Ages with stiffened joints, A new mutation in *Enpp1*

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Mutation (allele) symbol: *Enpp1*^{asj}

Mutation (allele) name: ages with stiffened joints

Gene symbol: *Enpp1*

Strain of origin: C57BL/6J

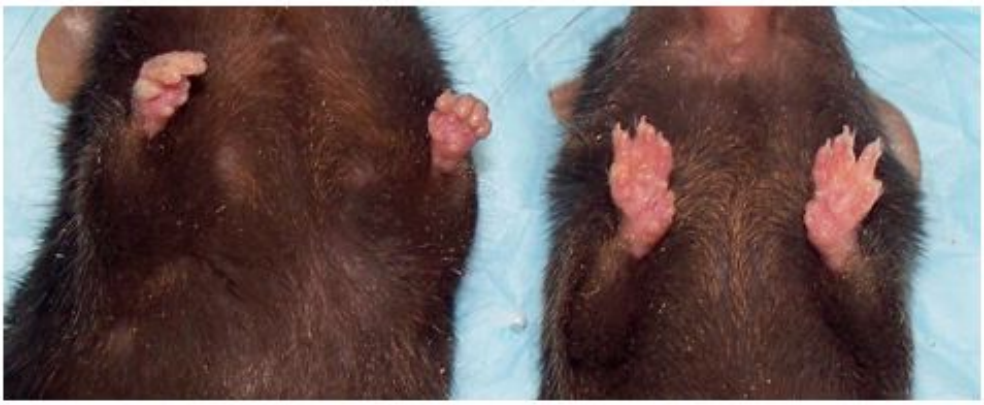
Current strain name: C57BL/6J-*Enpp1*^{asj}/GrsrJ

Stock #012810 (jaxmice.jax.org)

Phenotype categories: skeletal

Abstract
We have identified a new, recessive, mouse mutation that causes affected mice to develop stiffening of the joints by two months of age. Homozygotes have normal activity for the first weeks of their life, but by approximately 2 months of age they begin to change the manner in which they hold their forepaws, bending them in closer to the body, and develop a resultant slow, hobbling gait which worsens as they age. The front paws appear to have shortened digits, though the rear paws are phenotypically normal. Homozygotes of both genders do breed, but fertility is short lived and may provide 1 to 4 litters before the homozygote is too stiff to move around much or be able to take care of a litter. This mutation was named ages with stiffened joints (*asj*), mapped to Chromosome 10, and subsequently found to be a point mutation in ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*).

Origin and Description
The *asj* mutation was discovered by Linda Jorgenson of the neuromutagenesis program at The Jackson Laboratory in 2004 in the C57BL/6J progeny of an ENU treated C57BL/6J male. She first noticed the stiff posture of these mice and the abnormal way they held their front legs as they got older.



Paws of an adult control (left) or asj homozygote (right) after stiffening has developed

The digits of the front paws appear shorter than normal, but those of the hind paws are normal. Auditory brainstem response testing revealed moderate to severe hearing loss by 3 months of age. Homozygotes do live to 6 months or older and may give 1 to 4 litters before breeding stops. Heterozygotes live a normal life span and have normal fertility. The *asj* colony is maintained by mating a homozygote *asj* mouse of either gender to a C57BL/6J mouse or by intercrossing heterozygotes.

Pathology

A standard pathological screen done on four mutants at 11 weeks of age showed dilated joint spaces in the knees and elbows with hyperplastic synovium. Two affected mice were found to have arthritis of the spine at 12 weeks of age. Two mutants assessed at 13 weeks of age were found to have severe osteomyelitis of the joints with unusual synovial cysts. Two mutants assessed at 7 months of age had very stiff and unbendable joints with severe osteoarthritis in many joints, mineralization of the capsule of the follicles of the vibrissae, and one showed compression of the spinal cord and the other had stones in the urinary bladder. Eye examinations of two homozygotes were normal except for a cataract in the left eye of one.

Genetic Analysis

Using standard mapping protocols, the *asj* mutation was found to be recessive and mapped to Chromosome 10 using an intercross to CAST/EiJ. No affected mice were produced in the F1 generation but this mutation did segregate in the F2 generation. The *asj* mutation mapped between *D10Mit213* (NCBI 37 position 20.1Mb) and *D10Mit137* (NCBI 37 position 29.7 Mb) and was non-recombinant with *D10Mit183* (NCBI 37 position 22.2 Mb). This positioned the *asj* mutation in the area of *Enpp1* (NCBI 37 position 24.3 Mb). Based on map position and phenotypic similarities to other mutant alleles, *Enpp1* was considered a good candidate gene. Total RNA was isolated from kidney and testis of mice at 4 months of age with the TRIzol LS Reagent and the SuperScriptTM preamplification system (Invitrogen Life Technologies, Carlsbad, CA) was used to make first strand cDNA. We designed four pairs of PCR primers (Table 1) to amplify overlapping cDNA fragments based on the murine *Enpp1* mRNA sequence (NM_008813).

Table 1. PCR Primers used for Murine *Enpp1* cDNA sequencing and genotypic analysis. All primers were ordered from Integrated DNA Technologies, Coralville, IA.

Designation	Sequence (5'-3')	Fragment size	Exons
Enpp1-1F	GCGAGCCTATTAAAGCTCGG	811	1 -8
Enpp1-1R	ATTATGCCATGGGATTCCGG		
Enpp1-2F	CATTACAGCATCGTCACAGG	787	7 - 15
Enpp1-2R	AACGCAAGTTGCCACTGAGG		
Enpp1-3F	AGCATTTCCGGCCTTACCTG	839	15-23
Enpp1-3R	GACCGCTGACAACATTGATG		
Enpp1-4F	ATAGTGCCAATGTATCAGAG	693	22-25
Enpp1-4R	AGGAACACTCTGC AGATGTG		
Enpp1-GF	CTATGTACCCTACCAAGAC G	76	7
Enpp1-GR	AACACAG AGTGTGCCTAGTG		

For direct sequencing, the PCR reaction was scaled up to 20 µl; amplification was done for 36 cycles with a 15 s denaturing step at 94 ¡C, a 2 min annealing step at 60 ¡C, and a 2 min extension step at 72 ¡C. PCR products were purified from 1.5% SeaKem agarose gels using a Qiagen kit (Qiagen Inc., Valencia, CA). Sequencing reactions were carried out with automated fluorescence tag sequencing.

asj was found to be a missense mutation caused by a single base substitution of T to A at position 737 in exon 7 of the *Enpp1* gene, resulting in an amino acid substitution from valine to aspartic acid at residue 246.

Figure 1. Data from sequencing indicates a single base pair substitution at position 737 (T to A) in exon 7 between the *asj* and wildtype *Enpp1* genes. This mutation changes codon 246 from GTC to GAC, changing the amino acid from valine (Val, V) to asparitic acid (Asp, D) in the predicted mutant protein product

This point mutation creates a new *TaqI* restriction site (TCGA). To confirm the presence of the missense codon in the mutant *Enpp1* gene, we examined the DNA from the parental strains and 2 affected mice from our linkage analysis for the *TaqI* RFLP. Using the primers CTATGTACCCTACCAAGACG and AACACAGAGTGTGCCTAGTG we amplified a 76 base pair genomic fragment that contains the *TaqI* site introduced by the *asj* mutation. Digestion of the PCR amplified products with *TaqI* from homozygous, heterozygous, and wildtype DNA revealed the predicted RFLP pattern: homozygotes yielded two bands of 43 and 33 bp; heterozygotes yielded three bands of 76, 43, and 33 bp; and wild-type yielded one band of 76 bp. The RFLP pattern thus provides a tool for verification of the presence or absence of the asj allele. This genotyping method also

verified the lack of the mutation in the C57BL/6J parental strain and mapping partner CAST/EiJ.

Discussion

We report a new mutation in the *Enpp1* gene that causes stiffening of the joints and arthritis as the mice age to adulthood. Existing *Enpp1* mutants that have been characterized have similar or related phenotypes include calcinosis, with calcification of tendons and cardiac muscle, and ankylosis of the verebral column and limb joints, which results in an abnormal gait and early death. The association of a hearing deficit with an *Enpp1* mutation is a novel finding and warrants further characterization.

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