Fischer-344 Tp53-knockout rats exhibit a high rate of bone and brain neoplasia with frequent metastasis

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ABSTRACT
Somatic mutations in the Tp53 tumor suppressor gene are the most commonly seen genetic alterations in cancer, and germline mutations in Tp53 predispose individuals to a variety of early-onset cancers. Development of appropriate translational animal models that carry mutations in Tp53 and recapitulate human disease are important for drug discovery, biomarker development and disease modeling. Current Tp53 mouse and rat models have significant phenotypic and genetic limitations, and often do not recapitulate certain aspects of human disease. We used a marker-assisted speed congenic approach to transfer a well-characterized Tp53 mutant allele from an outbred rat to the genetically inbred Fischer-344 (F344) rat to create the F344-Tp53tm1(EGFP-Pac)Qly/ Rrrc (F344-Tp53) strain. On the F344 genetic background, the tumor spectrum shifted, with the primary tumor types being osteosarcomas and meningeal sarcomas, compared to the hepatic hemangiosarcoma and lymphoma identified in the original outbred stock model. The Fischer model is more consistent with the early onset of bone and central nervous system sarcomas found in humans with germline Tp53 mutations. The frequency of osteosarcomas in F344-Tp53 homozygous and heterozygous animals was 57% and 36%, respectively. Tumors were highly representative of human disease radiographically and histologically, with tumors found primarily on long bones with frequent pulmonary metastases. Importantly, the rapid onset of osteosarcomas in this promising new model fills a current void in animal models that recapitulate human pediatric osteosarcomas and could facilitate studies to identify therapeutic targets.

KEY WORDS: p53, Rat, Osteosarcoma, Sarcoma, Cancer

INTRODUCTION
Cancer researchers have extensively studied the Tp53 gene, as it is the most frequently mutated gene in human cancers (Hollstein et al., 1991; Petitjean et al., 2007). Discovered in 1979 and labeled the ‘guardian of the genome’, the Tp53 gene encodes a transcription factor (Tp53) that has tumor suppressor roles (Lane, 1992; Levine et al., 1991; Linzer et al., 1979). In response to DNA damage, the Tp53 protein specifically binds to DNA, transcribing genes that halt cell replication, initiate DNA repair and, at times, result in cell senescence or apoptosis (Mirza et al., 2003; Rivlin et al., 2011; Tokino and Nakamura, 2000). Mutations resulting in the absence of normal Tp53 in a cell lead to further genetic instability and improper cell cycle regulation. This ultimately results in the propagation of additional mutations, leading to malignancy (Lane, 1992). Although rare, germline mutations in TP53 result in a condition known as Li-Fraumeni syndrome (LFS), a familial disorder characterized by the development of a wide variety of tumors in the first through third decades of life (Malkin et al., 1990). The most frequently identified tumors in LFS individuals are breast carcinomas, soft-tissue sarcomas, osteosarcomas, and central nervous system (CNS) tumors, although the spectrum varies with age as well as the specific mutation (Bougeard et al., 2015).

Recently, knowledge surrounding the biochemical and physiological processes of Tp53 has advanced more rapidly with the development of transgenic rodent models. Mice have been used to study both the loss of Tp53 function and dominant-negative forms of the protein (Donehower and Lozano, 2009; Rivlin et al., 2011). A potential drawback of many of the mouse models is the relatively high rate of malignant lymphoma in the Tp53-null state that is strongly dependent upon the genetic background (Donehower et al., 1995; Harvey et al., 1993). This leads to early morbidity and mortality and subsequently, due to the shortened lifespan, the full tumor spectrum seen in human LFS is not seen in the mouse. As a possible alternative rodent model, the rat has many advantages. The rat has a larger body size and more human-like metabolism than the mouse, making rats a better model species for preclinical drug and device development (Aitman et al., 2008). With recent advances in genome modification technologies, previous barriers to making genetically engineered rats no longer exist. In fact, in 2010, Tp53 gene targeting was accomplished in the rat using some of these new technological advances (Tong et al., 2010). Currently, there are three well-characterized mutant rat models of Tp53, each developed in outbred stocks, and two additional inbred models that have yet to be extensively characterized (Table 1). These rat models show a decreased incidence of lymphoma and better representation of human cancers than many of the Tp53 mouse models.

Often, Mendelian diseases are greatly influenced by the genetic background of the individual, creating highly variable phenotypes between individuals. Tp53 mutations have been generated in a wide range of inbred mouse strains to investigate the influence of genetic background as a factor in phenotype variability (Donehower et al., 1995; Harvey et al., 1993). Tp53-deficient mice on a C57BL/6 background rarely develop mammary neoplasia, whereas BALB/c mice carrying the same mutant allele have a 55% incidence of mammary carcinoma in the heterozygous state (Kuperwasser et al., 2000). Rat models of human genetic disease similarly show...
phenotypic variability that depends on genetic background (Amos-Landgraf et al., 2007; Kantachuvesiri et al., 1999). Likewise, variability is seen across the spectrum of human disease categories, including but not limited to neurofibromatosis type 1, cystic fibrosis, breast cancer, Fanconi anemia, Li Fraumeni syndrome and hypertrophic cardiomyopathy (Boyle, 2007; Lopes et al., 2015; Metheny et al., 1995; Neveling et al., 2009; Nichols and Malkin, 2015; Szabo and King, 1995).

The outbred genetic nature of the existing rat \( Tp53 \) knockouts might contribute to tumor variability, complicating reproducibility of studies. We transferred the \( Tp53 \)-gene-knockout allele from an outbred stock to the Fischer-344 (F344) inbred strain of rat to stabilize the genetic background and determine if the genetic background would have an impact on the disease phenotype. We show that the phenotype of the inbred F344-\( Tp53 \text{tm1(EGFP-Pac)Qly/Rrrc} \) rat differs from that of previously reported \( Tp53 \) mutants. On the inbred genetic background, there is a high incidence of brain tumors, and bone tumors that are accompanied by frequent pulmonary metastasis. This new strain provides a refined model for cancer research to investigate the role of \( Tp53 \) in cancer initiation, propagation and metastasis.

### RESULTS

#### Generation of a congenic \( Tp53 \) rat model on the Fischer-344 genetic background

Beginning with the original STOCK-\( Tp53 \text{tm1(EGFP-Pac)Qly/Rrrc} \) rat, we backcrossed to F344/NHsd using a marker-assisted speed congenic approach to transfer the \( Tp53 \) mutation onto the F344 background (Fig. 1). \( Tp53 \) heterozygous males carrying the highest number of tested loci that were homozygous for F344 alleles were selected at each generation to be backcrossed to F344 females. The process was repeated through five generations, resulting in a congenic strain that carries the \( Tp53 \text{tm1(EGFP-Pac)} \) null allele on a F344 genetic background. Once congenic, the strain was maintained by continued backcrossing to F344. Included in the final phenotype characterization were 25 heterozygous rats (12 male, 13 female) and seven homozygous rats (six male, one female), the progeny from matings of N5-N7 heterozygous F344-\( Tp53 \text{tm1(EGFP-Pac)Qly/Rrrc} \) parents. Sixty-nine offspring were produced from heterozygous intercross matings (18 wild-type, 40 heterozygotes and 11 homozygous mutant animals). The genotypic ratios were as expected for Mendelian inheritance of an autosomal allele. However, there was a significant sex bias in the number of Table 1. Summary of \( Tp53 \)-mutant rat models

<table>
<thead>
<tr>
<th>Model name</th>
<th>Strain and/or stock</th>
<th>Method</th>
<th>Tumor spectrum (number of animals in each group)</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>F344-( Tp53 \text{tm1(EGFP-Pac)Qly/Rrrc} )</td>
<td>F344/NHsd</td>
<td>Speed congenics</td>
<td>( n=7 ) Meningeal sarcoma (54%) Osteosarcoma (36%)</td>
<td>Tong et al., 2010; Yan et al., 2012</td>
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<tr>
<td>STOCK-( Tp53 \text{tm1(EGFP-Pac)Qly/Rrrc} )</td>
<td>DA and Sprague-Dawley</td>
<td>Homologous recombination</td>
<td>( n=40 ) Hepatic hemangiosarcoma (80%) Lymphoma (18%)</td>
<td>van Boxtel et al., 2011</td>
</tr>
<tr>
<td>Crl:Wt(UL)-( Tp53 \text{tm1Hubr} )</td>
<td>Wistar</td>
<td>ENU mutagenesis</td>
<td>( n=11 ) Hemangiosarcoma (70%) Lymphoma (10%)</td>
<td>Hermens et al., 2015</td>
</tr>
<tr>
<td>( Tp53 \text{tm1f/f} )</td>
<td>Sprague-Dawley</td>
<td>Zinc-finger nuclease</td>
<td>( n=24 ) Osteosarcoma (18%) Malignant meningioma (15%)</td>
<td>McCoy et al., 2013</td>
</tr>
<tr>
<td>F344-( Tp53 \text{tm1KyO} )</td>
<td>F344/NSLc</td>
<td>ENU mutagenesis</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>LEA-( Tp53 \text{tm1(AmCyan1)Ncco} )</td>
<td>LEA</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

F344, Fischer 344; LEA, Long–Evans Agouti; LFS, Li Fraumeni syndrome; n.a., not applicable.

The outbred genetic nature of the existing rat \( Tp53 \) knockouts might contribute to tumor variability, complicating reproducibility of studies. We transferred the \( Tp53 \)-gene-knockout allele from an outbred stock to the Fischer-344 (F344) inbred strain of rat to stabilize the genetic background and determine if the genetic background would have an impact on the disease phenotype. We show that the phenotype of the inbred F344-\( Tp53 \text{tm1(EGFP-Pac)Qly/Rrrc} \) rat differs from that of previously reported \( Tp53 \) mutants. On the inbred genetic background, there is a high incidence of brain tumors, and bone tumors that are accompanied by frequent pulmonary metastasis. This new strain provides a refined model for cancer research to investigate the role of \( Tp53 \) in cancer initiation, propagation and metastasis.
F344-Tp53 rats experience early-onset tumor development

Previously reported Tp53-mutant mice and rats have a decreased lifespan compared to their wild-type counterparts owing to early tumor development (Donehower et al., 1995; McCoy et al., 2013; van Boxtel et al., 2011; Yan et al., 2012). We aged our animals until signs of tumor or illness were noted during daily evaluation, at which time we performed CO2 euthanasia and complete necropsy. The median time to morbidity for homozygous rats was 124 days (18 weeks), with a range of 16 to 21 weeks (Fig. 2). All homozygous animals developed one or more tumors within this time frame, including the single homozygous female that was euthanized at 16 weeks owing to dystocia (Table S1). The heterozygous rats developed disease, on average, by 47 weeks, with a range of 16 weeks to 62 weeks. One outlier was a heterozygous female rat euthanized at 16 weeks after presenting with exophthalmos, head tilt, ataxia and lethargy. She was determined to have a pituitary adenoma. We repeated genotyping on tissue from this animal and confirmed that it was indeed heterozygous, despite the unexpected early onset of morbidity. Overall, the F344 time to morbidity was longer than that reported for the original STOCK-Tp53 line, where half of the STOCK-Tp53 homozygous developed tumors by 10 weeks, and half of heterozygotes were euthanized or died by 32 weeks (Yan et al., 2012).

The tumor spectrum varies on different genetic backgrounds

In our study, homozygous F344 rats developed primarily osteosarcomas, meningeal sarcomas or a combination of the two tumor types (Fig. 3). One hemangiosarcoma was identified in a superficial tumor on a homozygous animal also bearing an osteosarcoma and meningeal sarcoma (Table S1). Heterozygous rats experienced a wider range of tumor types, with 9 out of 25 (36%) animals developing osteosarcoma. Seven heterozygous rats (28%) had meningeal sarcomas. Three heterozygotes that had similar clinical presentations of labored breathing and a hunched posture were diagnosed with lymphoblastic lymphoma, exhibiting a space-occupying thymic mass as well as metastasis throughout the lungs, kidneys, spleen, liver, vasculature and lymph nodes throughout the body. Three heterozygous animals developed pituitary adenomas, of which two exhibited changes in mentation (head tilt, ataxia, moribund). The third was an incidental finding in an animal with an osteosarcoma of the tibia. Pituitary gland tumors have been noted to occur in aged (1-2 years old) wild-type F344 rats with a rate of 30.4% in males and 54.2% in females (Haseman et al., 1998). The STOCK-Tp53 study reported only one brain tumor in mutant animals out of a large cohort, although the exact location within the brain was not specified (Yan et al., 2012). Three cases of lymphoma were seen in our study (9%), compared to 40% reported in the STOCK-Tp53 mutants (Yan et al., 2012). Although the original STOCK-Tp53 and F344-Tp53 rats both developed primarily sarcomas, the target tissue was vastly different. For example, 80% of homozygous STOCK-Tp53 knockout rats developed hepatic hemangiosarcoma, a tumor not seen in F344-Tp53 rats in our study.

Two heterozygous F344-Tp53 females presented with mammary adenocarcinoma, representing a similarity between the two models, in that 6 out of 31 female STOCK-Tp53 heterozygous rats displayed mammary carcinomas, compared to 2 out of 13 cases of mammary adenocarcinoma in the F344-Tp53 rats (Yan et al., 2012). Also identified in heterozygotes were cases of papillary cyst adenocarcinoma, fibrosarcoma and mesothelioma; the latter exhibiting metastasis throughout the abdomen. Tumors were consistent with the types of sarcomas commonly seen in individuals affected with LFS – mainly bone, CNS and soft tissue (Malkin et al., 1990).
F344-Tp53 osteosarcomas were common with frequent pulmonary metastases

Osteosarcomas were identified in 57% and 36% of homozygous and heterozygous animals, respectively. F344 rats normally show a very low incidence of osteosarcomas, with 0.2-0.4% reported previously (Haseman et al., 1998). Most osteosarcomas were found on long bones, and resulted in rats presenting with lameness and limb swelling (Figs 4 and 5A). Tumors were identified on the proximal tibia, proximal humerus and distal femur. Radiographs of long-bone tumors exhibited considerable amounts of soft-tissue osteoid, starburst bone growth, normal bone destruction, ill-defined tumor margins and pathologic fractures (Fig. 4A-D). Bone tumors radiographically did not cross a joint and were limited to a single bone. Computed tomography analysis of hindlimb tumors revealed excessive soft-tissue osteoid development and bone destruction that was limited to the tibia (Fig. 4E,F). Exceptions in regards to long bone location included one tumor on the mandible and one on a lumbar vertebra; the rat with the latter presenting with ataxia that progressed to hindlimb paresis, which was worse on the right side. The tumor was identified on the right side of a lumbar vertebra. All osteosarcomas, with one exception, were high-grade and conventional in type, exhibiting the production of an immature osteoid matrix (Fig. 5B,C). The single exception was a limb tumor in a homozygous animal without osteoid matrix, consistent with a minimally osteogenic osteosarcoma or a fibroblastic subtype of osteosarcoma. Despite this tumor finding, osteosarcoma metastasis was present in the pulmonary parenchyma, indicating that the animal indeed harbored an osteosarcoma, although a slight variation from the other types. Each homozygous and 4 out of 9 heterozygous rats that presented with osteosarcoma also exhibited histopathologic evidence of pulmonary metastasis. Metastases could be identified grossly in some animals as large firm nodules of osteoid present within and on the surface of the pulmonary parenchyma (Fig. 5D). In other animals, pulmonary metastasis was identified histologically, with osteoid present throughout large perivascular regions of neoplastic cells (Fig. 5E,F).

Meningeal sarcomas are a predominant tumor in animals presenting with lethargy or sudden death

Brain tumors, specifically meningeal sarcomas, were identified in 6 out of 7 homozygous animals. The brain of the seventh rat was not available for evaluation owing to post-mortem autolysis following sudden unexpected death. Meningeal sarcomas were also identified in heterozygous animals, but to a lesser extent (7 out of 25 rats). Tumors were highly invasive, with extensive disruption of normal brain tissue (Fig. 6A-C). Animals with meningeal sarcomas were frequently found to either die suddenly after appearing clinically normal the previous day or they would present with depressed or dull mentation.

Other clinical observations

Three animals were euthanized based on clinical signs of illness or distress in the absence of observable tumors. The single female homozygous rat required euthanasia owing to dystocia. Following histopathologic evaluation, this female was identified to have a meningeal sarcoma. Interestingly, Tp53 homozygous mice have been noted previously to have an increased rate of dystocia (Donehower, 1996; Hirota et al., 2010). Two heterozygous rats developed lethargy and dull mentation with one being diagnosed post-mortem with pyelitis and pyelonephritis, and the other having no identifiable tumors upon histopathologic evaluation.
DISCUSSION

Advantages of rat models for biomedical research
For years, aging and carcinogenicity studies have been performed in the well-characterized F344 rat, with data on over 100,000 study animals archived with the National Cancer Institute and National Toxicology Program (ntp.niehs.nih.gov). These studies provide a vast amount of historical data on the Fischer strain, including rates of spontaneously occurring neoplasms and average life span under various housing and disease conditions. In addition, the rat’s increased body size is amenable to studies requiring invasive techniques and repeated sampling, such as device testing, serum biomarker detection and metabolomics. With the recent advances in the ability to manipulate the rat genome, it is now possible to generate a wide variety of genetically engineered rats (Bao et al., 2015; Geurts et al., 2009; Mashimo, 2014). The size, physiology and genetics of the rat, along with the ability to manipulate the genome, position the rat to be the premier species for modeling human disease.

Importance of genetic background
The use of outbred stocks in drug and toxicology studies is often justified as a means to mimic human genetic diversity. However, commonly used breeding strategies for genetically modified animals created on an outbred background rarely maintain this genetic diversity. In addition, closely linked loci surrounding the genetic modification can become fixed and have an unappreciated effect on phenotype. By generating or maintaining mutations on an inbred genetic background, phenotypic variability due to genetic variation can be avoided.

F344-Tp53 provides a refined model for the study of cancer
Similar to previous studies showing phenotypic variability in mice with Tp53-null mutations on various genetic backgrounds, transfer of the null Tp53 mutation from the original outbred STOCK to the inbred F344 rat results in a distinct phenotype shift (Aitman et al., 2008; Donehower et al., 1995). Although both strains primarily developed...
sarcomas, the F344-Tp53 rat had tendency to form bone and brain tumors, compared to the more frequent liver and blood neoplasia identified in the STOCK-Tp53 rat. This is likely to represent a direct effect of the genetic background on the tumor spectrum. Further genetic studies could identify candidate modifier genes that might explain the genotype-to-phenotype variation. A major advantage of the F344-Tp53 congenic is the ability to model osteosarcoma and other tumor types on a standardized inbred genetic background.

Sex bias in homozygous mutants
Although the Tp53-null state is not considered embryonically lethal, the finding of fewer Tp53 homozygous females is not surprising based on literature reporting lower recovery rates of Tp53-null female mice due to the occurrence of exencephaly (Armstrong et al., 1995; Sah et al., 1995). Likewise, it has been noted that fewer homozygous females were recovered in two previously published Tp53 rat models (McCoy et al., 2013; van Boxtel et al., 2011). It was shown in Tp53-null rats that the lack of live-born female homozygotes is due, at least in part, to neural tube defects that lead to embryonic lethality (Kawamata and Ochiya, 2012). This effect of Tp53 indicates its importance not only in prevention of tumors but in its role in basic embryonic development.

F344-Tp53 osteosarcomas recapitulate human disease
The F344-Tp53 rat is beneficial as it allows the study of bone tumors that develop in a tissue microenvironment more similar to that of humans, as the rat exhibits true lamellar bone structure, a feature lacking in the mouse. Owing to the genetic complexity of osteosarcoma development, it is difficult to model tumor initiation and progression (Hansen et al., 1985; Sampson et al., 2013; Poos et al., 2015). Previous studies have used significant manipulations—such as long-term parathyroid hormone [PTH(1-34)] administration or tumor transplantation in the soft-tissue environments—or have utilized immunocompromised animals (Schwartz et al., 2013; Tsukamoto et al., 2012; Vahle et al., 2002). The tumors that developed in our rats recapitulate the aggressive human osteosarcoma phenotype, with tumors that primarily affect long bones and that readily metastasize. This is in direct contrast to mouse osteosarcomas that develop primarily on the axial skeleton and metastasize about 20% of the time (Walkley et al., 2008).

In our study, 57% of F344-Tp53 rats had osteosarcomas and meningeal sarcomas. In humans, meningeal sarcomas are rare and aggressive tumors that typically affect children (Buttner et al., 2001). The meningeal sarcomas in our model were histologically similar to human tumors in that they feature small round uniform cells with scant cytoplasm (Jing et al., 2014).

In summary, the F344-Tp53 knockout rat is highly suitable for oncology research. The mutation is now present in an inbred strain of rat frequently utilized in research. The inbred genetic background allows for easy maintenance of the model and introgression of additional mutations that already exist on the F344 background. The inbred F344-Tp53 knockout rat is a powerful model to investigate compelling questions surrounding the development of osteosarcomas and meningeal sarcomas.

MATERIALS AND METHODS
Ethical statement
All activities associated with this study received prior protocol approval by the University of Missouri Institutional Animal Care and Use Committee. The University of Missouri is United States Department of Agriculture (USDA) licensed and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited.

Housing and husbandry
Rats were housed in micro-isolator cages on ventilated racks (Thoren, Hazleton, PA) in an environmentally controlled animal facility maintained at 68-79°F, 30-70% humidity on a 14:10-h light:dark cycle in accordance with recommendations set forth in the Guide for the Care and Use of Laboratory Animals, 8th Edition (Animal Welfare Act, Animal Welfare Regulations, ILAR Guide). Breeders were fed Picolab 5058 breeder chow with ad libitum access to acidified autoclaved water. Study animals were fed Picolab 5008 chow. Routine health monitoring was performed by Charles River (Wilmington, MA).

Animals
The strain characterized in this study is F344-Tp531/EGFP-Pac/Qly/Rrrc, available from the Rat Resource and Research Center (RRRC), University of Missouri (RRRC# 661). This strain carries a Tp53 allele in which exons 2-5 have been replaced with a CAG-EGFP-IRESPac cassette (Tong et al., 2010). A marker-assisted speed congenic approach was used to generate the strain. Male rats from STOCK-Tp531/EGFP-Pac/Qly/Rrrc generated by Qin-Long Ying (USC) (Tong et al., 2010) and available through the RRRC (RRRC# 485) were backcrossed to F344/NHsd females from Harlan Laboratories (Indianapolis, IN). The resulting offspring were genotyped for the Tp531/EGFP-Pac/Qly/Rrrc allele, and heterozygous males were backcrossed to female F344/NHsd rats. For each successive generation, microsatellite analysis using a low-density panel of markers was performed, as previously described, on DNA from heterozygous males to identify those rats with the highest percentage of test loci that were homozygous for F344 alleles (Bryda and Riley, 2008). These males were used to produce the next generation. Heterozygous animals were intercrossed to generate homozygous animals. All rats used in the study were from generations N5-7.

Genotyping for the Tp53 allele
DNA was isolated from tail-snip biopsies that had been collected from 3-week-old rats extracted using the QiAGEN DNeasy Blood and Tissue Kit (QiAGEN, Valencia, CA) with minor modifications to the manufacturer’s protocol as follows: AW1 and AW2 wash steps were repeated once and followed by one elution into 200 μl of AE buffer. The primers used to identify the wild-type and Tp531/EGFP-Pac/Qly alleles were 5′-GGCTGTGCTCTGATGGTGAC-3′, 5′-GGGAAAGATTGGGAAGACATAAGC-3′ and 5′-CAGCGTGATGAGTGAAAGATTGGGAAGACATAAGC-3′. PCR was performed in 20-μl reactions containing: 10-20 ng genomic DNA, 2 μl 10× buffer with MgCl2 (Roche, Indianapolis,
IN), 3.2 µl of 1.25 mM dNTP mix (Promega, Madison, WI), 0.3 µl each of 25 µM primers and 1 μ FastStart Taq DNA polymerase (Roche). Reactions were performed in 200-µl thin-walled PCR tubes, and the thermocycling parameters were: 1 cycle at 95°C for 5 min, 35 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 30 s, and 1 cycle at 72°C for 10 min. Amplicons of 310 bp (wild-type allele) and 509 bp (mutant allele) were produced. Products were analyzed using the QIAxcel Advanced capillary electrophoresis system with the QIAxcel DNA Screening Kit, QX Alignment Marker 15 bp/1 kb, QX DNA Size Marker 50 bp-800 bp. The AM320 Injection Method with injection for 10 s at 5 kV and separation of 320× at 6 kV was used.

Endpoints
Rats were monitored daily for morbidity, as defined by: palpable tumors, lethargy, dyspnea, abnormal mentation or a combination of clinical signs of disease. When morbidity was noted, rats were euthanized.

Histopathology and phenotyping
Rats were euthanized by CO2 inhalation and subjected to a routine necropsy for gross evaluation of tissues and tumor collection. The following tissues were harvested and evaluated from each animal: any suspected masses, tumors or lesions, brain, heart, lung, liver, kidney, adrenal gland, pancreas and spleen, and any limb affected with lameness or swelling. Lesions were recorded, and tumor samples were dissected and submitted for routine histopathology preparation. Briefly, soft tissues were immersed in 10% buffered formalin for 48 h and processed for histologic examination. Tumors that were attached to bone were blocked in wax and cut into 5-µm sections. Tumors were fixed in 10% buffered formalin for 48 h followed by decalcification with a 50:50 solution of sodium citrate and formic acid until samples could easily be sectioned. Tumors that were attached to bone were processed for histological examination. Tumors that were attached to bone were blocked in wax and cut into 5-µm sections, which were stained with hematoxylin and eosin. Samples were evaluated by using brightfield microscopy in a blinded study method to determine tumor classification.

Imaging
Radiographs were obtained of long-bone tumors following tissue fixation but before decalcification. Tabletop images were obtained on an Innovet 64-slice Aquilion scanner with Quantum De-noising Software (Toshiba, Tustin, CA). Radiographs were obtained of long-bone tumors following fixation. CT scans were obtained on a Toshiba 64-slice Aquilion scanner with Quantum De-noising Software (Toshiba, Tustin, CA). 3D images were reconstructed using a volume rendering technique implemented in MIMICS 18.0. Radiographs and CT scans were used to evaluate bone involvement. Radiographs were obtained of long-bone tumors following tissue fixation and bone involvement. Anthracite 351 was used for bone modeling. Rats were monitored daily for morbidity, as defined by: palpable tumors, lethargy, dyspnea, abnormal mentation or a combination of clinical signs of disease. When morbidity was noted, rats were euthanized.

Supplementary information
Supplementary information available online at http://dmm.biologists.org/lookup/doi/10.1242/dmm.025767.supplemental


