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Genetics analysis of mouse mutations *Abnormal feet and tail* and *rough coat*, which cause developmental abnormalities and alopecia

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Abstract. Mutations in the mouse *Brachyury* (*T*) gene are characterized by a dominant reduction of tail length and recessive lethality. Two quantitative trait loci, *Brachyury-modifier 1* and *2* (*Brm1* and *Brm2*) are defined by alleles that enhance the short-tail *Brachyury* phenotype. Here we report on a genetic analysis of a visible dominant mutation *Abnormal feet and tail* (*Aft*) located in the vicinity of *Brm1*. Affected animals display kinky tails and syndactyly in the hindlimbs, both likely resulting from a defect in apoptosis. We observed an unusual genetic incompatibility between *Aft* and certain genetic backgrounds. We show that *Aft* and *T* are likely to interact genetically, since some double heterozygotes are tailless. In addition to the tail and hindlimb phenotypes, *Aft*-bearing mutants display characteristic late-onset skin lesions. We therefore tested for allelism between *Aft* and a closely linked recessive mutation *rough coat* (*rc*) and found that these two mutations are likely nonallelic. Our results provide a valuable resource for the study of mammalian skin development and contribute to the genetic analysis of *Brachyury* function.

Introduction

Mutations in the *Brachyury* (also known as *T* for tail) gene cause short, blunt tails in heterozygous mice (*T*/+), whereas *T*/*T* homozygotes die *in utero* (Dobrovolskaia-Zavadskaia 1927; Korzh and Grunwald 2001). Subsequent embryological investigations showed that mutant embryos had severe mesoderm abnormalities and failed to form a notochord (Chesley 1935; Gluecksohn-Schoenheimer 1944). The original allele was shown to be an almost 200-kb deletion (Herrmann et al. 1990), encompassing the *T* gene, which encodes a sequence-specific transcription factor (Kispert et al. 1995). To understand the molecular nature of *Brachyury* function, it is essential to identify and characterize molecules that act as targets and regulators of *T*, and elements of other genetic pathways interacting with *T* during embryogenesis.

Classical genetic analyses yielded several mutations whose functions modify the Brachyury phenotype. The first of these, originally designated as the *t*-alleles, produced a tailless phenotype in *T*/*t* mice, while not causing a tail-related phenotype on their own (Dobrovolskaia-Zavadskaia and Kobozieff 1932). It is now understood that the *t*-haplotypes are not alleles of *T*, but rather complex genetic entities on the proximal mouse Chromosome (Chr) 17 (Silver 1985). The tail-shortening effect of the *t*-complex is caused by the interaction of variant alleles at the *tct* (*t*-complex tail interaction factor) locus and the *T* gene (Lyon and Meredith 1964; Nadeau et al. 1989). Some of the *tct* alleles produce a short-tailed phenotype in the absence of the *T* mutation (Fujimoto et al. 1995). Currently the best candidate gene for *tct* is *Brachyury the Second* (*T2*), a gene closely linked to *Brachyury* and deleted in the original *T* mutation (Rennebeck et al. 1998).

Classical genetic analysis also identified unlinked *Brachyury*-interacting loci. One of them, *vestigial tail* (Michie 1956), is likely an allele of *Wnt3a* (Greco et al. 1996), which is a direct regulator of *Brachyury* (Yamaguchi et al. 1999). Another mutation, *t-int*, is an independent mutation on mouse Chr 17 affecting tail development (Artzt et al. 1987).

The observation that expressivity of the short-tail phenotype in *T*/+ mice is sensitive to the effects of genetic background (Wittman and Hamburgh 1968; Mickova and Ivanyi 1974) implies that there exist a number of *T* modifiers. Utilizing the background dependence of the Brachyury phenotype, Agulnik et al. (1998) identified two quantitative trait loci, *Brm1* and *Brm2* (*Brachyury-modifier 1* and *2*), with a tail-length effect. Certain alleles at these loci enhance the effect of the *T* mutation, causing extreme tail-shortening in carrier individuals. Although potentially useful in uncovering functional partners of *T*, these loci are not precisely mapped, because the 90% confidence intervals on their lengths exceed 10 cM. As a first step toward the identification of *Brachyury*-interacting genes within such large intervals, we conducted a genetic analysis of a mutation that affects tail development and is located in the vicinity of the *Brm1* region.

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Materials and methods

Mice. Founder-animals of both *Aft* and *rc* lines were derived from cryopreserved stocks imported from The Jackson Laboratory. Subsequent breeding was carried out in the mouse colony at Princeton. C57BL/6J and 129Sv wild-type animals were also obtained from The Jackson Laboratory. t^{h2} is a partial *t*-haplotype, which causes complete taillessness in T/t^{h2} mice, whereas t^{h2}/t^{h2} mice have wild-type tails. t^{h2}-carrying animals, originally described by Lyon and Meredith (1964), were bred in our colony. Data analyses were carried out using Microsoft Excel.

Molecular markers. To distinguish between Chr 9 derived from 129/Sv and C57BL/6J, we used two microsatellite markers that are polymorphic between these two strains: *D9Mit227* (23.0 cM) and *D9Mit48* (34.0 cM). Primers were purchased from Research Genetics (Huntsville, Ala.), PCR reactions were conducted as indicated by the manufacturer (Dietrich et al. 1994), and products were separated on 7% polyacrylamide gels. To distinguish between the wild-type (including *T*) and the t^{h2}-bearing chromosomes, we used a PCR-based identification method based on a polymorphism within the *Tcp1* gene (Morita et al. 1993). Amplification of wild-type chromosomes results in a 1.4-kb product, whereas *t*-chromosomes produce a 1.6-kb band. Products of PCR reactions (30 cycles: 94°, 40 sec; 62°, 1 min; 72°, 1 min) were separated on 1% agarose gels.

Preparation of samples. Preparations for Alcian Blue/Alizarin Red staining of tails and limbs were conducted according to standard protocols (Shen et al. 1997). Sectioning and hematoxylin/eosin staining of skin samples from *Aft*/+ and *rc*/*rc* mice was performed at Anmed/Biosafe, Inc. (Rockville, Md). All preparations were photographed with a Nikon E-600 microscope.

Results

As a first step toward the identification of a *Brachyruy*-interacting gene within the *Brm1* locus, mapped around 34 cM on Chr 9 (Agulnik et al. 1998), we conducted a genetic analysis of a little-characterized mutation *Abnormal feet and tail* (*Aft*) that arose spontaneously in the 129Sv strain (Lane 1987).

Phenotype of Aft-bearing mice. Heterozygous (*Aft*/+) mice exhibited kinks at the tip of the tail (Fig. 1A) and partial syndactyly of digits 3 and 4 on the hindlimbs; both phenotypes displayed variable expressivity among littermates. First, we investigated the morphological nature of the syndactyly (Fig. 1B), which was commonly unilateral. We observed a single animal with syndactyly of the forelimbs, and several animals in which digits other than 3 and 4 were fused. Upon examination of the Alcian Blue/Alizarin Red preparations of syndactylous limbs, we found that the bony elements of the digits were not fused (Fig. 1C). Instead, syndactyly was caused by the persistence of the skin web between digits 3 and 4, possibly as a consequence of incomplete apoptosis. Examination of Alcian Blue/Alizarin Red preparations from several mutant mice revealed that the tail kinks were caused by varying degrees of cartilage overgrowth and fusion, and a corresponding dysmorphogenesis of bony elements attached to the fusion site (Fig. 1D). These observations are consistent with abnormal bone/cartilage proliferation and/or cell death.

At the age of 6–8 months, many *Aft*/+ mice developed a phenotype not previously reported for this mutation. We first noticed local hair loss on the snout and behind the ears, as well as generally clumpy and unkempt fur. Over time, alopecia progressed into bleeding ulcerations (Fig. 1E). This phenotype was observed only in mutants and never in wild-type littermates. As in the case of tail kinks and syndactyly, expressivity of this trait was variable, its strength somewhat corresponding to the strength of the other two phenotypes. Generally, among the mutant animals the tail phenotype was most frequent, followed first by syndactyly and then by skin lesions.

The original report of the *Aft* mutation (Lane 1987) suggested that penetrance of this phenotype was low. Breeding data obtained in the course of this work allow an accurate estimate to be made. Two sets of reciprocal crosses (*Aft*/+ 129Sv × +/+ 129Sv) and (*Aft*/+ 129Sv × +/+ C57BL/6J) yielded nearly identical results with respect to the fraction of mutant animals. Of the total 441 offspring, 144 were mutants, suggesting a penetrance of approximately 65% on both genetic backgrounds.

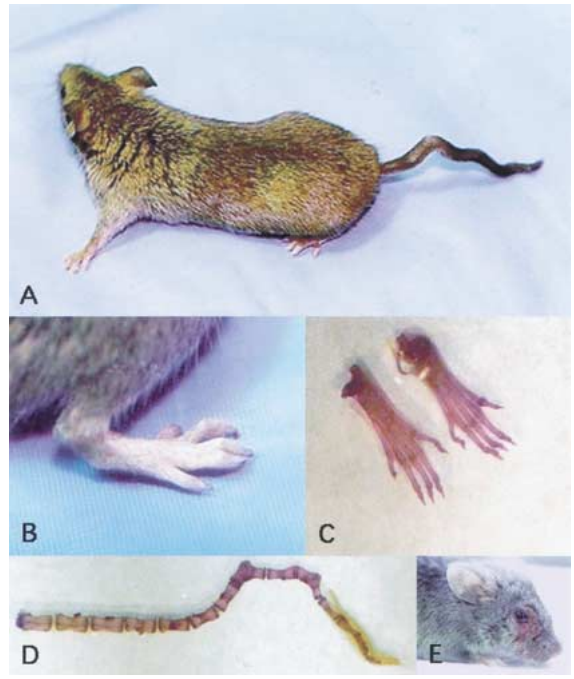

Fig. 1. Phenotype of the *Aft* mutation. *Aft*/+ animal **(A)**. Syndactylous limb of an *Aft*/+ animal **(B)**. Alcian Blue/Alizarin Red preparation of the limbs from an *Aft*/+ animal showing that the digits are fully articulated **(C)**. Alcian Blue/Alizarin Red preparation of the tail from an *Aft*/+ animal showing that kinks likely result from cartilage abnormalities **(D)**. Alopecia and ulceration in an *Aft*/+ animal **(E)**.

Map position of Aft. To refine the map position of *Aft*, we decided to use an "outcross –backcross" ((*Aft*/+ 129Sv × +/+ C57Bl/6J)F_1 × +/+ C57BL/6J) protocol (Silver 1995). Previous reports suggests that *Aft* is located around 32 cM on Chr 9 (Lane 1987); thus, we chose two markers, *D9Mit227* and *D9Mit48*, which flank this location and are polymorphic between the 129Sv and C57BL/6J strains. Although we observed no postnatal lethality or reduction of litter size in the F_1, we noticed that a substantial fraction of N_2 animals died soon after birth. Most deaths occurred within the first 9 days from birth and were distributed relatively uniformly throughout this period; there were no overt defects in the dying animals. Overall, of 350 animals recorded at birth, only 212 survived to 6–7 weeks of age; an additional 87 dead pups were collected. Despite concerted efforts to collect all dying animals, 51 animals were lost. We reasoned that the distribution of genotypes among the missing animals would likely be the same as that of the collected dead animals; thus, the total values were corrected for this missing population. All alive and collected dead mice were genotyped for both microsatellite markers (Table 1). Given incomplete penetrance of the Aft phenotype, it is impossible to determine with precision the map position of *Aft* with respect to *D9Mit227* and *D9Mit48*. However, the ratios of wild-type/mutant/dead animals were very similar between $D9Mit227^{129}/D9Mit227^{B6}$, $D9Mit48^{129}/D9Mit48^{B6}$, and $D9Mit227^{B6}/D9Mit227^{B6}$, $D9Mit48^{129}/D9Mit48^{B6}$ mice (columns one and three in Table 1), and between $D9Mit227^{129}/D9Mit227^{B6}$, $D9Mit48^{B6}/D9Mit48^{B6}$, and $D9Mit227^{B6}/$

Table 1. Offspring of a cross (*Aft*/+ 129Sv × +/+ C57BL/6J)F1 × +/+ C57BL/6J.

Phenotype	*D9Mit227*: *D9Mit48*:	Genotype[a] 129/B6 129/B6	129/B6 B6/B6	B6/B6 129/B6	B6/B6 B6/B6
Wild-type		13	15	3	168
Mutant[b]		12	0	1	0
Dead[c]		65(+41)	0	12(+6)	10(+4)

[a]129 represents 129Sv, B6 represents C57BL/6J.
[b]Mutant phenotype defined as presence of tail kinks, syndactyly, or both.
[c]Numbers in parentheses represent correction for missing animals (see text for details).

D9Mit227^{B6}, *D9Mit48*^{B6}/*D9Mit48*^{B6} mice (columns two and four in Table 1). Therefore, it must be concluded that both the lethality and the appearance of the mutant phenotype are controlled by a locus closely linked to *D9Mit48*. Because of this close linkage, in the following crosses we will consider *D9Mit48*^{129} to be a marker for the mutant *Aft* allele and *D9Mit48*^{B6} a marker for the wild-type allele. Furthermore, of the 153 born mice known or presumed to be *Aft*/+, nearly 80% died, whereas only 7% of their +/+ littermates did so. This is an unusual case of highly penetrant lethality in the N2 animals and it can not be explained by the action of a single Mendelian modifier.

Aft mutation causes recessive lethality. To test whether *Aft*/*Aft* animals die, as suggested by Lane (1987), we performed an intercross between the mutant F_1 hybrids derived from an outcross *Aft*/+ 129Sv × +/+ C57BL/6J. All 125 resulting animals were collected at birth and genotyped at the *D9Mit48* locus. The following genotypes were observed: 3 *D9Mit48*^{129}/*D9Mit48*^{129}; 75 *D9Mit48*^{129}/*D9Mit48*^{B6}; and 47 *D9Mit48*^{B6}/*D9Mit48*^{B6}. These values are significantly different from a 1:2:1 expectation ($p < 0.0001$) owing to a paucity of *D9Mit48*^{129} homozygotes, whereas 75:47 is not significantly different from 2:1 ($p > 0.05$), suggesting that lethality affecting *Aft*/+ mice generated in the backcross to C57BL/6J (see above) does not affect animals of the same genotype resulting from an intercross.

Aft genetically interacts with T. To determine whether *Aft* and *T* interact genetically, we investigated the phenotypes of animals carrying both mutations by examining progeny in a cross (*Aft*/+ 129Sv × +/+ C57BL/6J)F_1 × *T*/*t*^{h2} (Table 2). A notable observation from this cross was the existence of nine tailless mice, seven of which were *Aft*/+,*T*/+. The most plausible interpretation of such enhanced phenotype in double heterozygotes is a genetic interaction, although with low penetrance (8.5%), between the two genes. The remaining two tailless mice could be explained by recombination events between markers and the loci of interest (several cM between *Aft* and *D9Mit48*, and 3.5 cM between the *t*-haplotype marker within *Tcp1* and *T*). Crosses involving different animals from our outbred *T*/*t*^{h2}-carrying population produced results similar with respect to the tailless phenotype, although in several crosses we observed lethality of *Aft*-carriers (data not shown). We also noted that, whereas *T*/*t*^{h2} females transmitted *T* and *t*^{h2} equally frequently (48:52), *T*/*t*^{h2} males, consistent with previous reports (Lyon and Meredith 1964; Garside and Hillman 1989), displayed strong transmission ratio distortion in favor of *T* (86:14).

Gross phenotype of rc-bearing animals. Because *Aft*-carrying mutants displayed characteristic skin lesions, we examined the vicinity of this locus for other visible mutations affecting skin and fur. We found that a mutation, *rough coat* (*rc*), was previously reported to be located at 32 cM on Chr 9, closely linked to *Aft* (Dickie 1966; Eicher 1977). Animals carrying *rc* had unkempt fur and gradually developed hair loss. This mutation arose spontaneously in the C57BL/6J strain. We

Table 2. Interaction of *T* and *Aft* as demonstrated by the phenotypes of the offspring of a cross (*Aft*/+ 129Sv × +/+ C57BL/6J)F1 × *T*/*t*^{h2}.

Phenotype	Genotype *Aft*/+,*T*/+	*Aft*/+,*t*/+	+/+,*T*/+	+/+,*t*/+
Total mice	82	37	95	45
Tailless	7	0	1	1

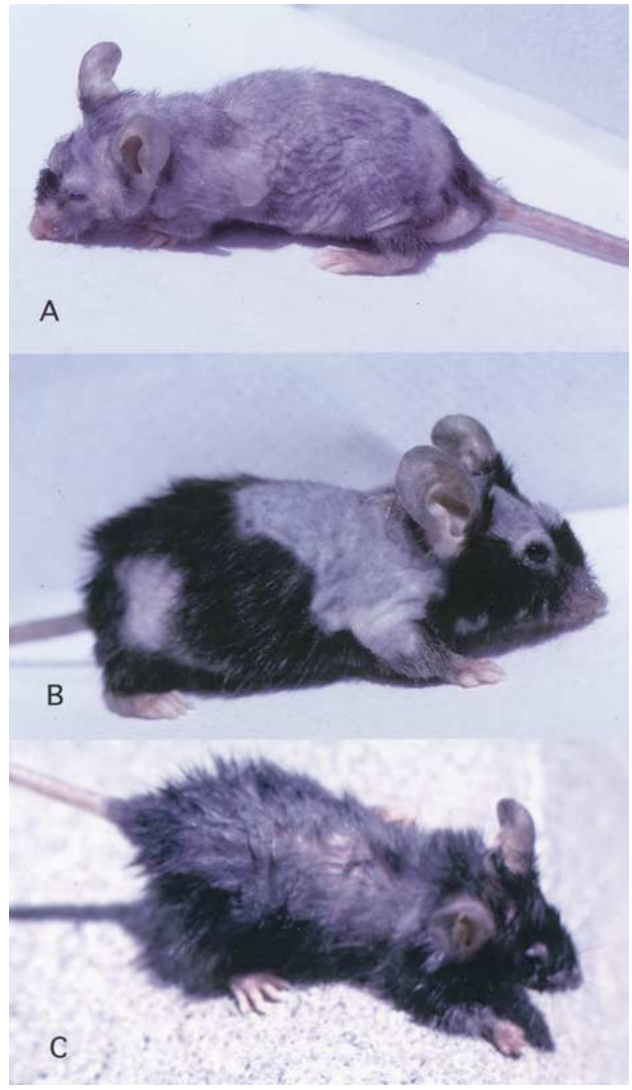

Fig. 2. Phenotype of the *rc* mutation. Panels **A**, **B**, **C** all show *rc*/*rc* animals; notice the differences in the extent and pattern of hair loss.

detected the onset of the mutant phenotype, manifested by uniform thinning of the fur, in animals as young as 2 weeks. Over time, the hair became brittle, clumpy, and oily in appearance. The extent of the alopecia was somewhat variable (Fig. 2A,B,C), but the trait was fully penetrant. Heterozygotes *rc*/+, derived from *rc*/*rc* × +/+ C57BL/6J, never displayed alopecia, implying that the mutation is fully recessive (not shown).

Histological defects in the skin of Aft- and rc-bearing mutants. To understand the structural defects underlying hair loss, we examined hematoxylin/eosin-stained preparations of the skin from affected animals. The skin of *Aft*/+ mice showed

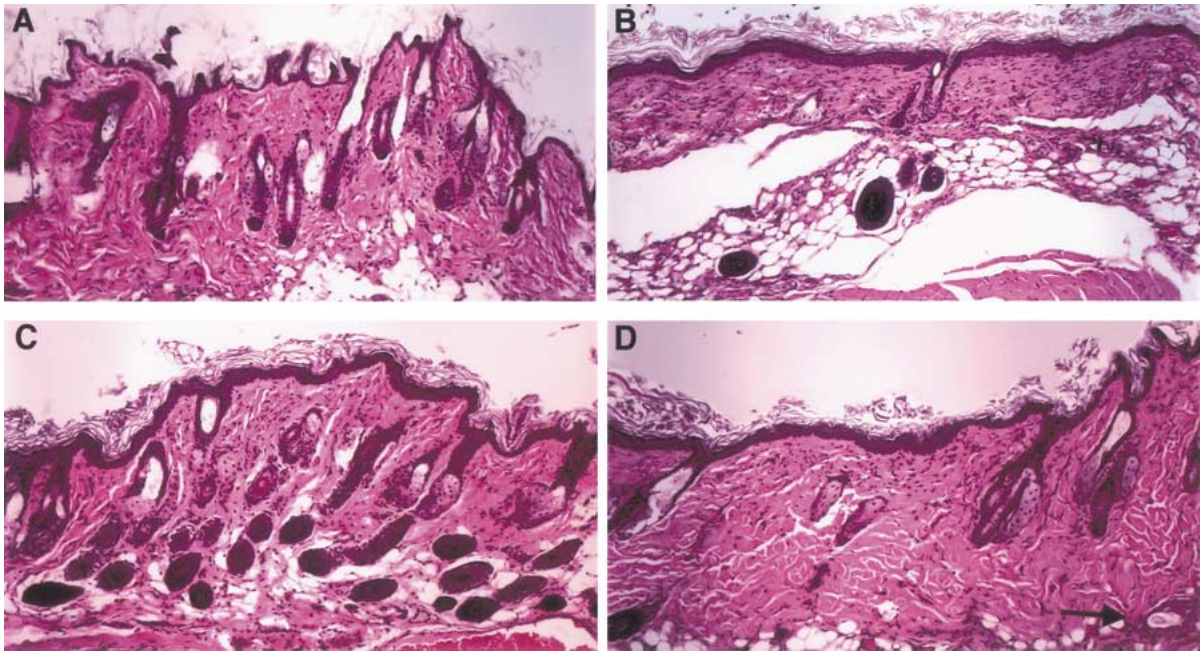

Fig. 3. Histology of Aft and rc mouse skin. Compared with unaffected skin **(A)**, the Aft mouse shows an area of alopecia with decreased follicle number and fibrosis in the dermis **(B)**. rc mouse skin shows similar changes between unaffected **(C)** and alopecia skin **(D)**. rc mouse showed additional findings of numerous foreign body giant cells and free hair shafts in areas of alopecia (**D,** arrow).

focal areas of decreased hair follicle density with fibrous tracts, pigment incontinence, and increased mast cells (Fig. 3 A,B). These features suggest a degeneration or a failed regeneration of follicles. The *rc*/*rc* mice also showed a decrease in the number of follicles with fibrosis, pigment incontinence and mast cells (Fig. 3C,D). However, there were also fragments of free hair shafts in the deep dermis with a foreign body giant-cell reaction. In addition, there was a significant increase in the number of regressing (catagen) hairs in the areas of alopecia (Fig. 3D). These changes suggest a defect in the structural integrity of the hair follicle, which is possibly hair-cycle related.

Test of allelism between Aft and rc. Our observations of the etiology of hair loss in mice with *Aft* and *rc* mutations indicated that the two phenotypes were similar, suggesting that they might represent alleles of the same gene. To test this hypothesis, we performed a cross *Aft*/+ 129Sv × *rc*/*rc* C57B1/6J. Since the *Aft* mutation confers a dominant, incompletely penetrant, late-onset hair loss, whereas the *rc* mutation has a recessive, fully penetrant, early-onset hair loss, if these two mutations are allelic or if they interact genetically, animals carrying both mutations might be expected to develop alopecia with earlier onset and/or greater severity than that seen in *Aft*/+ animals. Of the 36 mice recorded at birth, eight clearly had the Aft phenotype, whereas none displayed any skin-associated phenotype. Thus, it is likely that these two mutations are not allelic and do not interact, at least on this genetic background.

Discussion

Aft was identified as a potential *Brachyury*-interacting gene based on its map position and a phenotypic similarity to *T*. To examine the validity of this prediction, we attempted to refine the map location of *Aft*. Owing to variable penetrance and expressivity of the mutation and its strong dependence on genetic background, we were unable to obtain a fine-resolution map position. We did, however, confirm its close linkage to the marker *D9Mit8* located at 34 cM on Chr 9, near the center of the 90% confidence interval for *Brm1*.

We conducted a detailed examination of the phenotype of *Aft*-carrying mutants. We found that tail kinks are likely due to cartilage overgrowth and the corresponding distortion of bony elements, whereas syndactyly is caused by persistence of the connective tissue between the digits. Since the hair follicles in adult mice continuously cycle, they repeatedly undergo a type of morphogenesis, and the apparent dissolution of follicles could be caused by a defect in any of the cellular processes involved in fetal development. These observations are consistent with two possible interpretations: either apoptosis does not proceed properly, or there is an abnormally high degree of cell proliferation in a variety of tissues in Aft mutants.

We observed that few, if any, *Aft*/*Aft* animals survive to term. Penetrance of the mutant phenotype in *Aft*/+ mice is approximately 65% with variable expressivity. Unexpectedly, we discovered that nearly 80% of *Aft*-carrying mice produced in a cross (*Aft*/ + 129Sv × +/+ C57BL/6J)F_1 × +/+ C57BL/6J died soon after birth (Table 1). This lethality was closely associated with *D9Mit8*, i.e, the *Aft* locus, yet it was not observed in the intercross involving the F_1 progeny of an outcross *Aft*/+ 129Sv × +/+ C57BL/6J. One possible interpretation of this discrepancy is that within the C57BL/6J strain there exist multiple recessive modifiers enhancing the Aft phenotype to lethality. Alternatively, there may exist lethality-suppressing modifiers in 129Sv, which permit the survival of *Aft*/+ animals. Similar highly penetrant lethality on C57BL/6J background was recently reported for several deletions in the *t*-complex (Browning et al. 2002).

If *Aft* is a valid candidate locus for *Brm1*, it should enhance the short-tail phenotype of *Brachyury*. Since we found that taillessness is seen predominantly in animals carrying both *T* and *Aft* mutations, the most plausible interpretation is that it occurs as a consequence of an interaction between these two loci. This interpretation is further supported by the finding that *T* is expressed, and is likely an important regulator acting during cartilage formation in the tail and limbs of developing mouse embryos (Hoffmann et al. 2002), precisely the areas where Aft phenotypes are seen. The low penetrance of this "synthetic" phenotype (around 8.5%) may be explained by

weak association between the *T* and *Aft* pathways, or by effects of the genetic background which dampen the interaction.

Finally, we noticed two genes on Chr 9 which, when mutated, could cause the Aft phenotype. Animals mutant for the first gene, *Csk* (c-Src kinase), fail to turn and to close neural folds and hence die at midgestation (Imamoto and Soriano 1993; Nada et al. 1993). Since the syndactyly and tail kinks of the Aft animals could be interpreted as a consequence of abnormal apoptosis, we consider the *Dapk2* (Death-associated protein kinase 2; Kawai et al. 1999) gene to be a second possible candidate. The genes, mutations in which result in the Aft and rc phenotypes, will be identified by further molecular and genetic analyses of this region. The data presented here provide a resource that will be helpful in future studies of the *Brachyury* gene function, vertebrate development, and diseases of skin and hair.

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