Research Article

The Fusion Oncogene FUS-CHOP Drives Sarcomagenesis of High-Grade Spindle Cell Sarcomas in Mice

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Myxoid liposarcoma is a malignant soft tissue sarcoma characterized by a pathognomonic t(12;16)(q13;p11) translocation that produces a fusion oncoprotein, FUS-CHOP. This cancer is remarkably sensitive to radiotherapy and exhibits a unique pattern of extrapulmonary metastasis. Here, we report the generation and characterization of a spatially and temporally restricted mouse model of sarcoma driven by FUS-CHOP. Using different Cre drivers in the adipocyte lineage, we initiated in vivo tumorigenesis by expressing FUS-CHOP in Prx1+ mesenchymal progenitor cells. In contrast, expression of the oncoprotein in more differentiated cells does not form tumors in vivo, and early expression of the oncprotein during embryogenesis is lethal. We also employ in vivo electroporation and CRISPR technology to rapidly generate spatially and temporally restricted mouse models of high-grade FUS-CHOP-driven sarcomas for preclinical studies.

1. Introduction

Soft tissue sarcomas are tumors of the connective tissue that can arise anywhere in the body and are fatal in nearly 1/3 of patients. Myxoid liposarcoma (MLPS) is a malignant liposarcoma that accounts for approximately 30% of all liposarcomas, the most common soft tissue sarcoma subtype. Clinically, MLPS is distinguished by its remarkable response to radiation therapy compared to most other soft tissue sarcoma subtypes [1, 2]. Additionally, these tumors metastasize to bone, liver, and other soft tissue sites, whereas most other soft tissue sarcomas most commonly metastasize to the lung. MLPS occurs most often in the extremities, specifically within the thigh musculature [3].

The genetic hallmark of MLPS is the t(12;16)(q13;p11) translocation that is present in nearly 95% of cases [4, 5] and generates a novel fusion protein, FUS-CHOP [6]. The TERT promoter is also mutated in nearly 80% of MLPS, suggesting that the activation of telomere maintenance is important in MLPS tumorigenesis [7]. Exome analysis of MLPS has revealed few other recurrent mutations in coding sequences compared to other solid tumors [8]. These data suggest that the FUS-CHOP translocation is the predominant driver in MLPS tumorigenesis.

In the FUS-CHOP fusion oncoprotein, the N-terminus of FUS is joined via a unique linker region with the entire CHOP protein. Interestingly, experiments in transgenic mice showed that expression of the truncated form of FUS in the presence of aberrant CHOP was sufficient to generate tumors [9]. However, neither truncated FUS nor aberrant CHOP alone was sufficient for tumorigenesis. These experiments suggest a synergistic mechanism of tumorigenesis resulting from abnormal FUS and CHOP expression in MLPS. CHOP (also known as DDIT3/GADD153) is a C/EBP family protein that plays a role in adipogenesis and is induced by ER stress, growth arrest,
and DNA damage [10–12]. Despite the roles of CHOP in cellular stress responses, it is not induced by ionizing radiation [13]. FUS homozygous knockout mice are radiosensitive and exhibit genomic instability [14, 15]. FUS, Fused in Sarcoma, is a RNA-binding protein (RBP) that is a member of the FUS/EWS/TAF15 (FET) protein family of RBPs containing prion-like domains (PrLDs) [16, 17]. Other FET family members are commonly translocated in sarcoma and may transform mesenchymal progenitor cells that normally differentiate into bone, fat, cartilage, and muscle cells. At least four FUS-CHOP transcript variants exist, but the transcript structure does not affect prognosis [4]. Therefore, FUS-CHOP appears to be the primary oncogenic driver in MLPS, but the precise mechanism of MLPS tumorigenesis remains to be elucidated.

A critical question in MLPS biology is the identity of the cell of origin. It is possible that oncogenic fusions only transform cells if expressed in the appropriate cell type at a permissive stage of development. In this scenario, the expression of the oncogenic fusion in a different cell type or during the wrong developmental stage will not lead to transformation. In MLPS, the cell of origin is undefined. Previous mouse models of MLPS have expressed FUS-CHOP ubiquitously. While these studies reported generation of liposarcomas in mice, the tumors do not resemble human myxoid liposarcomas histologically [18–23]. To study the cell of origin of myxoid liposarcoma, we generated novel genetically engineered mice with a Cre-activatable FUS-CHOP translocation transcript. We crossed these mice to lines that express Cre recombinase in mesenchymal and adipose tissue lineages because MLPS arises most commonly in the thigh and is likely of mesenchymal origin [20–23]. Finally, to more accurately model human disease with a cell of origin undefined, it is possible that oncogenic fusions only transform cells if expressed in the appropriate cell type at a specific time during development. It is possible that expression of the oncogenic fusion in a different cell type or during the wrong developmental stage will not lead to transformation. In MLPS, the cell of origin is undefined. Previous mouse models of MLPS have expressed FUS-CHOP ubiquitously. While these studies reported generation of liposarcomas in mice, the tumors do not resemble human myxoid liposarcomas histologically [18–19]. To study the cell of origin of myxoid liposarcoma, we generated novel genetically engineered mice with a Cre-activatable FUS-CHOP translocation transcript. We crossed these mice to lines that express Cre recombinase in mesenchymal and adipose tissue lineages because MLPS arises most commonly in the thigh and is likely of mesenchymal origin [20–23]. Finally, to more accurately model human disease with a cell of origin undefined, we attempted to use CRISPR technology for genome editing to generate the FUS-CHOP translocation at the endogenous mouse genes.

2. Materials and Methods

2.1. Mouse Model Generation and Animal Use. Rosa26 LSL-FUS-CHOP/+ mice were generated by taking the human 7-2 FUS-CHOP translocation variant cDNA from the NCBI database and generating a targeting vector for the Rosa26 locus [6]. The design of the targeting vector with a neomycin cassette flanked by attB and attP sites with a floxed transcription/translation “STOP” cassette upstream of FUS-CHOP is described further in Section 3. R1 mouse embryonic stem cells (ESCs) were electroporated using standard techniques, and ESCs were selected with neomycin. ESC clones were tested for successful targeting of the vector into the Rosa26 locus and confirmed via PCR. Upon successful founder line generation, mice were crossed with Rosa26 Phic31+/+ mice (JAX stock no. 007670 [24]) to recombine the attB and attP sites and delete the Neo cassette. Then, the mice were crossed with wild-type 129/SvJ mice to generate Rosa26 LSL-FUS-CHOP/+ mice. These mice were crossed with the following cell-type-specific Cre and CreER lines: Meox2-Cre (JAX stock no. 003755 [25]), PdgfRα-Cre (JAX stock no. 013148 [26]), Prx1-Cre (JAX stock no. 005584 [27]), Prx1-CreER-GFP (JAX stock no. 029211 [28]), and aP2-CreER (a gift from the Yann Herault lab [29]).

2.2. In Vivo Cre Delivery or Activation. Mice lacking Cre expression were injected with an adenovirus expressing Cre, AdScMVCre (University of Iowa Viral Vector Core, VVC-U of Iowa-5), to activate recombination via Cre recombinase. Virus was prepared by mixing 25 μL of Ad-Cre with 600 μL minimal essential medium (Sigma-Aldrich, M4655). 3 μL of 2 M CaCl2 was added to each virus preparation, mixed, and incubated for 15 min at room temperature before injection of 50 μL into the gastrocnemius muscle. To activate CreER in a spatially restricted manner, a 5 mg/mL 4-hydroxytamoxifen (4-OHT, Sigma, H7904) solution in 25% ethanol/75% corn oil was administered via intramuscular injection. The solution was prepared by dissolving 4-OHT in ethanol and incubating at 60°C for 2 hours with shaking. Next, corn oil was added at a 3:1 corn oil to ethanol ratio, with continuous shaking at 65°C for 1 hour. Aliquots were stored at −80°C. 20 μL was injected into the gastrocnemius of experimental mice. All mice were anaesthetized with 2% isoflurane prior to any injection or procedure. All animal studies were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

2.3. Plasmid Constructs and Cloning. The pSECC vector was kindly provided by Tyler Jacks (Koch Institute at MIT) [30]. For cloning pSECC-sgp53, BbsI was used to digest pSECC and annealed sgp53 (Table S1) was cloned into the vector. sgp53 is a previously reported sgRNA sequence targeting exon 7 of p53 in mice [31, 32]. Sanger sequencing of the vectors was used with a sequencing primer targeting the U6 promoter in pSECC to confirm successful cloning.

2.4. Genotyping. Mice were genotyped using tails collected from mouse pups. Tail genomic DNA was extracted with a KAPA Mouse Genotyping Kit (KAPA Biosystems, KK7352), and PCR was performed using primers listed in Supplementary Table 1. PCR products were visualized after electrophoresis in 1% agarose gels.

2.5. Adenovirus Constructs and Generation. The Ad5-ΔE1ΔE3 vector backbone was constructed from the pX333 vector backbone [33]. For cloning pX333-FC, sgFus (Table S1) and sgChop (Table S1) were cloned into the pX333 vector. pX333-FC vector including the Cas9 transgene and the 2 sgRNAs was subcloned into the Ad5 adenoviral shuttle vector as described by Maddalo et al. [33] using XhoI and EcoRI sites to generate the adenoviral shuttle vector for adenovirus generation. All restriction enzymes and T4 ligase were purchased from New England Biolabs. Recombinant adenoviruses were generated by Virasport Inc. and validated in vivo prior to use in vivo.
2.6. In Vivo Electroporation. After mice were anesthetized, 50 μg of naked DNA plasmid diluted in sterile saline was injected into the gastrocnemius using a 31-gauge insulin syringe. A final DNA concentration of 1 μg/μL was used, and 50 μL was delivered in each injection. Electroporation was administered as previously described [32]. Briefly, a pair of needle electrodes with a 5 mm gap was inserted into the muscle to encompass the injection site, and electric pulses were delivered using a BTX Electro Square Porator ECM830 (BTX, San Diego, CA). Three 100 V pulses were administered to each injection site. The duration of each pulse was 200 μs, and the three pulses were given 50 μs apart.

2.7. Immunohistochemistry. Tumors were fixed in formalin overnight, transferred to 70% ethanol, paraffin-embedded, and sectioned to 5 μm thickness. Deparaffinized and rehydrated slides were blocked with 3% hydrogen peroxide. Antigen retrieval was performed by boiling in 2% citrate-based antigen unmasking solution (VECTOR H-3300) for 15 min. Slides were blocked with 5% normal serum in PBS + 0.25% Tween-20 and incubated with primary antibodies: 1: 500 mouse anti-CHOP (Cell Signaling #2895), 1: 50 rabbit anti-CD3 (Abcam ab28364), 1: 100 mouse anti-desmin (Agilent M076029-2), 1: 200 rabbit anti-vimentin (Abcam ab92547), 1: 1 rabbit anti-S100 (Dako GA504), and 1: 100 rabbit anti-cytokeratin (Abcam ab9377) diluted in PBS + 0.25% Tween-20 + 5% normal serum overnight at 4°C. Protein concentration was determined for the lysate supernatant by a bicinchoninic acid (BCA) assay (Pierce, 23225). Samples were boiled in 4X Laemmli sample buffer (Bio-Rad, 1610747) at 95°C for 5 min and then cooled to room temperature before loading in a 4–20% Tris-glycine polyacrylamide gel. Samples were electrophoresed at 200 V for 30 min before transfer to nitrocellulose. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS, Corning, 46-012-CM). Next, the membranes were incubated overnight at 4°C with primary antibodies diluted in TBS-T (0.1% Tween-20): FUS, 1: 1000 (Abcam, ab84078); CHOP, 1: 1000 (Cell Signaling Technology, 2895S); actin, 1: 10000 (BD Biosciences, 612656); p53, 1: 1000 (Cell Signaling Technology, 32532S); and p21, 1: 200 (Santa Cruz Biotechnology, sc-6246). The membranes were washed three times in TBS-T for 5 min before secondary antibody incubation with goat anti-rabbit IRDye800 (Li-Cor Biosciences, P/N 925-32211) and goat anti-mouse IRDye680 (Li-Cor Biosciences, P/N 925-68070) both at 1: 10,000 dilutions in TBS-T for 1 h at room temperature. The membranes were washed three times in TBS-T for 5 min and imaged using an Odyssey CLx (Li-Cor Biosciences). Image analysis for normalization and quantification was performed using Image Studio (version 5.2, Li-Cor Biosciences, P/N 9140-500).

2.9. Quantitative RT-PCR. Cells were lysed with TRIzol reagent (Thermo Fisher, 15960-026). RNA was isolated from samples using a Direct-zol RNA MiniPrep kit (Zymo Research, R2051). RNA samples were reverse transcribed to cDNA using iScript Advanced cDNA Synthesis Kit (Bio-Rad, 1725038). TaqMan probes from Thermo Fisher were used for PCR: Gapdh (MM99999915), Trp53 (Mm01731290), Cdkn1a (Mm00432448), Bbc3 (Mm0051926), Mdm2 (Mm01233136), and Bax (Mm00432051). Plates were run on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher), and data were analyzed using the comparative C_T method to generate expression fold-change values.

2.10. Radiation Treatments. Cells were cultured at least 24 hours prior to irradiation experiments. The X-RAD 160 (Precision X-ray) cell irradiation system was used at 160 kVp × 18 mA energy. An F1 filter (2 mm aluminum) was used for beam conditioning. Sample distance was set to 40 cm. For a dose of 10 Gy, calculations based on annual dosimetry determined a treatment duration of 230 seconds.

2.11. Tissue Culture and Cell Line Generation. NIH-3T3 cell lines were purchased from ATCC (CRL-1658) and cultured in DMEM (Thermo Fisher Scientific, 11965092) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, 16000044) and 1% antibiotic-antimycotic (Thermo Fisher Scientific, 10091148) and incubated at 37°C with 5% CO_2 in a humidified cell culture incubator. Tumor cells were isolated from primary mouse sarcomas and expanded in vitro using standard tissue culture methods. Briefly, tumor tissue was minced in the cell culture hood and digested by dissociation buffer in PBS (Thermo Fisher Scientific, 14040133) containing collagenase type IV (5 mg/ml, Thermo Fisher Scientific, 16000044) and 1% diacytomicin (Thermo Fisher Scientific, 10091148) and incubated at 37°C with 5% CO_2 in a humidified cell culture incubator. Tumor cells were isolated from primary mouse sarcomas and expanded in vitro using standard tissue culture methods. Briefly, tumor tissue was minced in the cell culture hood and digested by dissociation buffer in PBS (Thermo Fisher Scientific, 14040133) containing collagenase type IV (5 mg/ml, Thermo Fisher Scientific, 17104-019), dispase (1.3 mg/ml, Thermo Fisher Scientific, 17105-041) as well as trypsin (0.05%, Thermo Fisher Scientific, 25200056) for about 1 h at 37°C. Cells were washed with PBS (Thermo Fisher Scientific, 10010023) and filtered using a 40 mm sieve (Corning, 431750) and cultured for at least four passages to deplete stroma before being used for experiments.

2.12. Gene Editing and Validation. For in vitro editing, cells were transfected with plasmid DNA using TransIT-LT1 transfection reagent (Mirus Bio, MIR2300) as per the manufacturer’s instructions. Cells were harvested 48–72 hours later, and genomic DNA was extracted with a DNeasy Blood & Tissue Kit (Qiagen, 69504). For in vivo
2.13. Soft Agar Transformation Assay. 1.8% Bactoagar was made with dH2O and autoclaved. 2x and 1x DMEM were prepared using DMEM powder (Thermo Fisher Scientific, 12100046), fetal bovine serum (Thermo Fisher Scientific, 16000044), and antibiotic-antimycotic (Thermo Fisher Scientific, 10091148). 0.6% agar was made by diluting 1.8% agar with DMEM and kept in a 42°C water bath. 3 mL of 0.6% agar was poured per well of 6-well plates and allowed to solidify in a hood for 10 minutes before transferring to an incubator. Cells were trypsinized and counted using trypan blue solution (Thermo Fisher Scientific, 15250061). 250 µL of cells and 500 µL of 0.6% agar were mixed and gently pipetted onto the bottom agar in each well to create the 0.4% agar top layer. 5000 cells were plated per well in triplicate for each cell line. After plating, plates were placed in an incubator and allowed to grow for 3-4 weeks. Medium was supplemented each week by adding 0.5 mL of DMEM per well to prevent drying.

2.14. Detection and Analysis of Chromosomal Rearrangements. PCR and ddPCR amplification of mouse Fus-Chop was performed using primers spanning the translocation junction (Supplementary Table 1). AccuPrime Taq DNA Polymerase, High Fidelity (Thermo Fisher Scientific, 12346086), was used as per the manufacturer’s instructions for PCR. QX200 ddPCR EvaGreen Supermix (Bio-Rad, 1864034) was used for ddPCR as per the manufacturer’s instructions. PCR products were either used directly for gel electrophoresis or cloned into the pCR4-TOPO TA vector (Thermo Fisher Scientific, K457501) for Sanger sequencing. ddPCR samples were analyzed on a QX200™ AutoDG™ Droplet Digital™ PCR System.

3. Results

3.1. Design and Generation of Conditional FUS-CHOP Mice. To genetically engineer a mouse model with conditional expression of FUS-CHOP, we employed standard transgenic mouse methods starting with FUS-CHOP cDNA (Figure 1(a)) [6]. We used the 7-2 translocation variant of human FUS-CHOP, which has been reported to form tumors in mice when expressed in all tissues under an EF-1α promoter that is also used in this mouse model [18]. The 7-2 translocation variant, which was the first reported variant of the translocation to be discovered in MLPS, joins the first 7 exons of FUS in frame with exon 2 of CHOP via a short translated linker region originally part of the 5′UTR of CHOP. The FUS-CHOP cDNA was inserted into a vector targeting the mouse Rosa26 locus downstream of an LSL cassette—a transcriptional STOP cassette flanked by two loxP sites (floxed). The Rosa26 locus is widely expressed across cell types and tissues in mice. The LSL cassette enables temporally and spatially restricted activation of FUS-CHOP expression by Cre recombinase. A woodchuck hepatitis virus (WHV) posttranscriptional regulatory element with a pol-yadenylation signal (WPRE-pA) was placed downstream of the FUS-CHOP cDNA to enhance transgene expression. Finally, we included a Neo cassette flanked by attB and attP sites at the 3′ end of the targeting vector for positive selection of embryonic stem (ES) cell clones with neomycin.

After positive selection of ES cell clones, they were injected into blastocysts, which were subsequently transplanted into foster mothers. The chimeric pups that were born were genotyped to confirm the presence of FUS-CHOP cDNA in somatic genomic DNA (Figure 1(b)). Positive chimeras were crossed with wild-type 129/SvJ mice and genotyped to confirm germline transmission of the targeting vector (Figure 1(c)). Mice with confirmed germline transmission of the LSL-FUS-CHOP/+ allele became founders that were crossed with Rosa26 PhiCNeo/+ mice to delete the Neo cassette. Finally, these mice were crossed with wild-type 129/SvJ mice to cross out the PhiCNeo allele, which resulted in the experimental genetically engineered mouse model with the genotype Rosa26 LSL-FUS-CHOP/+ (LSL-FC) mice (Figure 1(a)). To confirm successful knock-in of the LSL-FUS-CHOP allele into the Rosa26 locus and document fidelity of the cDNA sequence, we primer walked and Sanger sequenced the Rosa26 locus in these mice (Supplementary Figure 1).

To restrict FUS-CHOP expression to specific mesenchymal and adipocyte precursor cell populations, we crossed Rosa26 LSL-FUS-CHOP/+ mice with various mouse lines expressing Cre recombinase (Table 1). These mice either expressed Cre recombinase in a tissue-specific manner, or ubiquitously starting in early embryogenesis. The selection of these Cre driver candidates was based on the expression of Cre at different stages of development and adipogenesis [34-36]. Merox2-Cre mice express Cre in the entire animal early in embryogenesis [25, 37]. PdgfRa-Cre mice express the recombinase at a later stage in early mesoderm development. Prx1 expression occurs later than PdgfRa expression during mesoderm development, but before commitment to the adipocyte lineage. Importantly, the Prx1-Cre mice restrict Cre activity mostly to white adipose tissue in both male and female mice [35]. Finally, aP2 (or Fabp4) is highly expressed in mature adipocytes, and undifferentiated cells have low levels of aP2 expression.

3.2. FUS-CHOP Expression in the Prx1 Lineage Drives Sarcomagenesis. Primary sarcomas developed in Rosa26 LSL-FUS-CHOP/+ mice when the mice expressed Cre in specific mesenchymal lineages during development (Figure 2 and Table 2). Rosa26 LSL-FUS-CHOP/+ mice crossed with Merox2-Cre and PdgfRa-Cre mice were embryonic lethal; Rosa26 LSL-FUS-CHOP/+ mice crossed with aP2-CreER mice did not form any tumors up to one year after delivery of either intramuscular 4-hydroxytamoxifen (IM 4-OHT) or intraperitoneal tamoxifen (IP Tam) to activate Cre in mature adipocytes (Table 2). However, tumors formed in Prx1-Cre; Rosa26 FUS-CHOP/+ mice. Prx1-
**Cre; Rosa26 FUS-CHOP/+** mice developed keratoacanthomas shortly after birth (Figure 2(a), Supplementary Figure 2). Some of these masses resolved on their own, and 36% of the mice developed sarcomas in the gastrocnemius or bone (Figure 2(b)). Histologically, these tumors were high-grade undifferentiated sarcomas with spindle cells (Figure 2(c)). Immunohistochemistry shows that these tumors express FUS-CHOP in nuclei (Figure 2(d)). In contrast, tumors from a mouse model of undifferentiated pleomorphic sarcoma in *LSL-KrasG12D; p53fl/fl* mice do not
express FUS-CHOP (Figure 2(e)) [38]. Therefore, Prrx1-expressing cells are permissive for initiation of sarcoma-genesis by FUS-CHOP.

Due to the formation of keratoacanthomas and to spatially restrict generation of tumors, we crossed Rosa26 LSL-FUS-CHOP/+ mice with Prrx1-CreER-GFP mice to allow site-specific tumor generation via activation of CreER by intramuscular 4-OHT. These mice formed tumors with similar penetrance (33.3%) compared to mice with Cre expressed from the Prrx1 promoter from birth (Table 2). However, the tumors we observed were not restricted to injection sites, possibly due to the leakiness of the CreER system. Further evidence of leakiness was the formation of tumors in noninjected Prrx1-CreER-GFP, Rosa26 LSL-FUS-CHOP/+ mice.

Intramuscular injection of an adenovirus expressing Cre recombinase (AdCre) into mice with conditional activation of oncogenic Kras\textsuperscript{G12D} and deletion of p53 (LSL-Kras\textsuperscript{G12D}; p53\textsuperscript{fl/fl}) generates primary soft tissue sarcomas with high penetrance as we previously reported [38]. Injection of AdCre into the gastrocnemius muscle of Rosa26 LSL-FUS-CHOP/+ mice to activate FUS-CHOP expression was not sufficient to form tumors (Table 2). Similarly, when two copies of FUS-CHOP were activated with p53 co-deletion, tumors also formed. Most importantly, because tumors did not form in Rosa26 LSL-FUS-CHOP/+ or Rosa26 LSL-FUS-CHOP/LSL-FUS-CHOP mice in the presence of intact p53, these data suggest that FUS-CHOP-driven sarcomas in mice are dependent on inactivation of p53 or the p53 pathway.

### 3.3. Molecular Characterization of FUS-CHOP-Driven Tu-mors Shows p53 Pathway Dependency.

To avoid keratoacanthomas and to temporally and spatially restrict tumor formation to develop a preclinical model to study FUS-CHOP-driven sarcomagenesis in vivo, we generated the Rosa26 FUS-CHOP; p53 (FCP) model, which delivers a plasmid (pSECC-sgp53) that contains Cre to activate expression of FUS-CHOP, and Cas9 and a p53 single guide RNA (sgRNA), sgP53, to create insertions/deletions (indels) in p53 (Figure 3) [30]. Delivering pSECC-sgp53, but not a control pSECC plasmid with a scrambled sgRNA (sgScr), into the gastrocnemius muscle via electroporation generates primary sarcomas with 100% penetrance in approximately 9–12 weeks (Figures 3(b) and 3(c), Table 2). CD31 staining of tumors revealed branching “crow’s feet” vasculature in this tumor model (Figure 3(f)). The “crow’s feet” vasculature is a pathognomonic feature of MLPS [2]. However, the tumors lack the myxoid stroma background found in human MLPS (Figure 3(d)). Rather, histologically, these tumors resemble high-grade sarcomas with high cellularity. To further characterize the FUS-CHOP-driven tumors, we performed IHC for markers expressed in different tissue lineages (Figure 3(g)).

### Table 1: Cre and CreER drivers of adipocytic lineage.

| Mouse line     | Cre or CreER driver | Purpose                      |
|----------------|---------------------|------------------------------|
| Meox2-Cre     | Embryonic/epiblast  | Whole animal expression      |
| PdgfRa-Cre    | Embryonic/mesoderm  | Early mesoderm-specific expres-
| Prrx1-Cre     | Mesoderm progenitor | Mesoderm-specific expression |
| Prrx1-CreER-GFP| Mesoderm progenitor | Mesoderm-specific expression |
| aP2-CreER     | Mature adipocyte    | Differentiated fat-specific expression |

Figure 2: Tumor generation in the adipocytic precursor lineage and immunohistochemical characterization of Prrx1-Cre; Rosa26 FUS-CHOP/+ tumors. (a) Gross image of keratoacanthomas that developed on the skin of the torso, hind limbs, and tails of mice. (b) Gross hind limb sarcomas were observed in mice after spontaneous resolution of keratoacanthomas. (c) Hematoxylin and eosin-stained section of soft tissue sarcoma. (d) FUS-CHOP IHC with CHOP antibody. (e) Section of primary mouse sarcoma from LSL-Kras\textsuperscript{G12D}; p53\textsuperscript{fl/fl} mice stained with CHOP antibody.
murine undifferentiated pleomorphic sarcomas were negative for cytokeratin and were intermixed with scattered dendritic type cells appearing as focal positive detection of S100. In contrast, the FUS-CHOP tumors were negative for cytokeratin and S100, which shows that these tumors likely do not originate from epithelial or neural crest lineages. Furthermore, because CD31 IHC (Figure 3(f)) was only positive in the vasculature and not in tumor cells, the tumors are unlikely to be derived from endothelial cells. However, both the KP and FUS-CHOP tumors were positive in the vasculature and not in tumor cells, the further investigation of the role of p53 in FUS-CHOP-driven sarcomas, we interrogated p53 signaling in cell lines derived from primary tumors with different p53 status (Figure 4(a)). For example, tumors 0592 and 1536 represent two tumors that formed without intentional deletion of p53. In contrast, tumors 1650, 2148, and 2149 were formed in the 3T3 positive control cell line, which has functional p53. We also observed high baseline p21 levels in one cell line derived from a FUS-CHOP-driven tumor (1650). This cell line did not express p53 protein, yet p21 activation was observed after irradiation through a p53-independent mechanism. Taken together, these data suggest that, in FUS-CHOP-driven tumors in mice, either p53 itself or its downstream signaling must be compromised for tumorigenesis. In the model initiated with CRISPR/Cas9 gene editing using a p53 sgRNA, p53 was inactivated at tumor initiation. In the other models in which Cre/loxP technology activates expression of FUS-CHOP without directly targeting p53, it appears that clonal selection drives p53 pathway inactivation during tumorigenesis in vivo.

3.4. Generation of Endogenous Translocations In Vitro. In addition to generating genetically engineered mouse models for the conditional expression of FUS-CHOP in a tissue-specific manner, we also devised a strategy to
generate endogenous Fus-Chop chromosomal rearrangements in vivo using CRISPR/Cas9 technology (Figure 5). This approach would enable the rapid, site-specific generation of autochthonous tumors in mice driven by the FUS-CHOP translocation expressed from the endogenous Fus locus with haploinsufficiency for FUS and CHOP. To generate the FUS-CHOP fusion, we modified the dual-guide plasmid system described by Maddalo et al. [33], hereafter referred to as pX333-FC, to deliver sgRNAs targeting Fus and Chop at intron 7 and intron 1, respectively (Figure 5(a)). To aid in selection and enrichment of cells harboring the translocation, we cloned versions of pX333-FC...
containing either a puromycin resistance marker or GFP to use in transfections.

NIH-3T3 cells were transfected with pX333-FC, and genomic DNA was assayed using Surveyor endonuclease to detect mutations and confirm Cas9 activity at the targeted loci (Figure 5(b)). At sites of Cas9-mediated double-stranded cleavage, indels form as a result of nonhomologous end-joining (NHEJ) repair. PCR for the amplicon of interest followed by repeated cycles of denaturing and annealing will generate amplicon heterodimers that have a mismatch. Surveyor endonuclease specifically cleaves DNA at sites of mismatch and will produce two fragments when run on a gel. Here, the presence of cleavage products demonstrates successful and specific CRISPR/Cas9 activity. Furthermore, the t(7;10) translocation was detected by PCR (Figure 5(c)) and confirmed with sequencing (Figure 5(d)). This experiment demonstrated that pX333-FC can be used to generate the Fus-Chop translocation in NIH-3T3 cells. We have successfully used this approach to generate the fusion in other cell lines derived from KP mouse sarcomas [38] (Supplementary Figure 3(a)).

| Tumor or cell line | Mouse background | Tumor generation |
|--------------------|------------------|------------------|
| 3T3                | N/A              | N/A              |
| 0592               | Rosa26 LSL-FC/+; Prx1 Cre | Cre             |
| 1536               | Rosa26 LSL-FC/+; Prx1 Cre | CreER           |
| 1650               | Rosa26 LSL-FC/LSL-FC | pSECC-p53      |
| 2148               | Rosa26 LSL-FC/LSL-FC | pSECC-p53      |
| 2149               | Rosa26 LSL-FC/LSL-FC | pSECC-p53      |
| 2150               | Rosa26 LSL-FC/LSL-FC | pSECC-p53      |
| 3T3-FC             | N/A              | N/A              |

Figure 4: p53 signaling and FUS-CHOP expression in FUS-CHOP-driven primary tumor cell lines. (a) Mouse background and tumor generation methods for primary tumors. (b) FUS-CHOP expression in primary mouse tumors and cell lines. (c) p53 and p21 expression 1 hour post-10 Gy irradiation in primary mouse tumors generated in different mouse backgrounds. 3T3 cells are mouse fusion negative controls, and 3T3-FC are mouse fusion positive controls for p53 signaling. (d) mRNA expression of p53 target genes 1 hour after treatment with 10 Gy ionizing radiation in NIH-3T3 and FUS-CHOP-driven tumor cell lines.
Using this approach, we generated a stable Fus-Chop-expressing NIH-3T3 cell line using CRISPR/Cas9 technology. First, we transfected the pX333-FC-GFP plasmid into NIH-3T3 cells and flow sorted single GFP-positive cells into 96-well plates. Colonies that formed from single cells were screened by PCR for the fusion transcript junction. Furthermore, western blotting using two different antibodies to Fus and Chop demonstrated successful costaining at approximately 70kDa, consistent with previously published data on the FUS-CHOP oncoprotein and predicted size (Figure 5(e)).

To assess the transformation capability of these cell lines, we performed transformation assays in soft agar. Only NIH-3T3 cells that were positive for FUS-CHOP after genome editing could form colonies in soft agar (Figure 5(f), Supplementary Figure 3(b)). These cells also formed tumors by 20 days after intramuscular injection into the hind limb of 3 of NCr nude mice (Supplementary Figure 3(c)). No tumors formed following allograft of control NIH-3T3 cells lacking FUS-CHOP for the duration of the allograft experiment up to 6 weeks.

3.5. CRISPR-Mediated Chromosomal Rearrangements In Vivo. Determining the efficiency of generating endogenous chromosomal rearrangements via CRISPR/Cas9 may have important implications for establishing an in vivo model using this approach because targeting a critical number of progenitor cells to induce the oncogenic translocation may be a technical obstacle for initiating a tumor in mice. To assess the efficiency of generating the endogenous Fus-Chop translocation in vitro, we used pX333-FC-GFP to generate KP sarcoma cell lines expressing endogenous Fus-Chop. We screened 229 single KP cells to isolate 9 single cell clones that were positive for the FUS-CHOP translocation (approximately 3.9% efficiency) (Supplementary Figure 3(a)).

We attempted to generate the Fus-Chop translocation at the endogenous locus in wild-type mice by using an adenovirus, AdFC, to deliver two sgRNAs with Cas9 to target the respective introns of Fus and Chop (Figure 5(a)). We cloned and validated AdFC to generate autochthonous tumors expressing Fus-Chop in wild-type mice. AdFC successfully generated the endogenous Fus-Chop translocation in vitro and in vivo, which was detected by translocation junction-specific PCR (Figure 6), but we never observed tumors in wild-type 129/SvJ mice injected with AdFC (Table 2). Because we were able to detect the Fus-Chop translocation in genomic DNA from the gastrocnemius muscles of mice injected with AdFC, this suggests that the t(7;10) translocation was successfully generated in muscle cells but was not sufficient for transformation. Because our
prior experiments suggested that p53 inactivation is important for FUS-CHOP-driven tumorigenesis in mice, we attempted to initiate tumors by generating a t(7;10) in p53fl/fl mice by co-administering AdCre, to delete p53, and AdFC, to engineer the translocation. None of the mice inoculated with these viruses developed tumors (Table 2). These data and the results from our transgenic mouse models suggest that translocation efficiency may have been too low in the in vivo setting to generate tumors. To more precisely measure the efficiency of generating CRISPR-mediated translocations, we used droplet digital PCR (ddPCR). We transduced NIH-3T3 cells in vitro at different multiplicities of infection (MOI) of AdFC virus and harvested genomic DNA for ddPCR quantification (Supplementary Figure 4). Translocation efficiency was confirmed to be approximately 3% in vitro (Figures 6(c) and 6(d)). We hypothesize that the efficiency may even be lower in vivo and is a bottleneck for generation of some cancers that require multiple genetic mutations for tumorigenesis, such as FUS-CHOP-driven tumors.

4. Discussion

Specific sarcoma subtypes harbor specific gene fusions, which likely drive sarcomagenesis in these tumors [39]. Chromosomal rearrangements have long been recognized as important for diagnosis and treatment of sarcomas, but the biological role of these gene fusions in sarcomagenesis is less well understood. Here, we generated and characterized multiple genetically engineered mouse models of FUS-CHOP-driven sarcoma to dissect the molecular events required for sarcomagenesis in vivo.

We successfully generated sarcomas in mice that are driven by FUS-CHOP, recapitulate the “crow’s feet” vasculature of human MLPS histologically, and resemble high-grade soft tissue sarcomas. These FUS-CHOP-driven tumors are primarily mesenchymal in origin and do not express markers of differentiation for epithelial, vascular, or neural crest tissues based on IHC (Figures 3(f)–3(g)). In mice, we find that alterations in the p53 pathway are required for FUS-CHOP-mediated tumorigenesis. Using in vivo electroporation with CRISPR/Cas9 technology, we also generated a spatially and temporally restricted FUS-CHOP-driven sarcoma model that forms with high penetrance. Because different sarcomas subtypes appear to arise from different mesenchymal cell lineages, we hypothesized that FUS-CHOP-driven sarcomagenesis may be tissue-specific.

To approach this question, we used a conditional gene expression strategy that allowed us to restrict FUS-CHOP oncogene expression to specific tissue lineages in vivo (Figure 1 and Table 1). Our mouse models showed that specific tissues and developmental stages are permissive for FUS-CHOP-driven tumor formation. Specifically, tissues expressing Prx1, a marker of early mesoderm and mesenchymal progenitor cells in several tissues [27], were permissive for FUS-CHOP-driven tumors. We discovered that early embryonic expression of FUS-CHOP in Meox2-Cre and PdgfRa-Cre mice was lethal. Furthermore, expression of FUS-CHOP in aP2-expressing cells, such as mature adipocytes, did not form tumors. Importantly, although our mouse models identify Prx1-expressing cells as a source of FUS-CHOP-driven tumors, cells expressing PdgfRa cannot be ruled out as a potential tumor-initiating cell for FUS-CHOP-driven tumors. In PdgfRa-Cre mice, all cells expressing PdgfRa in the early embryo express FUS-CHOP resulting in embryonic lethality, but adult mouse tissues that express PdgfRa may still be permissive for transformation by FUS-CHOP. Despite this caveat, we demonstrate that not all tissues are permissive for transformation by FUS-CHOP through experiments using three mouse models (Table 2). First, the aP2-CreER, Rosa26 FUS-CHOP/+ mouse

![Figure 6: Determination of endogenous chromosomal rearrangement efficiency in vitro and in vivo. (a) Schematic of in vitro and in vivo translocation generation via adenovirus. (b) In vitro and in vivo PCR validation of AdFC-generated t(7;10). (c) ddPCR quantification of translocation events in vitro. (d) ddPCR quantification of translocation efficiency in vitro.](image-url)
demonstrates that, in differentiated adipose tissue expressing aP2, FUS-CHOP is not sufficient to initiate tumorigenesis. Second, we show that, in Rosa26 LSL-FUS-CHOP/+ mice, activation of FUS-CHOP in gastrocnemius muscle via AdCre administration is also not sufficient for transformation. In human MLPS tumors, in addition to the formation of the FUS-CHOP translocation, one wild-type copy of both FUS and CHOP is lost and the stoichiometric ratio of FUS to CHOP to FUS-CHOP is 1:1:1. Thus, we used a third model of FUS-CHOP-driven sarcoma to ask if gene dosage was important for FUS-CHOP-driven transformation. Activation of two copies of FUS-CHOP in the gastrocnemius muscle of Rosa26 LSL-FUS-CHOP/LSL-FUS-CHOP mice via AdCre administration did not form tumors. Taken together, these models demonstrate that FUS-CHOP interferes with normal development when expressed early in embryogenesis and that FUS-CHOP expression alone in differentiated cells is not sufficient to initiate sarcomagenesis. Additionally, gene dosage cannot overcome genetic or epigenetic barriers that restrict the tissue-specific nature of FUS-CHOP-driven sarcomagenesis.

We conclude from our in vivo experiments that FUS-CHOP is not sufficient to drive tumorigenesis in all cell types and that the Prrx1 lineage is permissive for transformation. Next, we hypothesized that, in addition to tissue specificity, there may be other molecular determinants of FUS-CHOP-driven sarcomagenesis. p53 has been reported to be mutated in MLPS and is the most frequently mutated gene across all sarcomas [23, 40–42]. Overexpression of p53 correlates with reduced metastatic disease-free survival in localized MLPS [4], and models of MLPS have been generated on p53 null backgrounds [23, 43]. These studies suggest that p53 plays an important role in MLPS initiation and development. However, the earliest mouse models of MLPS did not require p53 deletion or inactivation, suggesting that inactivation of p53 is not required for sarcomagenesis [9, 44]. To clarify the role of p53 in FUS-CHOP-driven sarcomagenesis, we crossed Rosa26 LSL-FUS-CHOP/+ and Rosa26 LSL-FUS-CHOP/LSL-FUS-CHOP mice with p53fl/fl mice (Table 2). Administration of AdCre successfully generated tumors only in mice that had floxed p53 alleles and p53 deleted in tumors. Moreover, despite successfully modeling FUS-CHOP-driven sarcomas in the Prrx1 tissue lineage without targeting p53 for mutation, western blot analysis of primary tumor cell lines derived from these tumors revealed that p53 protein in these tumors was either absent or nonfunctional (Figure 4(c)). Induction of gene expression in several p53 downstream target genes after ionizing radiation was also absent in FUS-CHOP-driven tumor cell lines (Figure 4(d)). Thus, these data suggest that FUS-CHOP-driven sarcomagenesis in mice is p53 pathway-dependent and requires inactivation of p53. While it is well established that p53 is important for preventing tumorigenesis, conditional site-specific deletion or knockout of p53 is not sufficient for sarcomagenesis in mice [32, 38]. In contrast, the FUS-CHOP-driven sarcoma model achieves 100% penetrance in the Rosa26 LSL-FUS-CHOP/LSL-FUS-CHOP; p53fl/fl mice in 2-3 months. This difference indicates that FUS-CHOP expression contributes to the development of sarcomas in mice.

Genetically engineered mouse models expressing human transgenes are powerful tools for dissecting mechanisms of tumorigenesis. However, the regulation of an exogenous transgene may differ from genes expressed at the endogenous locus. To most accurately model FUS-CHOP-driven sarcomagenesis, we hypothesized that CRISPR/Cas9 technology could be applied to generate the t(7;10) translocation (equivalent to t(12;16) in humans) at the endogenous locus in mice. Advantages of this approach include expression of the gene fusion from its endogenous promoter at physiological levels under wild-type Fus promoter/enhancer regulation, and simultaneous editing of the genome to be haploinsufficient for wild-type Fus and wild-type Chop. We generated and validated an adenovirus, AdFC, to deliver Cas9 and sgRNAs for Fus and Chop to generate t(7;10) (Figure 6). While this approach was effective in vitro, no tumors formed in vivo (Figure 5, Table 2). Based on our prior findings, we also hypothesized that the lack of simultaneous p53 inactivation may have prevented tumor formation. To test this hypothesis, we co-delivered AdCre and AdFC into p53fl/fl mice, but we observed no tumors (Table 2). Therefore, we reasoned that low in vivo translocation efficiency may be a technical barrier for CRISPR-mediated tumorigenesis in this system. While CRISPR/Cas9 knockout mouse models require only one hit per gene, for translocation models, each cell that is edited only has two opportunities to form rearrangements before the PAM sites are destroyed and no further edits are possible. Additionally, NHEJ repair must produce a product that is in frame without a large deletion, which further decreases the efficiency of generating a functional fusion protein. ddPCR quantification of translocation events and in vivo detection of translocation suggest that although translocations are forming in mice, not enough cells that are permissive for FUS-CHOP-driven tumorigenesis are edited to form a tumor. Alternatively, though rearrangements are occurring in cells, these translocations are not occurring in a permissive cell type. Additional studies into the role of DNA topology and species-specific differences between humans and mice may further illuminate the biology of translocation-mediated sarcomagenesis.

In summary, we report the generation of multiple conditional mouse models of FUS-CHOP-driven sarcoma and show that Prrx1-expressing cells are a potential source of myxoid liposarcoma. We also show that differentiated fat cells are not permissive for transformation by FUS-CHOP and that p53 pathway inactivation is required for sarcomagenesis in mice. We demonstrate that CRISPR/Cas9 can be used to rapidly generate the FUS-CHOP translocation in vivo and in vitro, but translocation efficiency, developmental stage, and tissue specificity can be barriers to tumorigenesis. These genetically engineered mouse models and novel CRISPR tools will accelerate the study of FUS-CHOP translocation-driven sarcomagenesis and can serve as valuable preclinical models of FUS-CHOP-driven sarcomas to study the response to radiation therapy, chemotherapy, and novel therapeutics.
Data Availability
The data used to support the findings of this study are included within the article and supplementary information file(s).

Conflicts of Interest
All authors declare that there are no conflicts of interest regarding the publication of this paper. David G. Kirsch receives financial compensation from and serves on the scientific advisory board of Lumicell, Inc. He is also a co-founder of and receives stock from XRAD therapeutics. These interests are not directly related to this work.

Authors’ Contributions
M. C., A. V., and D. G. K. designed experiments. M. C., E. S. X., N. H. L., L. L., and Y. M. performed experiments. M. C. and E. S. X. performed in vivo electroporation. M. C. and N. L. performed immunoblots and tissue culture. M. C., E. S. X., and Y. M. performed histology, and D. M. C. analyzed H&E and IHC slides. M. C. and L. L. bred and maintained animals. A. V. and D. G. K. interpreted results and provided critical advice for the manuscript. M. C. and D. G. K. drafted the manuscript. All authors edited the manuscript.

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Supplementary Materials
Supplementary Table 1. Oligo sequences for all primers and sgRNAs used. Supplementary Figures 1–4. Sanger sequencing, histology, clone screening, transformation assays, and ddPCR raw counts. (Supplementary Materials)

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