A Mutation in *Af4* Is Predicted to Cause Cerebellar Ataxia and Cataracts in the Robotic Mouse

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The robotic mouse is an autosomal dominant mutant that arose from a large-scale chemical mutagenesis program. It has a jerky, ataxic gait and develops adult-onset Purkinje cell loss in the cerebellum in a striking region-specific pattern, as well as cataracts. Genetic and physical mapping of the disease locus led to the identification of a missense mutation in a highly conserved region of *Af4*, a putative transcription factor that has been previously implicated in leukemogenesis. We demonstrate that *Af4* is specifically expressed in Purkinje cells, and we hypothesize that the expression of mutant *Af4* leads to neurodegeneration. This function was not identified through knock-out studies, highlighting the power of phenotype-driven mutagenesis in the mouse to identify new pathways involved in neurological disease.

Key words: cerebellum; Purkinje cell; neurodegeneration; ENU mutagenesis; mouse models; positional cloning

Introduction

Mouse mutants are an important resource for understanding mammalian gene function and disease and will play an increasingly significant role as we attempt to assign functions to all human genes. Of the possible routes to mutagenizing the mouse genome, gene-trapping vectors and chemical mutagenesis are being used on a large scale to provide a significant number of new mutants for gene function analysis (Hrabe de Angelis et al., 2000; Nolan et al., 2000; Stanford et al., 2001). An advantage of phenotype-based mutagenesis is that no assumptions are made about the possible function of the genes involved; instead we rely on sensitive phenotype analysis of individual animals. This approach therefore might be more likely to identify novel genes and pathways in processes of interest than genotype-driven mutagenesis, which identifies the mutated gene before phenotype analysis. In addition to the loss-of-function phenotypes generally seen in knock-out and gene-trap mutated mice, chemical mutagenesis using a point mutagen such as *N*-ethyl-*N*-nitrosurea (ENU) can generate partial loss of function, gain of function, and dominant negative mutations, providing a wider range of mutants that may reveal different aspects of the function of a gene (Justice, 2000; Brown and Balling, 2001).

We have used the phenotype-driven chemical mutagenesis approach coupled with a sensitive phenotype analysis protocol known as SHIRPA to screen for motor mutants (Rogers et al., 1997; Nolan et al., 2000). During this screen, we identified a novel cerebellar mouse mutant that develops Purkinje cell loss, and we predict that a gene that was previously implicated in leukemia, *Af4*, is responsible.

Translocations involving the mixed lineage leukemia (*MLL*) gene on chromosome 11 and some 30 loci on various chromosomes are associated with 5–10% of human acute leukemias. Of these, the t(4;11) translocation is the most common, accounting for ~50% of acute lymphoblastic leukemia (ALL) in infants under 1 year of age (Mitelman, 1994; Rowley, 1998). This translocation results in a fusion product coding for a chimeric protein derived from *MLL* and *AF4* (*ALL1*-fused gene from chromosome 4) (Gu et al., 1992; Dörmann et al., 1993). However, the function of *AF4* in leukemogenesis is not well understood, despite B and T cell development defects in the *Af4* knock-out mouse, suggesting a role in lymphopoiesis (Isnard et al., 2000). We present a potentially novel role for *Af4* in neurodegeneration, demonstrating the power of phenotype-driven mutagenesis in the mouse for ascribing new functions to genes.

Materials and Methods

Histology and immunohistochemistry. Brains were removed from mice transcardially perfused with 4% paraformaldehyde and embedded in paraplast wax, and 10 μ m sections were cut for histology and immunostaining. For cell counts, sections were stained in 0.5% aqueous cresyl violet, and Purkinje cells were counted in every 25th parasagittal section throughout the whole brain. Cells were only counted if part of their nucleus was visible, and the Hendry correction factor was used to account for double cell counting (Hendry, 1976). For individual cerebellar

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lobe counts, totals were calculated from a 40 μ m region around the central vermis. For immunohistochemistry, sections were incubated with one of the following primary antibodies: rabbit anti-calbindin (SWant, Bellinzona, Switzerland) diluted 1:15,000 for 2 nights at 4°C; rabbit anti-parvalbumin (SWant) diluted 1:10,000 for 3 nights at 4°C; rabbit anti-GFAP (Dako, Glostrup, Denmark) diluted 1:400 overnight at room temperature; rabbit anti-ubiquitin (Novocastra, Newcastle-upon-Tyne, UK) diluted 1:4,500 overnight at room temperature. After incubation, sections were immunoperoxidase labeled using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), and peroxidase activity was detected using diaminobenzidine (Vector Laboratories) as the substrate.

Genetic and physical mapping. DNA for genotyping was extracted from mouse tail biopsies by standard procedures, and the PCR products derived from each marker were analyzed on ethidium bromide-stained 4% Nusieve GTG agarose (Flowgen, Ashby de la Zouch, UK) gels. For those markers that could not be resolved on conventional gels, one primer was radiolabeled before amplification, and the products were run on denaturing 6% polyacrylamide gels. The RPCI-23 bacterial artificial chromosome (BAC) library filters (obtained from the Human Genome Mapping Resource Centre, Hinxton, UK) were screened initially with combined radiolabeled 35-mer oligonucleotides specific for markers *D5Mit93*, *D5Mit23*, and *D5Mit10*. BAC end sequences were obtained from The Institute for Genomic Research database (http://www.tigr.org/tdb/bac_ends/mouse/bac_end_intro.html) or determined by direct sequencing. To construct the contig, PCR was used to confirm the presence of a marker in an individual BAC clone. New polymorphic markers were identified by cloning and sequencing products derived from Interspersed Repeat Element PCR (Detter et al., 1998) or by screening genomic sublibraries with a radiolabeled (CA)₁₅ repeat probe. Exon trapping was performed using the Exon Trapping System (Invitrogen, San Diego, CA) with three BAC clones that spanned the robotic candidate region. Purified BAC DNA was completely digested with *Bam*HI/*Bgl*II or *Pst*I and shotgun cloned into the pSPL3 vector. COS-7 cell transfection, exon amplification, subcloning, and sequencing were performed according to the manufacturer's instructions.

Candidate gene analysis. Cerebellum and liver cDNA for candidate gene sequencing was generated from RNA extracted using RNeasy Midi columns (Qiagen, Hilden, Germany) or Tri reagent (Sigma, St. Louis, MO). Five overlapping PCR products were generated to span the 1887 bp coding region of *Klhl8*, and eight products were designed to cover the 3636 bp coding region of *Af4* from cerebellum cDNA. Two overlapping products were generated to span the 900 bp coding region of *Hsd17b12* from liver cDNA. Primer sequences are available from K.E.D. RT-PCR products were purified using the Qiaspin gel extraction kit (Qiagen) before sequencing.

In situ hybridization. A 309 bp region of mouse *Af4* (*mAF4*) (3194–3502 bp of GenBank accession number AF074266) was PCR amplified and cloned into the pCR4-TOPO vector (Invitrogen). Whole mouse brains were frozen in OCT (Merck, Darmstadt, Germany) on dry ice, and 14 μ m parasagittal cryosections were cut and mounted on positively charged slides. Digoxigenin-labeled riboprobe synthesis and hybridization were performed essentially as described previously (Wilkinson, 1992).

Flow cytometric analysis. Single-cell suspensions of thymocytes from 3-week-old mice ($n = 3$) were obtained by gentle disruption of the thymus organ, and four-color staining with anti-CD8 Tricolor (Caltag, San Francisco, CA), anti-CD4 PE, anti-CD25 APC, and CD44 FITC (Becton Dickinson, Rutherford, NJ) was performed. Samples were analyzed on a Becton Dickinson FACS machine.

Results

Robotic mice develop region-specific Purkinje cell loss

The robotic mouse was identified from the progeny of an ENU-injected BALB/c male and a C3H/HeH female because of its jerky, ataxic gait, which is apparent from ~3 weeks of age. Mutants are also generally 50% of the size of their littermates from this time and remain so into adulthood. Robotic animals also develop cataracts that become complete and bilateral.

Figure 1. Progression of Purkinje cell loss in the robotic cerebellum. *a–d*, Calbindin-stained vermal parasagittal sections at 3 (*a*), 10 (*b*), 20 (*c*), and 42 (*d*) weeks. Patchy Purkinje cell loss occurs at 10 weeks in the anterior lobes, which is more obvious by 20 weeks of age. At 42 weeks most of the Purkinje cells have been lost, although lobe X is spared, and the anterior lobes are more severely affected. The lobes of the cerebellum indicated (*a*, I–X) are morphologically unaltered at all time points. Scale bar, 1 mm. *e*, *f*, Calbindin-stained transverse sections from control littermate (*e*) and robotic (*f*) mice at 20 weeks of age. Parasagittal bands of resistant Purkinje cells in the robotic cerebellum are indicated (arrowheads). Scale bar, 1 mm.

Spinal cord, peripheral nerve, and brain were analyzed using histological stains, and no morphological abnormalities were observed in any tissue or brain region other than the cerebellum. Further analysis of the robotic cerebellum using calbindin and cresyl violet-stained sections at 3, 5, 10, 20, and 42 weeks of age showed that its lobular structure was unaltered at all time points (Figs. 1*a–d*, 2*a,b*). Purkinje cell loss was observed in a striking, region-specific pattern in the vermis, with the anterior cerebellum much more severely affected and lobe X spared throughout (Fig. 1*a–d*). Cell loss was first visible at 8 weeks of age (data not shown), although the compartmentalized Purkinje cell death was obvious by 20 weeks (Fig. 1*c*). By 42 weeks of age, only 30% of Purkinje cells remained, with lobe X completely spared and almost no cells surviving in lobes I–IV (Fig. 1*d*). The total Purkinje

Figure 2. Neuropathological findings in the robotic cerebellum. Cresyl violet-stained parasagittal vermal sections at 3 (*a*) and 42 (*b*) weeks show shrinking of the robotic cerebellum over time attributable to a decrease in size of the molecular layer, whereas the general morphology of the cerebellar lobules remains intact. Scale bar, 1 mm. *c*, Calbindin immunostaining shows the Purkinje cell bodies, dendrites, and axons at 5 weeks of age. There is no Purkinje cell loss in robotic mice, but dendrites are thickened (arrowhead) and swellings of the axons are also apparent (arrow). Scale bar, 100 μ m. Parvalbumin immunostaining from littermate control (*d*) and robotic (*e*) mice shows Purkinje cell loss from the Purkinje cell layer (PCL) at 10 and 20 weeks in parasagittal sections. The decrease in size of the molecular layer (ML) and the resulting increase in cell density are also apparent. Scale bar, 100 μ m. *f*, GFAP immunostaining shows a marked increase in staining density in the robotic cerebellum at 10 weeks of age. The increase occurs in the Bergmann glia of the molecular layer (ML) and in astrocytes in the granule cell layer (GL). Scale bar, 400 μ m. *g*, Ubiquitin immunostaining at 10 weeks of age reveals dot-like deposits in the cerebellum of robotic mice in the granule cell layer (arrowhead) and axon tracts (arrow).

cells numbers and lobe-specific counts for the vermis are shown in Table 1.

Analysis of calbindin immunostaining on transverse sections revealed parasagittal bands of surviving Purkinje cells in the anterior cerebellum at 20 weeks of age. The cell loss was mainly confined to the central vermis, and the surviving cells were in three clear bands: one at the midline with one on either side (Fig. 1*e,f*) in a pattern similar to that of zebrin-positive parasagittal bands in the anterior cerebellar vermis (Hawkes and Herrup, 1995; Herrup and Kuemerle, 1997). At 42 weeks of age the banding was disrupted, indicating that at least a subset of the resistant cells at 20 weeks of age can only delay but not prevent cell death (data not shown).

Neurodegenerative changes in the robotic cerebellum

Other pathological features were also apparent using calbindin immunostaining. In a subset of mutants, swelling of Purkinje cell dendrites was apparent at 3 weeks of age. By 5 weeks, this feature was more pronounced, with additional swelling of the Purkinje cell bodies and focal swellings of Purkinje cell axons, known as axonal torpedoes (Fig. 2*c*). These were also apparent at 8 and 10 weeks of age. In addition, there was a marked decrease in size of the molecular layer of the cerebellum from 10 weeks onward, presumably caused by the loss of Purkinje cell dendrites that normally fill this region. This phenomenon is clearly demonstrated using parvalbumin immunostaining, which also revealed a concomitant increase in the density of basket and stellate cells that reside in the molecular layer (Fig. 2*d,e*) and may account for the reduction in size of the cerebellum observed at 42 weeks of age (Figs. 1*d, 2b*). Qualitative assessment of the granule cell population on cresyl violet-stained sections revealed no obvious loss in granule cells at any time point, although subtle cell loss in the granule cell population cannot be ruled out (Fig. 2*a,b*).

GFAP immunostaining revealed marked upregulation of GFAP, indicative of gliosis, at 8 weeks onward (Fig. 2*f*). Ubiquitin immunostaining revealed no ubiquitinated aggregates in Purkinje cells that have been reported in human spinocerebellar ataxias (Kaytor and Warren, 1999); however, dot-like ubiquitin staining was observed in the molecular layer and axon tracts from 8 weeks of age, similar to that reported in a number of neurodegenerative conditions (Fig. 2*g*) (Leigh et al., 1989). Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling staining was performed to identify single-stranded DNA termini indicative of an apoptotic mode of cell death, but no positive staining was identified other than on positive control sections (data not shown).

Additional features of the robotic phenotype

To investigate the effects of the robotic mutation on T lymphocyte development in relation to the *Af4* knock-out phenotype (Isnard et al., 2000), the thymus was analyzed for cellularity, for weight, and by flow cytometry. Whole thymus weights were significantly lower in mutants compared with littermate controls, as was thymic cellularity (Table 2). Thymocyte development can be analyzed by quantifying changes in surface marker phenotypes as they move from the CD4⁺/CD8[−] coreceptor double-negative (DN) to the CD4⁺/CD8⁺ double-positive (DP) stages, followed by selection and regulation of coreceptor levels to become CD4⁺ or CD8⁺ single-positive (SP) T cells. The surface markers CD25 and CD44 further characterize the DN stage, and a significant increase in the population of CD25⁺/CD44[−] as well as a decrease in CD25[−]/CD44⁺ cells was observed (Fig. 3*a*). In addition, we found a significant increase of coreceptor expression on CD4⁺ and

Table 1. Corrected Purkinje cell (PC) number in the cerebellum of robotic and littermate control mice at 10 weeks ($n = 2$) and 42 weeks ($n = 3$)

	Vermal PC number ^a										Total PC number ^b
Mouse line	Lobe 1	Lobe 2	Lobe 3	Lobe 4,5	Lobe 6	Lobe 7	Lobe 8	Lobe 9	Lobe 10		
10 week control	1066 ± 125	2977 ± 319	3639 ± 19	6675 ± 588	3738 ± 350	1225 ± 250	2212 ± 206	3913 ± 238	2012 ± 313	99910 ± 2062	
10 week robotic	50 ± 19	1075 ± 25	1638 ± 6	3088 ± 150	3238 ± 88	963 ± 6	1988 ± 81	2900 ± 119	1925 ± 75	74673 ± 1744	
42 week control	1125 ± 189	2550 ± 95	3375 ± 160	5738 ± 331	3363 ± 259	912 ± 124	1938 ± 219	3963 ± 219	2275 ± 231	95731 ± 3792	
42 week robotic	25 ± 15	75 ± 37	100 ± 30	503 ± 140	1012 ± 110	350 ± 51	638 ± 137	1575 ± 155	2087 ± 260	26912 ± 7547	

^aMean number of Purkinje cells calculated for the cerebellar vermis ± SEM. Numbers were determined as described in Materials and Methods.

^bMean total number calculated for the whole cerebellum ± SEM.

Table 2. Thymocyte cell count numbers in control and robotic mice

	Control	Robotic
Weight (g)	15.4 ± 0.36	11.2 ± 0.81*
Thymus (mg)	83.3 ± 3.48	55.0 ± 7.37*
Thymocytes per lobe (10 ⁶)	67.5 ± 0.35	27.3 ± 1.78**
DP (%)	77.3 ± 0.88	80.0 ± 3.0
SP CD8+ (%)	5.0 ± 0.01	4.3 ± 0.33
SP CD4+ (%)	9.7 ± 1.45	8.5 ± 0.89

Results are expressed as mean ± SEM. Two-tailed Student's *t* test: * $p < 0.01$; ** $p < 0.001$.

Figure 3. Thymocyte development abnormalities in robotic mice. Flow cytometric analysis of littermate control (white bars) and robotic (black bars) thymus at 3 week of age ($n = 3$). Data for immature T cell markers CD25/CD44 (*a*) and average SP CD4/CD8 expression (*b*) are shown as the mean ± SEM. Two-tailed Student's *t* test: * $p < 0.05$, ** $p < 0.03$, *** $p < 0.01$.

CD8⁺ SP thymocytes in robotic (Fig. 3*b*). However, the proportion of the total thymic population at the DP and SP stages was not significantly different in mutant and control mice (Table 2).

Robotic mice breed poorly, and *in vitro* fertilization has been required to maintain stocks for genetic mapping purposes. No females have been observed that carry pups to full term, and small litters result from the mating of mutant males to wild-type females. A skewed ratio of wild-type to robotic mice that reach weaning age reflects a prenatal and postnatal death rate of ~20%; however, histological analysis of robotic mouse testis did not reveal any gross abnormalities.

A preliminary clinical biochemistry study was performed ($n = 5$) to determine possible defects in other organs in robotic mice (T. Hough, personal communication). A small but significant increase in creatinine ($p < 0.01$) levels was measured in mutant animals that may reflect renal dysfunction, although histologically the kidneys appeared normal. Levels of total cholesterol and high-density lipoprotein cholesterol in blood were also measured and were not elevated in robotic mutants.

To quantify the balance and motor coordination deficits of the robotic ataxic phenotype, latency before falling from an accelerating rotarod was measured at 5 weeks of age. Robotic and

Figure 4. Multiple amino acid sequence alignment of members of the AF4 protein family showing the highly conserved 18 amino acid region within which the robotic mutation lies (arrow). Mouse AF5q31 sequence was taken from an unpublished predicted mouse protein (GenBank accession number AAG17126). *Fugu rubripes* sequence was taken from an Ensembl predicted protein of 1123 amino acids, SINFRUP00000087356 (www.ensembl.org/Fugu_rubripes).

control mice both displayed improved performance over the trial period, although mutants showed a significant impairment in this task (data not shown). The observation that motor coordination is reduced before overt cell loss (which begins at ~8 weeks of age) indicates that Purkinje cell function is impaired early in the disease process, consistent with the early neuropathological findings described above.

Genetic and physical mapping of the robotic locus identifies a point mutation in *Af4*

A genome scan had previously mapped the robotic mutation to a 15 cM region on mouse chromosome 5 (Nolan et al., 2000). A high-resolution genetic map was generated using 515 backcross animals, and a BAC contig was constructed spanning the 1.2 cM candidate region between flanking microsatellites *D5Mit93* and *D5Mit10*.

Exon trapping isolated multiple clones representing three genes in the contig, confirming the distribution of genes predicted by annotation of the human and mouse genomic sequence from both the public and Celera (Celera Discovery System) databases. We cloned two novel mouse genes in the region, named hydroxysteroid 17- β dehydrogenase type 12 (*Hsd17b12*; GenBank accession number AY101768) and Kelch-like 8 (*Klhl8*; GenBank accession number AY101769), although no mutations were identified in either gene by sequencing the coding regions from robotic cDNA. *Af4*, the third gene in the robotic candidate region, was sequenced from cerebellum cDNA, and a T to C transition was identified in the second base of codon 280 leading to a valine to alanine amino acid substitution. Parental strains C3H/HeH and BALB/c were sequenced and shown to have a T at this position, implying that the mutation was a result of ENU administration. The mutation occurred in a highly conserved region of

Figure 5. Expression of *Af4* in the cerebellum. Bright-field images of *in situ* hybridization in littermate control (*a*) and robotic (*b*) animals at 5 weeks of age with an antisense *Af4* riboprobe. Expression is confined to the Purkinje cell layer (PCL) in both cases. The molecular layer (ML) and granule cell layer (GL) are marked. The corresponding sense probe is shown (*c*). Scale bar, 100 μ m. *d*, *e*, Vermal parasagittal cerebellum sections from littermate control (*d*) and robotic (*e*) mice at 5 weeks using the antisense *Af4* riboprobe. Reduced levels of expression are observed in lobe X. Scale bar, 1 mm.

AF4, within a run of 13 amino acids that share 100% identity with all known *AF4* homologs, suggesting that it plays a critical role in the function of the protein (Fig. 4) (Nilson et al., 1997; Taki et al., 1999; Britanova et al., 2002). No differences in expression level of any of the genes in the candidate region were observed between robotic and control animals by quantitative RT-PCR (data not shown).

Af4 expression in the cerebellum reflects the pathology of the mutant

In situ hybridization on robotic and control littermate sections showed that within the cerebellum *Af4* was expressed solely in Purkinje cells (Fig. 5*a–e*) and that the expression was clearly lower in lobe X of the cerebellum in both robotic and littermate control mice (Fig. 5*d,e*). Lobe X is completely spared from Purkinje cell loss in robotic mice even at 42 weeks of age, suggesting that higher levels of *Af4* expression are required to facilitate Purkinje cell death and implying that the robotic *Af4* allele is acting via a gain-of-function mechanism. RT-PCR was used to demon-

strate that *Af4* is expressed in the lens of control animals (data not shown), consistent with a role in the cataract formation observed in robotic mutants.

Discussion

The data presented here describe a new mutant mouse model for cerebellar ataxia that develops Purkinje cell loss in a stereotypical region-specific manner. High-resolution genetic and physical mapping reduced the robotic locus to a small region containing three genes, and sequencing revealed a point mutation in a highly conserved region of *Af4*.

Although the possibility of there being a second mutation in the region genetically linked to the robotic phenotype cannot be ruled out absolutely, there is compelling evidence that *Af4* is the causative gene. The robotic locus was reduced to a 0.5 Mb region that contained only three genes, as determined by exon trapping studies and analysis of genomic sequence annotation from both the public and Celera databases. All three genes in this region were sequenced, and no differences in their expression levels were observed between robotic and control animals; only one coding point mutation was isolated in the positional candidate *Af4*. Second, the *Af4* mutation occurred in the most highly conserved region of the *AF4/LAF4/FMR2* (ALF) domain, suggesting that the residue mutated in robotic mice is of critical importance to the function of the protein (Nilson et al., 1997). The evolutionary conservation of this motif is demonstrated by the presence of an *AF4*-related homolog from *fugu rubripes* containing the 13 amino acids that are identical to the mammalian proteins. Previously, the only nonmammalian homolog of this protein family described was the *Drosophila* protein Lilliputian, which shares identity only to the C-terminal homology domain of *AF4* (Tang et al., 2001; Wittwer et al., 2001). Such evolutionary conservation is important given the conservative nature of the valine to alanine amino acid change that occurs in robotic *AF4*. An analysis of 10121 disease causing missense mutations in humans shows that valine to alanine substitutions do cause disease but that such a change is only 1.43 times more likely to be disease causing than a nonharmful substitution (Goodstadt and Ponting, 2001). Finally, we have shown that *Af4* is expressed specifically in Purkinje cells in the cerebellum, the main site of pathology in the robotic mouse, and that expression is lower in the Purkinje cells that are not affected by the mutation.

AF4 is a member of a protein family containing *FMR2*, *LAF4*, and *AF5q31*, which are all nuclear proteins with transactivation activity, suggesting that they act as transcription factors (Prasad et al., 1995; Gu et al., 1996; Ma and Staudt, 1996; Baskaran et al., 1997; Gecz et al., 1997; Nilson et al., 1997; Isnard et al., 1998; Li et al., 1998; Taki et al., 1999). In addition, it has been demonstrated that *LAF4* has nonspecific DNA binding properties (Ma and Staudt, 1996). Recent studies suggest that the ALF domain may act as a transcriptional repressor on the basis of increased levels of reporter gene expression from *AF4*, *LAF4*, and *FMR2* constructs in which this region of the protein was deleted (Hillman and Gecz, 2001). A mutation in the ALF domain could therefore alter transcriptional regulation of downstream target genes caused by a gain-of-function or dominant-negative effect. This mechanism would be consistent with the fact that the lower levels of *Af4* expression observed in lobe X of the cerebellum are not sufficient to cause Purkinje cell death in robotic mice. However, without detailed functional or structural information on the *AF4* family, it is still possible that the robotic mutation may influence protein–protein binding events specific to Purkinje cells in the brain.

A pattern of Purkinje cell loss similar to that observed in ro-

botic mice has been described in a number of other cerebellar mouse mutants. Three central bands of resistant cells and preferential loss of Purkinje cells in the anterior cerebellum with sparing of lobe X have been described in the leaner mutant (Herrup and Wilczynski, 1982) and in the mouse models of Niemann-Pick disease types A/B [acid sphingomyelinase knock-out (ASMKO)] and C (Higashi et al., 1993; Sarna et al., 2001). These changes are preceded by granule cell loss in the leaner mouse (Herrup and Wilczynski, 1982; Heckroth and Abbott, 1994), distinguishing the pathological findings from those seen in robotic mutants. However, the similarity of the region-specific cell loss in robotic mice to the Niemann-Pick models is more striking, and dot-like ubiquitin staining has also been noted in the type C disease model (Higashi et al., 1993; Sarna et al., 2001).

Niemann-Pick disease is an autosomal recessive lysosomal storage disorder that is characterized by hepatosplenomegaly and lipid accumulation as well as retarded physical growth and severe neurologic disturbances, including ataxia in many cases (Pentchev et al., 1995; Schuchman and Desnick, 1995). Types A and B are caused by deficient acid sphingomyelinase activity (Levran et al., 1991), and type C is caused by mutations in *NPC1*, a gene thought to be involved in intracellular trafficking of cholesterol (Carstea et al., 1997). Mouse models of related lipid storage disorders such as Tay-Sachs disease also exhibit reproductive impairment (Trasler et al., 1998), and recent studies of the ASMKO line revealed defects in sperm physiology attributable to lipid-filled vacuoles present in the testis and epididymis (Butler et al., 2002).

Considering that a number of these pathological features are observed in robotic mice, it might be hypothesized that AF4 is involved in pathways important for cholesterol homeostasis and metabolism. However, we have found no direct evidence for cholesterol accumulation in the brain of robotic mice or other organs, including the kidney, spleen, liver, and testis. Moreover, the clinical biochemistry data indicate that levels of blood total cholesterol and HDL cholesterol were not elevated in mutants, as is characteristic of the ASMKO line (Horinouchi et al., 1995). Despite the neurodegenerative features linking the robotic phenotype to lysosomal storage disorders, expression levels of *Smpd1* and *Npc1* were not altered in robotic mice compared with wild-type animals when examined by quantitative RT-PCR (data not shown), suggesting that the AF4 mutation may have an influence on related, but not identical, pathways. The stereotyped pattern of cell death in these mutants therefore may reflect a more general underlying susceptibility of biochemically distinct subsets of Purkinje cells to a range of CNS insults.

Although the role of AF4 in leukemogenesis is unclear, it is generally thought that the fusion protein derived from the N-terminal portion of MLL and the C-terminal portion of AF4 is responsible for causing the leukemic phenotype (Borkhardt et al., 1994; Downing et al., 1994). The mutation that we have described occurs upstream of known translocation breakpoints, suggesting that the function of AF4 in robotic mouse may not reflect its potential oncogenic role. The *Af4* knock-out mouse (*mAf4* $-/-$) displayed lymphoid development problems but was not reported to have any motor abnormalities indicative of a cerebellar defect, although 20% had a reduced size up to 6 weeks of age, a phenotype shown by all robotic mice throughout life (Isnard et al., 2000). Heterozygous (*mAf4* $+/-$) animals showed no such abnormalities, suggesting that the robotic phenotype is unlikely to be caused by a loss-of-function mutation.

Consistent with a role for AF4 in lymphocyte maturation, we observed abnormalities in T cell development in robotic mice

that were similar but distinct from those seen in the *Af4* knock-out mice. A reduction in thymic cellularity and weight as observed in robotic mice was described as part of the *Af4* knock-out phenotype, although no data from wild-type (*mAf4* $+/+$) mice were reported (Isnard et al., 2000); however, we did discover distinct differences between the knock-out and robotic lines. The phenotypic changes in the DN population as indicated by the CD25 and CD44 marker profiles of robotic mice indicate that the seeding of prothymocytes at this stage is significantly affected (Godfrey and Zlotnick, 1993). In the knock-out line, however, the subsets of DN precursor thymocytes were normal (Isnard et al., 2000). At the DP stage, upregulation of coreceptors as well as intermediate expression of the CD3/T cell receptor complex facilitates positive and negative selection events. Before thymocytes then enter the periphery as mature T cells, downregulation of one coreceptor occurs, and they become either CD4⁺ or CD8⁺ SP. Because both coreceptors play an essential role in signaling via the T cell receptor, the higher levels of expression observed in robotic mice might indicate that cells have undergone a more stringent negative selection (Lee et al., 1992; Robey et al., 1992). Alternatively, it may be the direct or indirect result of the transcriptional regulatory activities of the mutant *Af4* gene. This effect on thymocyte development in the robotic mouse is once again distinct from the *Af4* knock-out phenotype. Taken together, these data are consistent with our hypothesis that the *Af4* allele is acting through a gain-of-function mechanism.

The identification of novel gene functions is inherent to the phenotype-driven mutagenesis strategy used here because no *a priori* assumptions are made concerning the underlying genetic defect. The hypothesis that a gain-of-function mutation in *Af4* causes neurodegeneration may lead to the identification of new pathways that are important for neuronal survival and adds to the list of cerebellar mutants that have provided a wealth of information regarding CNS function (Heintz and Zoghbi, 2000).

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