGAGCGGTTTGGCTATG3′; and pBlight R, 5′AAATAAGGATCCCTCGAGGTCGACATCGATAAGCTTGAATTCAGTAGTTCTAGAGCGGCCGCCGCGGAACCCCTATTTGT3′. PCR conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min for 25 cycles, using 10 ng pBluescript SK(−) as the template. We then ligated *NheI*/BamHI-digested PCR product with an *NheI*/BamHI fragment containing the thymidine kinase cassette from PL253 (14) to give rise to pBlight-TK. Two homology arms flanking the genomic area of *Zfp423* to be subcloned by gap repair were PCR amplified (Expand High Fidelity; Roche) using 1 μg BAC DNA from clone 398E19 as the template, digested with *BamHI* and *HindIII* (5′ arm) or *HindIII* and *NotI* (3′ arm), and three-way ligated into *BamHI*- and *NotI*-digested pBlight-TK. The primers used for amplification of the 5′ and 3′ homology arms were as follows: 5′ arm F, 5′AATTTTGGATCCGCCTCCAGCTC ATGCTGT3′; 5′ arm R, 5′AATTTTAAGCTTCTCTGGGTCTCCAGGACAT3′; 3′ arm F, 5′AATTTTAAGCTTGTGCTGACCCATGGAGTGAA3′; and 3′ arm R, 5′AATTTTGCAGGCCGCTGCCCATATGGACAGATGT3′. A total of 500 ng of this retrieval vector was linearized by *HindIII* digestion, gel purified, and transformed into heat-shocked and electrocompetent DY380 cells containing the 398E19 BAC clone. The genomic 13.8-kb region thus subcloned was modified in two targeting rounds using the neomycin (*neo*) cassettes from PL452 and PL451 as described previously (14). First, to insert the single 5′ *loxP* site, a floxed *neo*/kanamycin cassette (from PL452) containing homology to a region 5′ of *Zfp423* exon 4 was inserted. The primers used to amplify the homology arms were as follows: 5′ targeting 5′ arm F, 5′AAATAAGTCGACGCCCTCGGCTTGCTTCAT3′; 5′ targeting 5′ arm R, 5′AAATAAGAATTCTCCACAGGCCGAAAAGGAAT3′; 5′ targeting 3′ arm F, 5′AAATAAGGATCCCAAGCTTCTGTGGTCAATTTCCGGTGAC3′; and 5′ targeting 3′ arm R, 5′AAATAAGCGGCCGCGACAGAGCCAGCTTCTCCA3′. *Sall*, *EcoRI*, *BamHI*, *HindIII*, and *NotI* sites are underlined. Homology arms were digested with *Sall* and *EcoRI* (5′ arm) and *BamHI* and *NotI* (3′ arm) and four-way ligated with the *EcoRI*- and *BamHI*-digested *neo* cassette from PL452 and *Sall*- and *NotI*-digested pBluescript. The targeting cassette was released by *Sall* and *NotI* digestion and inserted into the retrieved *Zfp423* fragment by recombineering, as described previously (14). The *neo* cassette was then removed by transformation into arabinose-induced *Cre*-expressing EL350 cells (11) to leave behind only a single *loxP* site along with the extra *HindIII* site used for genotyping. Next, using the same procedure, a *Frt-neo-Frt-loxP* cassette (from PL451) was inserted 3′ of exon 4. The primers (*Sall*, *EcoRI*, *BglII*, *BamHI*, and *NotI* sites are underlined) used for amplifying the homology arms for the 3′ targeting vector were as follows: 3′ targeting 5′ arm F, 5′AAATAAGTCGACGCCATTGATAGGGAAGTAGGAT3′; 3′ targeting 5′ arm R, 5′AAATAAGAATTCTCAGATCTCCAGAGAGAGCTCTCTG3′; 3′ targeting 3′ arm F, 5′AAATAAGGATCCGGGCGCCGCCACCTTCTTTGA3′; and 3′ targeting 3′ arm R, 5′AAATAAGCGGCCGCCGACCAACCTG3′. PCR conditions for amplification of all homology arms were 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 25 cycles, using 1 μg BAC clone 398E19 DNA as the template. All primers were purchased from Integrated DNA Technologies (IDT). PCR was done using Expand High Fidelity

(Roche). The transitions between genomic DNA and inserted sequences were confirmed by sequencing, and the conditional gene-targeting vector was tested for functionality by transformation into arabinose-induced EL250 (*Flp* expression; to test for *neo* removal) and EL350 (to test for deletion of exon 4). The conditional targeting vector was then linearized by *NotI* digestion and electroporated into 129-derived CJ7 embryonic stem (ES) cells, using standard procedures. G418 (200 μg/ml) and FIAU [1-(2-deoxy-2-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil] (0.5 μM) double-resistant clones were analyzed by Southern blot hybridization, using external probes I and III (Fig. 1A), and correctly targeted clones harboring both the single 5′ *loxP* site and the 3′ *Frt-loxP*-flanked *neo* cassette were injected into C57BL/6 blastocysts, using standard procedures. After germ line transmission of the targeted (*neo*) allele, the null (knockout [ko]) and conditional ko (cko) alleles were obtained by crossing to β-actin-*Cre* and β-actin-*Flp* transgenic mice, respectively. The line with the cko allele was maintained by intercrossing homozygous cko/cko mice, and the line with the null allele was maintained by intercrossing ko/+ heterozygous mice. The mice were kept on a mixed genetic background (129, C57BL/6, and CD1). Nutra-Gel (Bio-Serv, New Jersey) was put in all mating cages with ko/ko pups, and the Nutra-Gel was changed daily. If the ko/ko pups were too small at normal weaning age, they were kept with the female for 2 or 3 weeks longer. If needed, after weaning, the ko/ko animals were given saline injections intraperitoneally (0.9%; Sigma-Aldrich) but not more frequently than every other day.

***Zfp423* gene trap clone XH542.** The BayGenomics search engine (<http://baygenomics.ucsf.edu>) was used to identify an ES cell clone with an *Zfp423* gene trap allele (XH542). The ES cell clone was obtained from BayGenomics (funded by the National Heart, Lung, and Blood Institute), tested for the lack of mouse antibody production, and injected into C57BL/6 blastocysts by using standard procedures. After germ line transmission, the allele was backcrossed for two generations to C57BL/6, and the line was then maintained either by homozygous (gt/gt) intercrosses or by gt/+ × C57BL/6 backcrossing. The exact integration site of the *beta-geo* cassette (derived from the pGT11x vector) was identified using inverse PCR on *SacI*-digested and self-ligated genomic tail DNA, followed by sequencing. The primers (IDT) used for inverse PCR were as follows: F, 5′CTGTATGAACGGTCTGGTCTT3′; and R, 5′GATTGACCGTAATGGGATAGGT3′. The PCR (Expand High Fidelity; Roche) conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 2 min for 35 cycles.

Genotyping. Mice were genotyped by Southern blot analysis of tail DNA, using standard methods. ES cells were analyzed using the external probes I and III, and tails were analyzed using either probe I, II, or III. The probes were PCR amplified using 1 μg BAC as the template. Primers (Fig. 1A) were as follows: 5′ external probe F, 5′CCTTATCTATGAGAACCTCTAGTA3′; 5′ external probe R, 5′CTTACAGTGTACATGAGGATTTGT3′; 3′ external probe F, 5′GAGATGATGGCTTTGTGTG3′; and 3′ external probe R, 5′CCCAAAGGACCTAGCATGT3′. The internal probe (Fig. 1A) is the 5′ targeting 5′ homology arm. Probes I, II, and III were amplified using BAC clone 398E19 DNA as the template; XH542 probe (BAC clone RP23-60C20 as the template; Invitrogen) F, 5′CTGGCCATCCTGTCTACTTT3′; XH542 probe R, 5′CTCGGGTGTGTGCAACAAT3′; Cre probe (genomic tail DNA from a Cre transgenic line as the template) F, 5′ATCTGGCATTCTGGGGATT3′; and Cre probe R, 5′TTATTCGGATCATCAGCTACAC3′. PCR conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 25 to 30 cycles. Embryos (yolk sac DNA) and early postnatal pups (tail DNA) were genotyped by three-primer PCR analysis with the following primers: *Zfp423* F, 5′GCCTGTACCCAAAGGACCA3′; *Zfp423* wt R, 5′GCAGTGACACCTGCCTCAA3′; and *Zfp423* mut R, 5′GGACCATAAGAGGACGTGAT3′. The wild-type band is 264 bp, and the mutant band is 438 bp. The following primers were also used: genetrp F, 5′GGTCGGTAGCTGGCAACA3′; genetrp wt R, 5′CCAGAGGAGTATTTCTCCTT3′; and genetrp mut R, 5′CCTCTACATAGTTGGCAGT3′. The wild-type band is 422 bp, and the mutant band is 568 bp. PCR conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles. All primers were from IDT. PCR was done using Expand High Fidelity (Roche).

Expression analysis. Total RNA was purified from dissected brains, using a handheld homogenizer (OMNI TH 115; OMNI International) and TRI Reagent (Sigma-Aldrich) following the manufacturers' directions. Poly(A) RNA was isolated using the Micro Poly(A) Purist kit (Ambion) according to the manufacturer's directions. First-strand synthesis was done using poly(A)-selected RNA and the Superscript III kit (Invitrogen) and random hexamer primers. Northern blot analysis was done according to standard procedures. Full-length *Zfp423* cDNA (27) was used as a probe, and a plasmid containing murine *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) was labeled using a nick translation kit (Roche) and used as the control probe. Reverse transcription (RT)-PCR analysis was done with the following primers (IDT): exon3 F, 5′GAGCCAGAGTGATGATCGGAA3′; exon4 R, 5′GCGTAGGTGACGCAACATC3′, and

FIG. 1. Generation of null and conditional *Zfp423* alleles. (A) *Zfp423* genomic region encoding exon 4. Restriction fragment sizes are indicated as well as the positions of the internal and the two flanking probes used for genotyping analysis (roman numerals). The shaded area indicates the part of the genomic region included in the targeting vector, and the three different alleles described in this study are shown. *neo* is the result of the gene-targeting event. *cko* is the conditional knockout allele derived from the *neo* allele after Flpe-mediated excision of the PGK-*neo* cassette. One *Frt* site and two *loxP* sites remain in the locus. *ko* is the null allele derived from the *neo* or from the *cko* allele by Cre-mediated recombination between the two *loxP* sites. Only a single *loxP* site remains in the modified locus. *loxP* and *Frt* sites are indicated as black and red triangles, respectively. Neo, PGK-em7-neomycin dual selection cassette for bacteria and ES cells; TK, thymidine kinase cassette for counterselection in ES cells; H, HindIII; B, BglII; N, NotI. The genomic region is not drawn to scale. (B) Results of a Southern blot analysis of BglII-digested tail DNA, probed with the internal probe (II). The genotypes are indicated above each lane, and the sizes of the bands are indicated to the left. (C) Results of a poly(A) Northern blot analysis of whole-brain RNA from 3-week-old mice, using a full-length *Zfp423* cDNA probe. After decaying, the blot was rehybridized with a *Gapdh* probe as a control for RNA quality. Genotypes are indicated as well as the positions of the different bands. wt, wild type.

lacZ R, 5'CCAGTCACGACGTTGTAAA3'. PCR conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 25 cycles, (Expand High Fidelity; Roche). The wild-type product (exon3 F plus exon4 R) is 258 bp, and the gene trap product (exon3 F plus lacZ R) is 318 bp.

Fixation, Histochemistry, lacZ staining, and pictures. Embryos and whole brains from postnatal pups were dissected and fixed overnight in 4% paraformaldehyde-phosphate-buffered saline (PFA-PBS). Adult mice were perfused intracardially with cold 1× PBS followed by 4% PFA-PBS, and the dissected brains were postfixed overnight in 4% PFA-PBS. After fixation, the brains were stored in PBS with 0.005% sodium azide. For hematoxylin-eosin (H&E) staining, the brains were processed, embedded, sectioned, stained, and mounted using standard procedures. Staining for beta-galactosidase was done as follows: dissected brains or embryos were fixed for 1 h in glutaraldehyde-PFA, washed in PBS overnight, and then embedded in 3% agarose. Sections (1 mm) were cut, using a vibratome, and stained in beta-galactosidase staining solution overnight at 37°C. The stained sections were embedded in paraffin, cut in 8-μm (microtome) sections, and counterstained with 0.1% neutral red for 1 minute before being mounted. Pictures were taken using a Zeiss Axiophot microscope (for slides), a Nikon SMZ1500 microscope (whole brains), a Zeiss AxioCam HRC digital camera, and OpenLab software (Improvision). Pictures of wild-type and mutant mice and video of the *Zfp423* mutant mouse (see the supplemental material) were taken using a Nikon Coolpix 8800 digital camera.

Immunostaining and confocal microscopy. Brains were fixed as described above, and 50-μm sections were embedded in agarose and cut using a vibratome. The sections were blocked for 1 hour in 1× Tris-buffered saline (TBS) with

0.005% azide (Sigma-Aldrich), 10% normal goat serum (NGS; Jackson ImmunoResearch), 2% bovine serum albumin (Sigma-Aldrich), and 1% Triton X-100 (Sigma Aldrich) and then stained for 2 days at 4°C with primary antibodies diluted in 1× TBS with 0.005% azide, 1% NGS, and 1% Triton X-100 (rabbit Calbindin D28K, 1:10,000; Swant, Bellinzona, Switzerland). The samples were washed in 1× PBS with 0.005% azide for 2 hours and incubated overnight in 1× TBS with azide, 1% NGS, and 1% Triton X-100 with secondary antibody, goat anti-rabbit Cy3 (1:500; Jackson ImmunoResearch), and DAPI (4',6'-diamidino-2-phenylindole) (1:10,000). After being washed in PBS with azide, the samples were mounted using ProLong Gold (Molecular Probes, Eugene, OR). A Carl Zeiss LSM510 Meta (Jena, Germany) confocal microscope (×40 magnification) and Carl Zeiss LSM Image Browser software were used for confocal microscopy.

RESULTS

Generation of null and conditional *Zfp423* alleles. To study the biological effects of a genetic disruption of *Zfp423* in vivo, we simultaneously generated a conditional and a null allele by using a novel and efficient recombineering strategy recently developed in our laboratory (Fig. 1A) (14). We decided to delete *Zfp423* exon 4, since this exon encodes 25 of the 30 zinc finger repeats (27). Deletion of exon 4 furthermore results in a

frameshift when exons 3 and 5 are spliced together, and the resulting mRNA, if made, is expected to encode a protein of only 132 amino acids, including only the first zinc finger. To construct the conditional targeting vector, a 13.8-kb *Zfp423* genomic region spanning exon 4 was subcloned, by plasmid gap repair, from a mouse 129 strain-based BAC into a vector with a thymidine kinase cassette for counterselection in ES cells. Next, a *loxP*-flanked *neo* cassette was inserted 5' of *Zfp423* exon 4. The *neo* cassette was then deleted by Cre-mediated excision in bacteria, leaving behind a single *loxP* site and an introduced HindIII site for genotyping. Finally, a *neo* cassette flanked by two *Frt* sites and a single *loxP* site was inserted 3' of exon 4, deleting a genomic HindIII site and inserting a BglII site for genotyping.

Correct targeting of ES cells was verified using Southern blot analysis and external probes (data not shown). Seven of 64 clones (11%) analyzed contained both the single *loxP* site and the *Frt-neo-Frt-loxP* cassette, and three of these correct clones were injected into blastocysts. After germ line transmission of the *neo* allele, the ko allele was obtained by crossing heterozygous *neo* mice to mice with general *Cre* expression. Mice with the cko allele were obtained by crossing *neo* mice to mice with general *Flp* expression. The cko allele was maintained by intercrossing homozygous cko/cko mice. Since homozygous cko/cko mice display no phenotype and since the ko and cko alleles are derived from the same *neo* allele, the phenotypes described below can be attributed to the designed deletion of exon 4, and the data described here therefore result from the analysis of mice derived from a single ES cell clone. Heterozygous ko/+ mice were intercrossed to obtain ko/ko null mice. An example of a genotyping result using Southern blot analysis and internal probe II is shown in Fig. 1B. Figure 1C shows the results of a Northern blot analysis of brain RNA from 3-week-old mutant and control mice. As expected, homozygous null mice do not express full-length *Zfp423* mRNA. A smaller and very weak band with the size expected for an exon 4 deletion can be observed both in ko/+ and ko/ko mice but not in +/+ mice.

Starting from before 2 weeks of age, and with 100% penetrance, homozygous *Zfp423* null mice show clear signs of ataxia, with wobbling gait and problems with balance and motor coordination (see Video S1 in the supplemental material). Homozygous null mice are noticeably smaller than their normal littermates (Fig. 2A), and after weaning, these mutants can be sustained only if given wet food in the bottom of the cage and saline injections, when necessary, to prevent dehydration. With this extra level of attention, however, null mice can live for more than a year in our hands, although a significant number of animals die before or around weaning.

The cerebellum is underdeveloped in *Zfp423* null mice. The early onset of ataxia suggests problems with normal cerebellar development, and we therefore decided to look at the developing cerebellum in more detail. As shown in Fig. 2B, the cerebellum in homozygous ko/ko mice is severely underdeveloped, and whereas the cerebellum from both ko/+ and +/+ littermates consists of two lateral hemispheres and a central vermis, the vermis is severely reduced in ko/ko mice and the two hemispheres are much smaller than normal. Figure 3 shows H&E-stained mid-sagittal cerebellar sections from ko/+ and ko/ko littermates from four different stages of postnatal development (P4, P7, P10, and P13). Normal postnatal cere-

FIG. 2. Runt size and gross cerebellar defects in *Zfp423* null mice. (A) Photograph showing the size difference between two 5-week-old male littermates. The genotypes are indicated. (B) Photographs of paraformaldehyde-perfused and fixed brains clearly showing the severely reduced cerebellar vermis in two 13-day-old *Zfp423* mutants. All four pictures are shown to the same scale. Ve: vermis; He: hemisphere.

bellar development results in a large increase in cerebellar size, accompanied by an increase in cerebellar cortical surface area due to extensive foliation. The normal increase in size is mediated partly by the rapid proliferation of granule cell neuronal precursors (reviewed in reference 8). Although cortical foliation is seen in ko/ko mice, the number and size of the lobes are much reduced. After the normal cerebellum reaches its final size, the granule cells stop proliferating and migrate inward past the developing Purkinje cells to give rise to the internal granule cell layer (IGL). In the mutants, an IGL is also formed,

FIG. 3. Cerebellar defects in postnatal development in *Zfp423* mutant and control mice. Mid-sagittal H&E-stained cerebellar sections from two littermates from four different stages of postnatal development (P4, P7, P10, and P13), clearly showing the decreased cerebellar size and reduced foliation. All pictures are shown to the same scale.

indicating that the signals controlling this process are still present in the mutants.

The observed overall reduced size of the cerebellum is not simply due to a delay in development, as *ko/ko* mice of even 1 year of age never develop a normal cerebellum (Fig. 4A). Similarly, the severe reduction of the vermis is seen clearly on frontal cerebellar sections from 1-year-old mice (Fig. 4B). The small cerebellar size and the almost complete absence of a vermis are therefore due to a significant defect in hindbrain development in these mice.

Developmental sequence of reduced brain size in *Zfp423* null mice. To evaluate the developmental sequence of reduced brain size in *Zfp423* null mice, we acquired a series of H&E-stained sections of *Zfp423* knockout brains at various ages (Fig. 5). At embryonic stages (E15 shown), small differences be-

tween normal and *ko/ko* brains were observed that became more noticeable at later ages. By birth (P1), structures from the diencephalon through the myelencephalon appeared hypomorphic, and this difference persisted throughout life, leading to the appearance of enlarged lateral ventricles (Fig. 5). The size differences between normal and knockout animals were most pronounced posterior to the mes/metencephalon (mid/hindbrain) border (Fig. 5).

Ectopic and poorly developed Purkinje cells. Normally, during the inward migration of granule cells, synapses are formed between the axons (parallel fibers) of the granule cells and the developing dendritic arbors of the Purkinje cells. After the formation of synapses, the Purkinje cells form a single cell layer between the outer molecular layer and the IGL. Even in *ko/ko* mice of 1 year of age, many of the Purkinje cells are ectopically positioned in the molecular layer (Fig. 4C). To analyze the ectopic positioning of Purkinje cells in *ko/ko* mice in more detail, we used confocal microscopy and a marker specific for Purkinje cells in the cerebellum (Calbindin). As shown in Fig. 6, the Purkinje cells have already started forming a single cell layer by P4 in the normal *ko/+* littermate, whereas Purkinje cells in *ko/ko* mice have not. Furthermore, the development of dendrites is delayed when *ko/ko* and *ko/+* mice are compared during early postnatal development. The Purkinje cells in the *ko/ko* mice eventually do develop a dendritic arbor, but the branching of the dendrites is less developed than in the *ko/+* littermates and the orientation of the Purkinje cell bodies appears disorganized.

***Zfp423* expression in the cerebellum.** To study the normal expression pattern of *Zfp423* in the cerebellum, we took advantage of a publicly available gene trap clone, XH542. In this clone, a splice acceptor- β geo (*lacZ* fused to *neo*) reporter gene is integrated in *Zfp423* intron 3 (Fig. 7A). To facilitate genotyping by Southern blot analysis and PCR, we used an inverse PCR strategy to precisely locate the insertion site and subsequently designed a probe for easy genotyping (Fig. 7B). Mice homozygous for the gene trap allele (*gt/gt*) do not display the ataxic phenotype observed in *ko/ko* mice (data not shown), indicating that the gene trap is not a null allele. To see whether we could detect wild-type *Zfp423* expression in *gt/gt* and *gt/ko* mice, presumably due to alternative splicing, we performed an RT-PCR analysis of brain RNA (Fig. 7C). The clear presence of a wild-type transcript in the absence of a wild-type allele confirms that the gene trap allele is not a null allele. Intron 3, where the gene trap is integrated, is 75 kb, and the insertion site is 11 kb downstream of exon 3. It is possible that the large intronic size and the large distance between the upstream exon and the splice acceptor 5' of the β geo reporter are the reason the intended disruption of normal splicing is not complete. The extra, larger band seen in the *gt/gt* and *gt/ko* lanes (Fig. 7C) was sequenced and shown to be the result of cryptic splicing involving exon 3, 206 bp of the gene trap vector plasmid backbone 3' to the poly(A) signal from the β geo reporter gene, and exon 4. Due to several in-frame stop codons, this product encodes only the amino acids from *Zfp423* exons 1 through 3. The extra band was not detectable in the *gt/+* lane, probably due to competition with the transcript from the normal allele in the PCR amplification. We next used beta-galactosidase staining as an indicator of normal *Zfp423* expression in heterozygous gene trap (*gt/+*) embryos and mice (Fig. 7D). *lacZ*

FIG. 4. Cerebellar defects in 1-year-old *Zfp423* mutant mice. H&E-stained sections from three female (A) or three male (B and C) littermates of 1 year of age. (A) Mid-lateral sagittal sections of the cerebellum showing that the reduced cerebellar size is still evident at 1 year of age. All sections were cut at the same distance from midline and shown at the same magnification. (B) Frontal half-cerebellar sections from three 1-year-old male littermates. Note the severe reduction in size of the cerebellar vermis and clearly reduced cerebellar size. All sections were cut at the same distance from the cerebellar/midbrain border and shown at the same magnification. He, hemisphere; Ve, vermis. (C) Higher-power magnification of the cerebellar cortex; all sections are shown at the same magnification. ML, molecular layer. White arrows point to the presence of some of the ectopic Purkinje cells in the molecular layer in the *ko/ko* mutant.

staining correlates with the developing single cell layer of Purkinje cells at postnatal day 4. It is therefore possible that the *lacZ* staining observed at E15.5 also correlates with that of migrating postmitotic Purkinje cells at this stage. This expression analysis suggests that *Zfp423* expression in the cerebellum is restricted to Purkinje cells. In addition, strong *lacZ* staining was also found in the thalamic region (data not shown).

Due to the presence of both *lacZ* expression and alternative splicing, we speculated that the gene trap allele could be hypomorphic, and with a significantly reduced amount of the

wild-type message, *gt/ko* mice might not have enough normal *Zfp423* expression to completely rescue the observed cerebellar phenotypes of *ko/ko* mice. We therefore analyzed the cerebella from mice resulting from *gt/+* $\times$ *ko/+* intercrosses (Fig. 7E). Whereas both *ko/+* and *gt/+* animals had normal cerebella, the cerebellum in *gt/ko* mice was significantly reduced in size with less extensive foliation. However, the Purkinje cells appeared to be localized normally in these mice (data not shown). This phenotype thus appears to be intermediate between the normal and the *ko/ko* mutant phenotype, supporting the finding that the XH542 allele is hypomorphic.

FIG. 5. Subtelecephalic structures of the *Zfp423* null brain are smaller than those in normal littermates. H&E-stained sagittal sections of brains from *ko/ko* and normal (*ko/+* or *+/+*) littermates were collected from E15 to 1 year of age. Note the normal size of the olfactory bulb and cerebral cortex at all stages of development. Met/ myelencephalic structures are hypomorphic, however, with the cerebellar dysgenesis most prominent. One-millimeter scale bars are indicated. Asterisks indicate enlarged ventricles in the mutant brains. hb, hindbrain.

DISCUSSION

Using recombineering and gene knockouts, we show here that the multi-zinc finger transcription factor gene *Zfp423* is required for normal cerebellar development. Mice homozy-

FIG. 6. Misorganization of Purkinje cells in *Zfp423* *ko/ko* mice. Calbindin (red)- and DAPI (blue)-stained sagittal cerebellar sections from four different stages of postnatal development, as indicated, analyzed by confocal microscopy. Note the developmental delay of Purkinje cells as well as the lesser degree of dendritic branching and general Purkinje cell body misorganization. EGL, external granule cell layer. The genotypes are indicated.

gous for a *Zfp423* null allele are ataxic, the cerebellum is severely underdeveloped with reduced size and decreased foliation, and the vermis is severely reduced and almost completely absent. The Purkinje cells develop more slowly in the mutants, and many are ectopically situated in the molecular layer of the cerebellar cortex. The early onset ataxia in these mice is therefore most likely a result of these cerebellar defects.

Zfp423 is highly related to *Zfp521* (26); however, at least in cerebellar development, *Zfp521* does not appear to be able to substitute for the absence of *Zfp423*, suggesting that these genes have nonoverlapping expression patterns and/or func-

tion. This has indeed been confirmed in previous experiments during B-cell development, in which *Zfp521* expression was detected at all developmental stages while *Zfp423* expression was not detectable at any stage (26). Not surprisingly, in our *Zfp423* null mice, we could not detect any hematopoietic phenotypes (data not shown).

Although the overall size of the cerebellum is reduced, the granule cells still migrate inward past the Purkinje cells and an IGL is formed in the mutants. The size reduction is therefore probably due mainly to a severe reduction in granule cell precursor proliferation that does not affect the subsequent migration and differentiation of granule neurons. It has recently been shown that the secreted mitogen Sonic hedgehog (Shh) is produced in Purkinje cells and is required for granule cell precursor proliferation but not for maturation and migration (12, 21). Mice lacking the Shh target gene *Gli2* die in late gestation/perinatally due to severe skeletal abnormalities (16). *Shh* expression correlates spatially and temporally with foliation, and *Gli2* mutants show a significant reduction in foliation and in the number of granule cell precursors at late gestation/birth (3). Interestingly, *Bmp2* has been shown to antagonize Shh-mediated proliferation of granule cell precursors through Smad5 signaling (17). *Zfp423* is also involved in *Bmp2* signaling through activated Smads (7). Using *lacZ* staining as a surrogate marker for *Zfp423* expression, our data suggest that *Zfp423* is expressed in Purkinje cells. Since the cerebellar size and degree of foliation in *Zfp423* null mice are severely reduced, without affecting the later formation of an inner granular layer, it will be very interesting to see whether *Zfp423* is involved in linking the two very important Shh/*Gli2* and *Bmp2*/Smad signaling pathways.

lacZ expression is only an indirect measure of *Zfp423* expression, and a more extensive expression analysis is needed to verify the expression pattern and to perhaps identify other cell types with *Zfp423* expression. Several attempts to analyze *Zfp423* expression on brain sections from adult mice by in situ hybridization have so far not been successful (data not shown), suggesting that the expression of *Zfp423* at the RNA level is very low.

Whether the mislocalization of Purkinje cells is simply an effect of the overall changes in cerebellar structure and size, or whether it is due to a cell-autonomous defect of Purkinje cells, remains to be tested. We are currently using several *Cre* lines to determine whether the cerebellar phenotypes observed in *Zfp423* null mice result from a Purkinje cell-intrinsic defect.

In addition to the reduced cerebellar size in *Zfp423* null mice, the cerebellar vermis is also severely reduced and almost

completely absent. It has been shown that the mid-hindbrain border is determined by reciprocal inhibition of *Gbx2* and *Otx2* expression (1, 15). Mice with a caudally shifted expression of *Otx2* are ataxic and lack most of the vermis due to lack of hindbrain specification (1). It is a very attractive hypothesis that the reduced vermis in *Zfp423* null mice could also be caused by improper mid-hindbrain organization. Our previous analysis of *Zfp423* expression in whole-mount embryos by using in situ hybridization showed that this gene is widely expressed throughout the brain, at least at stages E10.5 and E12.5 (27). This expression pattern is not inconsistent with the mid-hindbrain hypothesis.

Zfp423 was first identified as an interacting partner of *Ebf1* in rat olfactory epithelium (19). *Ebf1* null mice lack mature B cells and display defects in striatal development, but they have no cerebellar phenotype (5, 13). Genetic disruption of *Ebf2* results in hypogonadotropic hypogonadism and defects in motor nerve conduction, presumably due to hypomyelination (2). Disruptions of *Ebf2* and *Ebf3* further showed that although the expression of *Ebf* target genes and the appearance of olfactory epithelium are normal, *Ebf2* null, *Ebf3* null, and combined *Ebf2 Ebf3* heterozygous mutant animals have defects in the projection of olfactory axons, indicating only partial functional redundancy (23). Interestingly, in a report by Corradi et al. (2), it was also mentioned that *Ebf2* null mice are "mildly uncoordinated and walked with an unsteady, waddling gait," exhibited a "hunchback" posture, and displayed "substantial defects in cerebellar morphogenesis." The cerebellar phenotypes reported for the *Ebf2* null mice are similar to the phenotypes described in our study for *Zfp423*, although the *Ebf2* null mice appear to be somewhat less affected than *Zfp423* mice (G. Giacomo Consalez, personal communication). Although this remains to be tested, these data suggest a specific genetic interaction between *Zfp423* and *Ebf2*.

It was previously reported that mice with a genetic disruption of *Zfp423* have no obvious phenotype (6). This conclusion was based on the analysis of mice homozygous for a gene trap allele different from the one used in our study (W008G09). As with the gene trap allele described in this study (XH542), exons 1 to 3 were trapped in the W008G09 line. We have shown that the wild-type messenger is being produced from the trapped XH542 locus, and it is therefore highly likely that the same is the case for the W008G09 line. Only in the combination with the true null allele do the XH542 gene trap mice display a hypomorphic phenotype.

To our knowledge, this is the first report of a mouse with a conditional and a null allele created using the recombineering

FIG. 7. XH542 is a hypomorphic allele of *Zfp423*. (A) Intron 3, where the β geo reporter (blue box) is integrated. Both the wild-type (WT) and gene trap alleles are shown. Exons 3 and 4, the BglII sites (B), the genomic probe used for genotyping by Southern blot analysis, and the sizes of the restriction fragments are indicated. The sources of the three detectable splice products are shown: exon 3-exon 4 splicing (wild type; green); exon 3- β geo splicing (red), and the composite aberrant splicing event (exon 3-plasmid-exon 4; blue). The positions of the three primers used for RT-PCR are also indicated. SA, engrailed splice acceptor. The genomic map is not drawn to scale. (B) Genotyping by Southern blot analysis. gt, gene trap allele. (C) Results of RT-PCR analysis using the forward primer located in exon 3 together with a reverse primer in exon 4 (upper panel) or in *lacZ* (lower panel). The presence of a wild-type product in the absence of a wild-type allele clearly shows that the wild-type messenger is being made from this allele. (D) Mid-sagittal sections (heterozygous gt/+ mice) from embryonic day 15.5 and postnatal day 4, stained for *lacZ* expression and counterstained with neutral red. The position of the external granule layer (EGL) is indicated. *lacZ* staining correlates with the expected position of Purkinje cells. (E) H&E-stained mid-lateral sagittal sections from adult mice of the indicated genotypes. Note the hypomorphic phenotype of the gt/ko brain. All sections were cut at the same distance from the midline.

approach developed in our laboratory (14), and our data clearly illustrate the usefulness of this approach for functional genomic studies. The null allele has been very informative and used here to determine that *Zfp423* is required for normal cerebellar development. The conditional allele can now be studied using different *Cre* lines to further investigate which cell type(s) is responsible for the observed phenotypes, and an inducible *Cre* line can be used to determine the time point or time window in which *Zfp423* is required for normal cerebellar development. Using recombineering, it is no longer difficult to create a conditional allele that, in addition to the null allele, also allows for temporal as well as cell- and tissue-specific disruption of a gene of interest. Using a combination of BAC modification (25, 28) and subsequent gap repair, followed by the insertion of a positive selection marker (14), the recombineering approach also allows for more-sophisticated (conditional) gene targeting, including in-frame deletions and point mutations.

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