2. Material and methods

2. 1. Mouse strains and housing of animals

H2K-*c-fos*-LTR mice (in the following designated as *c-fos* tg) were maintained in C57/Bl6-background. H2K-*fra-1*-LTR (in the following designated as *fra-1* tg), *c-jun*$^{fl/fl}$ and *junD*-KO mice were backcrossed at least 8 times to obtain mice in pure C57/Bl6 background. Nude mice were in NMRI-background.
Animals were bred in a SPF (specific pathogen free) facility of the Bundesinstitut für Risikobewertung (Berlin) and screened regularly for viral, bacterial, fungal and parasite contamination. For animal experiments, the mice were kept in the animal facility of the DRFZ in individually ventilated cages (IVC)-cages.
All animal experiments have been approved by the Landesamt für Gesundheit und Soziales.

2. 2. Plasmids

MSCV-Cre-GFP and MSCV-GFP retroviral constructs were a kind gift from Dr. William E. Paul (NIH, Maryland, USA). MSCV-Cre-Neo and MSCV-Neo retroviral vectors were kindly provided by Axel Behrens (Cancer Research UK, London, UK).

2. 3. Molecular biology and biochemistry

2. 3. 1. Isolation of genomic mouse DNA

A small piece of tail (~2mm) was cut and incubated overnight at 55°C in 750 µl tail preparation buffer (1% SDS, 50 mM Tris-HCl pH 8, 100 mM EDTA, 100 mM NaCl) containing 0.5 mg/ml Proteinase K (Roth). After complete digestion 250 µl of saturated NaCl was added and tubes were shaken vigorously for 5 min. Samples were centrifuged at full speed in a table centrifuge (Heraeus Biofuge) for 10 minutes,

the supernatant was transferred to a new tube and DNA was precipitated with 500 µl Isopropanol. DNA was harvested at full speed, supernatant was discarded and DNA dissolved in 500 µl Tris-EDTA (10 mM Tris, 1 mM EDTA).

2. 3. 2. Genotyping

The genotype of the mice was determined by PCR.

	junD	**c-jun**	**c-fos**	**fra-1**
Initial Denaturation	94 °C 5 minutes	94 °C 5 minutes	94 °C 5 minutes	94 °C 5 minutes
Denaturation	94 °C 30 seconds	94 °C 30 seconds	94 °C 30 seconds	94 °C 30 seconds
Annealing	58 °C 40 seconds	62 °C 40 seconds	52 °C 40 seconds	58 °C 40 seconds
Extension	72 °C 1 minute	72 °C 1 minute	72 °C 1 minute	72 °C 1 minute
No. of cycles	35	40	35	40
Product size	315 bp WT-band, 822 bp KO-band	380 bp WT-band, 440 bp fl-band, 580 bp	600 bp tg-band	1200 bp tg-band

Primer sequences:

c-fos: forward primer 5`agtctggcctgcgggtctct 3`, reverse primer 5`gtcggctggggaatggtagtagg 3`
fra-1: forward primer 5`cgatcaccaagaaccaatcag 3`, reverse primer 5`gggattaaatgcatgcctagct 3`
c-jun: forward primer (P1) 5`ctcataccagttcgcacaggcggc3`, reverse primer (P2) 5`ccgctagcactcacgttggtaggc 3`, reverse primer (P3) 5`cagggcgttgtgtcactgagct3`

junD: forward primer 5`tcgctcttggcaacagcggccgccaccagg 3`, reverse primer #1 5`ggccgctcagcgcctcctcgccatagaagg 3`, reverse primer #2 5`atcttccagataactgccgtcactccaacg 3`

PCR-reaction mix:

- 2.5 µl DMSO (Sigma)
- 2.5 µl 10 X PCR-buffer [670 mM Tris-Hcl, pH 8.8; 160 mM NH_4SO_4; 0.1 % Tween 20]
- 2.5 µl forward primer [10 pmol/µl]
- 2.5 µl reverse primer [10 pmol/µl]
- 1.5 µl $MgCl_2$ [25 mM]
- 0.5 µl dNTP [10 mM]
- 0.2 µl Taq-polymerase [5 U/µl]
- 1 µl DNA (150-400 ng)
- To 25 µl

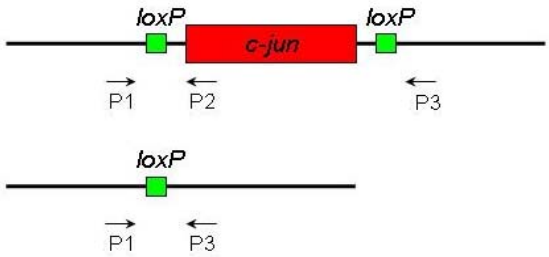

Figure 2.01. Strategy for genotyping of *c-jun*^{wt}, *c-jun*^{fl} and *c-jun*$^{\Delta}$ alleles. Primer 1 and 2 amplify the *c-jun*^{wt} allele and the wild type allele harboring the loxP sites which result in a bigger amplicon. In case of Cre-mediated recombination, the downstream part of *c-jun* where primer 3 binds is brought in vicinity of primer P1 and thereby a new amplicon is generated. Since binding site for P2 vanish after recombination, the floxed allele should not be amplified any more.

2. 3. 3. RNA isolation and cDNA synthesis

RNA from cell cultures was isolated with Trizol reagent (Invitrogen) according to the manufacturer`s recommendation. To obtain RNA from mouse soft tissues, tissue samples were dissected and immediately transferred into a 15 ml conical plastic tube

(Greiner) filled with 3 ml of TRizol and subsequently treated with an ultratorax. The following steps were performed according to the protocol of the manufacturer.
RNA from mouse bone was isolated by dissecting the bone and cleaning it from other tissues like muscles and tendons. The bone was crushed in a pre-cooled mortar by adding liquid nitrogen. The bone powder was then transferred to 3 ml of TRizol and processed further as outlined above.
Prior cDNA synthesis, RNA samples were treated with DNaseI (Fermentas) to exclude any DNA contamination. 1 µg of RNA sample was digested with 1 unit of DNaseI in a total volume of 10 µl at 37 °C for 30 minutes. DNaseI was deactivated by adding 1 µl of 25 mM EDTA and incubating at 65 °C for 10 minutes.
cDNA synthesis was performed with the reverse transcription kit from Roche as follow:

- 4 µl RNA (100 ng/µl)
- 2 µl TaqMan 10 X buffer
- 4.4 µl MgCl2 [25 mM]
- 4 µl dNTP [10 mM]
- 0.5 µl random hexamer [50 µM]
- 0.5 µl oligo dT [50 µM]
- 0.5 µl RNase inhibitor [20 U/µl]
- 0.5 µl reverse transcriptase [50 U/µl]
- 3.6 µl H_2O

The samples were incubated for 10 minutes at 25 °C, followed by 40 minutes at 48°C and a final step at 95°C for 5 minutes.

2. 3. 4. Conventional RT-PCR and Light Cycler PCR

Amplification of JunD and c-Jun by conventional RT-PCR:

- 2.5 µl 10 X PCR-buffer [670 mM Tris-Hcl, pH 8.8; 160 mM NH_4SO_4; 0.1 % Tween 20]
- 2.5 µl forward/reverse primer [10 pmol/µl]

- 1.5 µl MgCl2 [25mM]
- 0.5 µl dNTP [10 mM]
- 0.2 µl Taq Polymerase [5 U/µl]
- 1 µl cDNA
- To 25 µl

PCR-steps for conventional PCR (T3 Thermocycler, Biometra):

Initial denaturation: 2 minutes at 94°C
Denaturation: 30 seconds at 94°C
Annealing: 40 seconds at 58°C
Elongation: 1 minute at 72°C
Number of cycles: 35

Light Cycler analysis was performed with the Light Cycler 480 SYBR Green Kit from Roche according manufacturer`s recommendation on a Roche Light Cycler. Annealing temperature was for each primer 64°C and primer concentration was 5 pmol/reaction.
The following program was used for each primer pair:

	Temperature [°C]	**Time [sec]**
Initial denaturation	95	600
denaturation	95	10
annealing	64	10
extension	72	10

Specificity of the generated products was approved by melting curve analysis and agarose gel electrophoresis. Samples were run in duplicate and relative quantification was assessed by the formula:

$$mRNA\,ratio = \frac{2^{(cp_{reference\ gene})}}{2^{(cp_{target\ gene})}}$$

Cp: crossing point

The following oligonucleotides were used for conventional and semi quantitative RT-PCR:

c-Fos : forward 5`cgggtttcaacgccgactac, reverse 5` caggtctgggctggtggaga 206 bp
Fra-1: forward 5`gagacgcgagcggaacaag, reverse 5`cttccagcaccagctcaagg 167 bp
JunB: forward 5`tcacgacgactcttacgcag, reverse 5`ccttgagaccccgataggga
c-jun: forward 5`actcggaccttctcacgtc, reverse 5`cggtgtagtggtgatgtgcc 107 bp
junD: forward 5`catcgacatggacacgcaag, reverse 5`cggtgttctggcttttgagg 150 bp
c-fms: forward 5`ccggcccactcttggaattt, reverse 5`agaccgttttgcgtaagacct 151 bp
M-csf: forward 5`ctagtgagggatttttgacccag, reverse 5`tggtcctctgttgatttaggga 130 bp
RANK: forward 5`gcccagtctcatcgttctgc, reverse 5`gcaagcatcattgacccaattc 130 bp
RANKL: forward 5`accagcatcaaaatcccaag, reverse 5`tttgaaagccccaaagtacg 204 bp
OPG: forward 5`gctgctgaagctgtggaaac, ctctccatcaaggcacgaag 147 bp
Traf6: forward 5` aaagcgagagattctttccctg, reverse 5`actggggacaattcactagagc 125 bp
NFATc1: forward 5`gacccggagttcgacttcg, reverse 5`tgacactaggggacacataactg 97 bp
CathepsinK: forward 5`ggccagtgtggttcctgtt, reverse 5`cagtggtcatatagccgcctc 154 bp
TRAP: forward 5`cactcccaccctgagatttgt, reverse 5`catcgtctgcacggttctg 118 bp
mGlap: forward 5`aggcagactcacaggacacc, reverse 5`cgaaactccacaaccaaatg
Col X: forward 5`ggacagcagggacttacagg, reverse 5`ggaaagccgttttcacctctt 209 bp
RUNX2: forward 5`tgttctctgatcgcctcagtg, reverse 5`cctgggatctgtaatctgactct 146 bp
Collagen Iά1: forward 5`ctggcggttcaggtccaat, reverse 5`ttccaggcaatccacgagc 141 bp
sFRP1: forward 5`tggcccgagatgctcaaatg, reverse 5`ggttgtaccttggggcttaga 102 bp
PHEX: forward 5`attgctgataatgggggtct, reverse 5`tctctggcagcttctggtct 179 bp
SOST: forward 5`agccttcaggaatgatgccac, reverse 5`ctttggcgtcatagggatggt 134 bp
vegfά: forward 5`atcttcaagccgtcctgtgt, reverse 5`tatgtgctggctttggtgag 138 bp
vegfδ: forward 5`tcacgctcagcatcccatc, reverse 5`acttctacgcatgtctctctagg 125 bp
p15: forward 5`ccctgccacccttaccaga, reverse 5`cagatacctcgcaatgtcacg 169 bp

Dec1: forward 5`tacaagctggtgatttgtcgg, reverse 5`ctgggaagatttcaggtcccg 137 bp
c-Fos transgene: tgtgttcctggcaatagcgtgt, reverse 5`ggcaattccgcccatagtga 114 bp
cyclinD1: forward 5`cagaggcggatgagaacaag, reverse 5`gttgtgcggtagcaggagag 184
p53: forward 5`gccatggccatctacaagaa, reverse 5`tttccttccacccggataag 125 bp
p21: forward 5`cagtacttcctctgccctgct, reverse 5`gttttcggccctgagatgtt 131 bp
p27: forward 5`tctcttcggcccggtcaat, reverse 5`ggggcttatgattctgaaagtcg 116 bp
P8: forward 5`cccttcccagcaacctctaaa, reverse 5`tcttggtccgacctttccga 116 bp
HPRT: forward 5`gttaagcagtacagccccaaa, reverse 5`agggcatatccaacaacaaactt 131 bp

2. 3. 5. Protein extraction and Western Blot

Confluent cells (10 cm diameter dish) were washed twice with cold PBS and incubated with 500 µl buffer A for 10 minutes at room temperature. Cells were scraped with a cell lifter (Costar), the lysate was transfered to a pre-chilled microcentrifuge tube (Eppendorf) and centrifuged at top speed for 3 minutes. The supernatant (cytosolic fraction) was stored at -20 °C and the pellet was resuspended in 150 µl buffer B by pipeting up and down. Samples were then shaken vigorously at 4°C for 2 hours and afterwards centrifuged at 4°C at top speed for 5 minutes. Supernatant (nuclear fraction) was transferred to a new pre-chilled tube and stored at -20°C.

Buffer A:
10 mM HEPES, pH 7.9
10 mM KCl
0.1 mM EDTA
0.4 % IGEPAL
1 X Protease Inhibitor Cocktail (Sigma)

Buffer B:
20 mM HEPES, pH 7.9
0.4 M NaCl
1 mM EDTA
10 % Glycerol

1 X Protease Inhibitor Cocktail

Protein concentration was determined with the Pierce Micro BCA Protein Kit based on the method described by Bradford.
10 % SDS-PAGE were run at 130 V. Protein transfer was performed with a semi-dry transfer chamber (Novablot) at 100 mA for 1 hour on nitrocellulose. Blots were blocked with 7 % dry milk powder in TBS/Tween (Promega) for 1 hour at room temperature. Prior first antibody incubation, blots were washed in TBS/Tween for 5 minutes. Primary antibodies were incubated at 4°C overnight. Blots were washed 3 times with TBS/Tween and incubated with secondary antibody for 45 minutes at room temperature. After 4 washing steps, blots were incubated with ECL-detection reagents (Amersham Pharmacia) and visualized on Kodak X-Omat films.

2. 3. 6. Antibodies:

Polyclonal c-fos (clone sc-52, Santa Cruz Biotechnology, Santa Cruz, USA): 1:500
Polyclonal JunD (clone sc-74, Santa Cruz Biotechnology, Santa Cruz, USA): 1:500
Monoclonal c-jun (clone 60A8, Cell Signaling, Beverly, USA): 1:1000
Monoclonal Rsk2 (clone sc-9986, Santa Cruz Biotechnology, Santa Cruz, USA): 1:400
Monoclonal β-actin (clone AC-15, Sigma, Saint Louis, USA): 1:5000
Anti mouse (Promega, Madison, USA): 1:20000
Anti rabbit (Promega, Madison, USA): 1:20000

2. 4. Cell biology

2. 4. 1. Isolation of primary mesenchymal cells from mouse calvariae and osteoblast differentiation

Calvariae of 3-6 days old pups were cut out, cleaned in PBS with Penicillin/Streptomycin and subsequently digested in α-MEM media (PAN Biotech GmbH) containing 0.1 % collagenase I (Sigma) and 0.2 % Dispase II (Roche) (digestion media) for 10 minutes at 37°C. This first fraction was discarded and fresh

digestion media was added, incubated for 10 minutes at 37°C and this time the liquid phase was recovered. This step was repeated 3 times. The collected liquid phase was centrifuged and cell pellet was resuspended in α-MEM media supplemented with 10 % FCS (Sigma), Penicillin/Streptomycin (PAA) and Glutamine (PAA) (complete media).

Cells were grown until subconfluency and amplified by splitting the cells into 5 new dishes. The cells were counted with a CASY-cellcounter and plated at a density of 10^4 cells/cm^2. When the cells reached confluence the media was supplemented with 100 µg/ml ascorbic acid (Sigma) and 5 mM β-glycerophosphate (Fluka) to induce osteoblast differentiation.

Osteoblast differentiation was assessed by alkaline phosphatase staining kit (Sigma) according to the manufacturer`s recommendation around day 10. After 21 days, mature osteoblasts were fixed in 3.7 % Formaldehyde for 5 minutes and stained with alizarine red (Sigma).

2. 4. 2. Isolation of primary tumor cells from osteosarcomas

Tumorigenic tissues of bones were cut with a scalpel and cleaned in PBS with Penicillin/Streptomycin. Tumors were then transferred to a conical 50 ml plastic tube (Greiner) and minced in complete α-MEM with a scissor. The tumor pieces were centrifuged and plated on 6-well dishes (Greiner). When the outgrowing cells reached confluency, the cells were amplified by splitting in a ratio of 1 to 5.

2. 4. 3. Preparation of viral supernatants with Phoenix cells and viral infection

Phoenix cells were seeded at 10^6 cells/6-well in 2 ml complete DMEM (PAN Biotech GmbH) media. For transfection, 5 µg of plasmid DNA was pipeted in a 96-well (Greiner) with 100 µl of DMEM media (without serum and antibiotics). In another tube 15 µl of transfection reagent (Metafectene) was added to 100 µl of DMEM media (without serum and antibiotics). The two mixtures were united, incubated for 20 minutes at room temperature and afterwards put gently on the Phoenix cells. After 12-18 h, the media of the Phoenix cells was renewed. After additional 24 h the

supernatant was collected and filtered through a 0.45 µm filter device (Millipore) to get rid of any cell debris. HEPES (Gibco) (final concentration 20 mM) was added and the supernatant was stored at -80°C.

For viral infection 3 X 10^5 cells were plated on 10 cm diameter dishes (Greiner) one day before infection. Prior infection media of the target cells was reduced to 3 ml. The viral supernatant (~2 ml) was pipetted to the cells and 5 µl of Polybrene (Sigma) [8mg/ml] was added. After 18 h the viral supernatant was replaced by fresh α-MEM media. Depending on selection marker, 48 h after infection the cells were selected by culturing the cells in ά-MEM with 10 % FCS and G418 (Gibco) [400 µg/ml] or by sorting with FACS (FACS-Diva, Becton Dickinson).

2. 4. 4. FACS analysis and sorting

For proliferation analysis by means of FACS, cells were harvested and washed twice in PBS. 3 X 10^6 cells were incubated with 2.5 µM CFSE (Molecular Probes) in a total volume of 2 ml PBS for 10 minutes at 37°C. Staining reaction was stopped by adding 10 ml of complete media and incubation at 37 °C for 10 minutes. The cells were washed twice with complete media and plated at 5 X 10^5 cells/10 cm diameter dish. 24 h after staining of the cells, CFSE fluorescence which is detected in the FL-1 channel was acquired and defined as day 0 of the proliferation experiment. Prior each FACS-analysis (FACS-Calibur, Becton Dickinson and FCS Express 2.0, De Novo software) cells were incubated with Propidiumiodide (Sigma) to discriminate dead cells, which were gated and excluded from further analysis. After 3 days CFSE fluorescence was acquired again and proliferation rate was calculated according:

$$division/day = \frac{\left(\frac{(\log(GM\ day\ 0) - \log(GM\ day\ n))}{(\log(2))}\right)}{(n\ days)}$$

GM: geometric mean

To determine the cell cycle profile, cells were plated at a density of 5 X 10^5 cells/10 cm diameter dish and synchronised by serum starvation (growth in 0.5 % FCS α-MEM media) for 48h. Cell cycle was induced by replacing starvation media with complete media. 24 h later cells were harvested, washed in PBS and fixed with 70 % ethanol for 30 minutes. The cells were centrifuged, resuspended in 500 µl PBS and incubated with RNaseA (Fermentas) (100 µg/ml) to ensure only DNA is stained. 50 µl of propidiumiodid (20µg/ml) was used to stain DNA.

FACS sorting of infected cells was performed at the FACS core facility of the DRFZ with a FACS-DIVA sorter. GFP-positive cells with strong signals in the FL-1 channel were collected and plated on 10 cm dishes. 1 day after cell sorting, the cells were counted and plated for experiments.

2. 4. 5. TUNEL assay

For TUNEL assay (Roche) cells were grown on round coverslips (12 mm diameter). TUNEL assay was conducted according the manufacturer's protocol.

2. 5. Histology

2. 5. 1. Paraffin sections and immunofluorescence

Bone and osteosarcoma tissues were fixed in 3.7 % formaldehyde/PBS overnight at 4°C. After fixation, the samples were decalcified with 5 % EDTA (pH 7.25) for 10 days at room temperature. Decalcified sampled were dehydrated according standard protocols in increasing alcohol gradients and Xylol and finally embedded in paraffin. Samples were cut with a microtome (Leica) to obtain 4 µm thick sections.
For immunostaining, sections were rehydrated in decreasing alcohol gradients. Epitopes were unmasked by boiling the samples in citrate buffer (10 mM, pH 6.0) for 10 minutes. After cooling down, samples were blocked in PBS/0.1 % Triton X 100 (Sigma) +10 % FCS for 1 h at room temperature. Sections were washed in PBS and

incubated with primary antibody (1:50) in PBS/0.1 % Triton X 100 + 1 % FCS for 1 h at room temperature. After 3 times washing in PBS, secondary antibody (Rhodamine Red-X goat anti-rabbit, Molecular Probes, Eugene, USA; 1:500) in PBS/1 % Triton X 100 + 1% FCS was added and incubated for 45 minutes at room temperature. Sections were washed 3 times with PBS and sealed with mounting media and coverslips.

2. 5. 2. Bone Histomorphometry

For bone histomorphometry non-decalcified osteosarcoma, vertebral bodies and tibia were fixed overnight at 4°C in 3.7 % formaldehyde. Samples were dehydrated as mentioned above and subsequently treated with infiltration solution. Acrylat-polymerization was performed overnight at 4°C. Sections of 3-12 µM thickness were obtained by using a microtome.
Prior staining, sections were incubated 3 X 10 minutes with 2-(Methoxyethy)-acetate (Sigma) and afterwards rehydrated.
Von Kossa staining was performed according the following protocol:

- 5 minutes in silvernitrate (3%)
- 10 minutes in deionized water
- 5 minutes in sodaformol
- 10 minutes in tap water
- 5 minutes in sodiumthiosulfate (5%)
- 10 minutes in tap water
- 20 minutes in van Gieson solution
- Dehydration in alcohol gradients with final step in Xylol
- Mounting in DPX (Sigma)

Bone mineral density and trabecular thickness were quantified according standardized protocols with the OsteoMeasure histomorphometry system (OSTEOMETRICS Inc.).

2. 6. Animal experiments

2. 6. 1. Tumor cell transplantation

4-6 weeks old male nude mice were anaesthetized with Ketamin (Delta Select) (80 mg/kg body weight) and Xylazin (Bayer) (16 mg/kg body weight) intraperitoneally. 2 X 10^6 cells were injected subcutaneously in both sides of the back. Developing tumors were monitored weekly and mice were sacrificed 4 weeks after transplantation for determining tumor size.

2. 6. 2. Ovariectomy:

Female mice were anaesthetized as described above. A small incision at the back of the animals was conducted to get access to the ovaries. Ovaries were cut off and wound was closed with a hot fine forceps. Animals were clipped and kept warm. 8 weeks after surgery, animals were sacrificed and analyzed.