Supporting information for Tomatsu *et al.* (2002) *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.232570999

Supporting Methods

Methodology for Screening Homologous Recombinants. To identify homologous recombinants from genomic DNA, the PCR method uses a forward primer in intron 9 (Sc1: 5'-GACTGACTGCTACGAGCTGCAGATTGAACCTGG-3') and a reverse primer in intron 10 outside the targeting sequences (Sc12R: 5'-TCATCAAGGTGGCCCATGTCTTTAATCCAAGAG-3'), which produces a 3,910-bp fragment and retains an *Eco*RI restriction site in the normal allele but not in the mutant allele. After cleavage with *Eco*RI, the 3,580- and 330-bp restriction fragments present in the normal allele are distinguished from the uncleaved 3,910-bp PCR fragment from the mutant allele on a 1.2% agarose gel. Genomic DNAs of the clones positive by PCR screening were digested with *Eco*RI, transferred to nylon membranes, and hybridized with the 3' probe (probe 1,760 bp), generating 14.3-kb fragments in the recombinants instead of 9.5-kb fragments in the normal allele (data not shown).

This *neo*^{r} cassette removal leaves only the 34 bp of one loxP site in intron 9. The removal of the *neo*^{r} was diagnosed by PCR of tail DNA by using primers in intron 9 (forward - BGCre2: 5'-TTTGCCTGCATGTGTGAGGGTGTCGATCC-3') and (reverse - BGO5: 5'-GATGGTGATCGCTCACCAAATC-3'), which amplified a 650-bp fragment whereas a non-*neo*^{r}-excised allele did not reveal any fragments under these conditions: The reaction mixture was heated for 5 min at 94°C followed by 35 cycles of 45-sec denaturation at 94°C, 40-sec annealing at 63°C, and 45-sec extension at 72°C.

Methodology to Identify Missense Mutations at the Genomic Level. To amplify the murine *Gus* gene, including the E536A and E536Q mutations, a forward primer (TMO22: 5'-CCTGTGTCATTTGCATGTGACTATT-3') and a reverse primer (TMO40R: 5'-TGTGGGTGCTGGGAACCAGACTGAG-3') were used, resulting in amplification of a 665-bp fragment. Digestion with *Bst*UI revealed the 217- and 448-bp restriction

fragments in the E536A mutant allele and the uncleaved 665-bp PCR fragment in the normal allele, whereas digestion with *Rsa*I resulted in the 80-, 139-, and 446-bp restriction fragments in the E536Q mutant allele and the 80- and 585-bp PCR fragments in the normal allele (Fig. 5 *A* and *B*). To amplify the 650-bp fragment of murine *Gus* gene containing the L175 residue, a forward primer (TMO11: 5'-TCTGGCCATTTACCCTGTGCTCATTC-3') and a reverse primer (TMO30R: 5'-CTTGGTCCACATTAGTGATCACAGTGATAT-3') were used. Digestion with *Mse*I showed the 158-, 180-, and 312-bp restriction fragments in the L175F mutant allele and the 180- and 470-bp PCR fragments in the normal allele (Fig. 5*C*).

Methodology to Identify Missense Mutations in Mouse *Gus* Gene at the mRNA Level. To identify mutations in mouse *Gus* at the mRNA level, RT-PCR was done on mRNA of liver. Five micrograms of total RNA was mixed with oligo(dT) primer in a total volume of 20 μl, including RNase inhibitor, dNTPs, reverse transcriptase, DTT, and reverse transcriptase buffer according to the instruction manual (GIBCO/BRL). Reaction mixtures were incubated at 42°C for 50 min to make the first strand of cDNA, followed by inactivation of enzyme at 70°C for 15 min. The remainder of the total RNA was eliminated by RNase at 37°C for 20 min. Two microliters of the products of one reverse transcriptase reaction was annealed to 50 pmol each of sense and antisense primers. After heating of the reaction mixtures for 5 min at 94°C, PCR amplification was carried out for 35 cycles as follows: 45-sec denaturation at 94°C, 40-sec annealing at 63°C, and 1.5-min extension at 72°C. The PCR products were directly sequenced. To amplify mouse *Gus* cDNA, including the E536A and E536Q mutations, a forward primer (TMO60: 5'-TCTGTGGCCAATGAGCCTTCCTCTG-3') and a reverse primer (TMO4R: 5'-GAACGTGTGAACGGTCTGCTTCCG-3') were used, resulting in amplification of a 617-bp fragment. Digestion with *Bst*UI revealed the 280- and 337-bp restriction fragments in the E536A mutant allele and the uncleaved 617-bp PCR fragment in the normal allele, whereas digestion with *Rsa*I resulted in the 61-, 137-, 145-, and 274-bp restriction fragments in the E536Q mutant allele and the 145-, 198-, and 274-bp PCR fragments in the normal allele (Fig. 6 *A* and *B*). To amplify the 297-bp fragment of *Gus* cDNA containing the L175 residue, a forward primer (TMO28: 5'-

TGGAACATGAGGGAGGTCACCT-3') and a reverse primer (TMO30R: 5'-CTTGCTCCACATTAGTGGTCACAGTGATAT-3') were used. Digestion with *Mse*I showed the 95- and 202-bp restriction fragments in the L175F mutant allele and the uncleaved 297-bp PCR fragment in the normal allele.

Fig. 7 shows the variation in the histopathology of the same stifle joint and associated bone in the three missense models.