4 Material and methods

4.1 Material

4.1.1 Chemicals

All chemicals, if not noted otherwise, were purchased from the companies Merck, Sigma or Roth.

- Acetic anhydride (Sigma)
- Agar (Difco)
- Agarose (Gibco BRL)
- Betaine (N,N,N –trimethylglycine) (Sigma)
- CTP, GTP, ATP, UTP (Roche)
- Culture grids (Wire Mesh Corporation, USA)
- Dextran Sulfat (Sigma)
- DNAse (deoxyribonuclease), RNAse free (Roche)
- dNTPs (2'-Deoxynucleoside 5'-triphosphates) (Roche)
- DPX Mountant for histology (Fluka)- DPX Mountant for histology (Fluka)
- Ethidiumbromide (Serva, Heidelberg)
- Fatfree milk (Instant-Magermilchpulver) (Neuform, Germany)
- Glass coverslips (Menzel-Glaeser, Germany)
- Glutaraldehyde (Sigma)
- Glycogen (Roche)
- Heparinase III (Sigma)
- Ficoll 400 (Pharmacia)
- Paraffin-paraplasts (Sherwood Medical Co, USA)
- PEG 4000: polyethylenglycol (MBI Fermentas)
- PFA (Paraformaldehyde) (Merck)
- Proteinase K (Roche)
- [P_{33}]-UTP (radioactive Uridintriphosphat) (Amersham, GB)
- Random primer: pd(N)6 Random Hexamer 5'-Phosphate (Amersham)
- Restriction endonucleases (NEB, Roche)
- Ribonuclease A, RNAse A (Roche)

- RNAse inhibitor (Roche)
- Superfrost plus slides (Menzel-Glaeser, Germany)
- Taq-DNA polymerase (Eppendorf)
- TEA, triethanolamine (Merck)
- Tissue culture dish 60x15 mm (Falcon)
- Toluidine Blue O (Sigma)
- Tryptone (Difco)
- T7, T3, SP6 RNA-polymerase (Roche)
- T4 DNA Ligase 400U/µl (New England Biolabs)
- Yeast extract (Difco)
- (3-aminopropyl)triethoxysilane (Sigma)

4.1.2 Buffers and Solutions

- Alcian blue solution: 15mg alcian blue; 80 ml 100% ethanol; 20 ml 100% acetic acid
- Denhardt's reagent (50x): 50g Ficoll; 5g polyvinylpyrrolidone; 5g BSA; H_2O to 500 ml - -
- EB: 10 mM Tris-HCl, pH 8,5
- Gel-louding-buffer (10x): 15% Ficoll (Type 400); 0,25% Bromphenolblue; 6 mM EDTA, pH 8.0
- Heparinase III digestion buffer: 50 mM Tris HCl, pH7,2; 100 mM NaCl; 1 mM CaCl; 0,1% TritonX100; 5 µg/ml BSA
- Hybridization buffer: 50% Formamid; 3 M NaCl; 20 mM Tris-HCl; pH 7.4; 5 mM EDTA; 10 mM NaH_2PO_4-H_2O; pH 8.0; 10% Dextran Sulfat (w/v); 1x Denhardt's; 0,5 mg/ml Yeast RNA total
- Kodak-developer D-19 (Kodak, USA)
- Kodak-fixer (Kodak, USA)
- Kodak Scientific Imaging Film (Kodak, USA)
- LB medium (Luria Bertrani): 1% tryptone; 0,5% yeast extract; 1% NaCl, pH 7.0
- LB agar: 15g agar per 1L LB-medium
- Ligase-Buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0,5 mM ATP, pH 7,8 (New England Bio labs).
- Limb culture medium: BGJ-b medium (Gibco BRL); 0.1% BSA (Bovine Serum Albumin) (Gibco BRL); 1% antibiotic-antimycotic (Gibco BRL)

- Lysis-buffer (50 mM Tris-HCl, pH 8,0; 100 mM EDTA; 1% SDS; 100 mM NaCl; 20 mg/ml Proteinase K)
- Paraformaldehyde 4% (w/v) in PBS
- PBS (Phosphat Buffered Saline): 1.5 mM KH_2PO_4; 140 mM NaCl; 3 mM Kcl; pH 7.4
- Photoemulsion (autoradiography emulsion type NTB2) (Kodak, USA)
- Proteinase K: 10mg/ml resuspended in 50 mM Tris-HCl, pH 8,0; 1 mM $CaCl_2$,
- RNAse free water (DEPC water) 0,1% DEPC (Diethylpyrocarbonat)
- Scott's buffer (10g $MgSO_4$, 2g Natriumbicarbonate in 1000 ml H_2O)
- SOC medium: 2% tryptone; 0,5% yeast extract; 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose
- Solution A: 25 mM EDTA, pH 8.0; 75 mM NaCl
- Solution B: 10 mM EDTA pH 8.0; 10 mM Tris-HCl, pH 8.0; 400 µg/ml Proteinase K; 1% (w/v) SDS
- SSC (standard saline citrate, 20x): 300 mM Sodiumcitrate; 3 M NaCl; pH 7.0
- TAE-buffer (50x): 2 M Tris, pH 7,5 - 8,0; 50 mM EDTA
- TBE-buffer (50x):
- TE: 10 mM Tris-HCl, pH 8,0; 1 mM EDTA, pH 8,0
- TN: 0.1M Tris-HCl, pH 7.5; 0.15 M NaCl
- TNB = TN + 0.5% NEN blocking reagent
- TNT = TN + 0.025%-0.05% Tween-20
- Transcription buffer (10x): 0.4M Tris-HCl, pH 8,0; 60 mM $MgCl_2$; 100 mM dithiothreitol, 20 mM spermidine (Roche)
- Trizol reagent (Gibco BRL)
- Washing buffer (10x): 4 M NaCl; 0.1M Tris-HCl; 0,05 M EDTA; pH 7.5

4.1.3 Kits

- Plasmid Midiprep kit (Qiagen)
- QIAprep Plasmid Miniprep kit (Qiagen)
- QIAquick PCR purification kit (Qiagen)
- pCR-2.1-TOPO cloning kit (Invitrogen)
- BrdU labeling and detection kit II (Roche, Germany)
- DAB-staining kit (Pierce)
- RT-PCR-Kit: Ready-To-Go You-Prime First-Strand Beads (Amersham)

- SYBR Green I PCR Master Mix (Applied Biosystems)
- DNA Purification kit (Biozym)
- TSA Biotin System kit (NEN Life Science Products)
- ImmunoPure Metal Enhanced DAB Substrate kit (Pierce, Rockford, IL)

4.1.4 Proteins and antagonists

- ShhAb80, kindly provided by Andrew P. McMahon (Yang et al., 1997)
- 3G10-biotinylated HS antibody (David et al., 1992) (Seikagaku Corporation)
- Fibroblast growth factor, Fgf2 (bFgf), (Sigma)
- Alkaloid cyclopamine, kindly provided by William Gaffield (Incardona et al., 1998)
- Transforming growth factor ß1, Tgfß1, human (Sigma)

Table 1: Protein names and their synonymes

Genetic nomenclature of the protein	Synonyms used in literature
Fgf2, fibroblast growth factor 2	bFgf/ Fgf2/ Fgfb
Pthlh, parathyroid hormone-like hormone	PTH-like; parathyroid hormone-related protein (PthrP); parathyroid hormone-related peptide; PTH-related peptide
Pthr1, parathyroid hormone receptor	PPR/PTH-related peptide receptor; PTH/PTHrPR
Bglap1, bone Gla protein	Osteocalcin mOC-A/ OG1

4.1.5 Mouse and rat DNA probes for in situ transcription

Probe, Vector, Insert size (b.p.), DNA Endonuclease (for antisense), RNA polymerase (for antisense), Reference

rCol II, pGEM-3Z, 550 bp, *Hind* III, T7, (Kohno et al., 1984)

mPthlh, pCR2.1TOPO, 423 bp, *HindIII*, T7, (Koziel et al., 2004)

rPthr1, pcDNAI, 500 bp, *Bam*H I, SP6, (Abou-Samra et al., 1994)

mCol X, pBSK+, 400 bp, *Cla* I, T3, (Jacenko et al., 1993)

mBmp7, pBSK-, 900 bp, *Hind* III, T3, (Bitgood and McMahon, 1995)

Mmp13, pCR-TOPO21, 1000 bp, *Hind* III, T7, (Yamagiwa et al., 1999)

Bglap1, pBSK, 500 bp, *Xba* I, T3, (Celeste et al., 1986)

mIhh, pBSK, 1800 bp, *Xba* I, T7, (Bitgood and McMahon, 1995)

mPtch, pBSK, 5000 bp, *BglII*, T7, (Goodrich et al., 1996)

mRunx2, pCR2.1TOPO, 640 bp, *SpeI*, T7, (Stricker et al., 2002)

mGli1, pBSK, 1600 bp, *NotI*, T3, (Hui et al., 1994)

mGli2, pCR2.1TOPO, 4761 - 5498 bp (737 bp), *SpeI*, T7

mGli3, pCR2.1TOPO, 40 – 891 bp (851 bp), *SpeI*, T7

mFgfr1, pBSK, 420 bp, *SacI*, T7 (Minina et al submitted)

4.1.6 Mouse lines

Ext1$^{Gt/Gt}$ mice (official designation, *Ext1Gt*(*pGT2TMpfs*) *064Wcs*) were provided by Olivia G. Kelly and William C. Skarnes (Mitchell et al., 2001). *Ihh* deficient mice, *Col-II-Gal4* and *UAS-Ihh* transgenic mice were kindly provided by Andrew P. McMahon (Long et al., 2001; St-Jacques et al., 1999). *Fgfr3*$^{Ach/+}$ mice were kindly provided by D. Ornitz (Naski et al., 1998). *Gli3-XtJ* mice (herein named as *Gli3*-mice) have a deletion of a 3' *Gli3* sequence, resulting in a null allele. These mice were provided by Ulrich Rüther (Hui and Joyner, 1993). Wild-type mice (NMRI) were derived from Charles River (Sulzfeld, Germany). Transgenic mice were identified by PCR of tail DNA.

4.1.7 Bacterial strain

Escherichia coli DH5a™. Genotype : F-f80dlacZDM15 D(lacZYAargF)U169 deoR recA1 endA1 hsdR17(r_{k-}, m_{k+}) phoA supE44 1-thi-1 gyrA96 relA1(Gibco BRL, Karlsruhe).

4.2 Methods

4.2.1 Quick preparation of genomic DNA for mouse genotyping

Small pieces of mouse tails were incubated in 500 µl lysis-buffer at 55 °C overnight. After cooling to room temperature (RT) 200 µl 6 M NaCl was added to the digested solution, mixed and incubated on ice for 10 minutes. The solution was centrifuged for 8 minutes at 7000 rpm. The supernatant was transferred to a clean tube and the DNA was precipitated by adding 2.5V of 100% ethanol. The precipitate was centrifuged at 14000 rpm for 15 minutes. The DNA pellet was resuspended in 100 µl TE.

4.2.2 Preparation of high quality genomic DNA from mammalian tissue

To receive high molecular genomic DNA, shear forces have to be avoided during the isolation procedure. 2-3 g of liver tissue was cut in small pieces and suspended in Solution

A. For 500 µg tissue, 2 ml of Solution A was used. The equal amount of Solution B was added to the suspension and carefully mixed. Digestion took place overnight at 55°C. To remove RNA, the suspension was incubated in RNAse A (10 µg/ml) for one hour at 37°C. Phenol-chloroform treatment was performed twice with careful inversion and low speed centrifugation steps of 5000 rpm for 10 minutes. 5 M NaCl was added to a final concentration of 250 mM. For precipitation 0.7V of isopropanol was used. Genomic DNA adhers to silica-glass and was therefore collected by rotating a glass stick in the tube. DNA was washed in 70% ethanol and airdried at the tip of the glass stick. DNA was solved overnight in TE at 4°C. The quality of genomic DNA was analysed by gelelectrophoresis after digestion with an appropriate restriction enzyme and the quantity was measured with a photometer (Pharmacia Biotech).

4.2.3 Restriction digest of genomic DNA

The restriction reaction contained 5 µg genomic DNA, 40 U of *BamHI* or *XbaI* enzyme and was buffered according to the manufacturer's instructions in a total volume of 400 µl. 0,1 mM DTT, 1 mM Spermidine and 1/100 V BSA (10 mg/ml) were added to the digestion mixture to stabilize the DNA and to facilitate complete digestion. The digestion reaction was incubated at 37°C for at least 6 hours. Efficiency of digestion was controlled by gelelectrophoresis. After complete digestion, the high molecular band of DNA disappears and a homogen smear of digested DNA is visible. The digested DNA was purificated by phenol-chloroform extraction and ethanol precipitation.

4.2.4 Isolation of RNA from limb tissue

To avoid contamination with RNAses usual precautions were taken (Ausubel, 1996; Sambrook, 1989). RNA was isolated using Trizol reagent. Four limbs of one embryo were collected and homogenized in 1 ml of Trizol. The RNA extraction was performed according to the manufacturer protocol. Total RNA was solved in 50 µl RNAse free H_20, supplied with 1x transcription buffer and treated with 2 µl DNAse at 37°C for 1h to digest residual DNA. The RNA solution was filled up with RNAse free water to a final volume of 100 µl and was precipitated with 10 µl 4M LiCl, 1 µl Glycogen and 250 µl ethanol for 1h at -20°C. After centrifugation the RNA-pellet was resolved in 20 µl RNAse free water. The quantity and quality of the isolated total RNA was determined by photometric measurement.

4.2.5 Determination of the concentration of nucleic acids

To quantify the amount of DNA or RNA, the optical density of probes was measured with a photometer at a wavelenght of 260 nm. For DNA (double strand) the OD_{260}= 1 corresponds to a concentration of 50 µg/ml, for RNA the OD_{260}= 1 corresponds to a concentration of 40 µg/ml. The relation of the absorption at 260 nm and 280 nm describes the purification grade of the nucleic acids and should not exceed 2.0.

4.2.6 Reverse Transcription - cDNA synthesis

Transcription of RNA into cDNA was performed with the RT-PCR Kit from Amersham Bioscience. 1 µg of total RNA was incubated with the first strand reaction mix beads containing buffer, dATP, dCTP, dGTP, dTTP, murine reverse transcriptase (Moloney Leukemia Virus), RNAguard and BSA according to the manufacturer instructions. The first-strand reaction was primed with random primers ($pd(N)_6$). Finally the cDNA was diluted 1:10 to a final volume of 300 µl.

4.2.7 Gelelectrophoresis

Agarose-gelelectrophoresis was carried out for analytical purposes and for isolation of specific DNA-fragments. Corresponding to the size of the DNA-probes 0.8% to 2% agarose gels were prepared with TAE buffer. To visualize the DNA fragments, 0.5 µg/ml ethidiumbromide was added to the liquid agarose. Ethidiumbromide intercalates into the stacked bases of the DNA molecule and emits orange light when illuminated with ultraviolet light (254 nm). For loading the DNA and to estimate the running time of the gel, gel-loading buffer was added to the DNA solution and standard size markers were used to determine the size of the isolated bands. For cloning DNA fragments were excised from the gel and extracted with glass milk according to the manufacturer instructions. Gelelectrophoresis of total RNA was performed in 1x TBE buffer.

4.2.8 Ligation of DNA

The T4 DNA-Ligase is used to ligate DNA fragments with compatible overhangs or blunt ends. To circularize digested genomic DNA fragments and to ensure self-ligation 5 different dilutions of the digested genomic DNA (60ng/µl) were made: 2x, 4x, 8x, 16x, 32x. The ligation reaction mixture was set up as follows: 2 µl of 10x ligation buffer, 2 µl of 50%PEG, 10 µl of diluted genomic DNA and 0,2 µl of T4 DNA-Ligase. PEG greatly increases the rate of ligaton. The reaction mixture was filled up with H_20 to a total volume

of 20 µl. The reaction was incubated at 16°C overnight. After ligation, the circularized DNA was precipitated with ethanol for 1 hour at -80°C. The pellets were resuspended in 20 µl EB buffer.

4.2.9 TOPO cloning

TOPO vectors were used according to the manufacturer's instructions to subclone PCR amplified DNA fragments. In brief, DNA fragments were amplified using taq polymerase, which produces single 5´A overhangs. These are complementary to T overhangs of activated TOPO vectors. Topoisomerases, covalently bound to the TOPO vector, facilitate the ligation reaction.

4.2.10 Heat shock transformation of bacteria

A heat shock of 42°C facilitates the uptake of plasmid DNA by bacteria. The plasmids contained ampicillin resistance genes as selection marker. Plasmid DNA was diluted to 50 ng/µl in H_2O and 1 µl was added to 200 µl of chemical competent *E. coli XL-1 blue* cells. The cells were incubated on ice for 20 minutes, then transferred to a water bath for heat shock at 42°C (30 seconds), and chilled on ice for another 5 minutes. Finally, 0.5 ml of SOC medium was added and the cells were incubated on a shaker for 1 h at 37°C. To distinguish single colonies after overnight incubation 10 µl were plated on selective agar plates and incubated at 37°C for 16 to 20 hours. Single colonies were selected and grown in a suitable amount of LB medium for plasmid preparation.

4.2.11 Sequencing

Sequencing was carried out with a BigDuy-Terminator Kit by Martin Meixner, Institut for Genetics, Humboldt University, Berlin

4.2.12 PCR-Methods

The polymerase chain reaction method allows the amplification of DNA fragments with specific oligonucleotid primers The PCR products can be fragmented by gel-electrophoresis, purified and used for cloning (Mullis et al., 1986; Saiki et al., 1985). For a standard PCR reaction the reaction mixture consisted of the following components: 10 ng of plasmid or 100 ng of genomic DNA; 2,5 µl 10x PCR-buffer; 0,25 µl 25 mM dNTPs, 1,25 µl of each primer (10µM) and 0,5 µl of 5U/µl Taq-polymerase. Amplification conditions were: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, an appropriate annealing

temperature for 30 seconds, 68°C for 1 minute and 72°C for 7 minutes. The PCR-products were analyzed in a 1% agarose gel.

4.2.12.1 Genotyping of mice by PCR

For genotyping of mice the standard PCR protocol was used, if not otherwise indicated.

Primers for *Ext1*$^{Gt/Gt}$ mice (ascertained by inverse PCR):
Ext1fw: 5'-CACATCAGGTGCCTCACAAC-3'
Ext1rv: 5'-CTCCCAGCACTTTTCCTGAC-3'
5'pgto: 5'-TACATAGTTGGCAGTGTTTGGG-3'

PCR conditions for genotyping of *Ext1*$^{Gt/Gt}$ mice were: 94°C for 5 minutes; 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1 minute; and 72°C for 7 minutes. The resulting 800 bp fragment indicated the transgenic allel and a 600 bp fragment indicates the wild-type allel.

Primers for *Gli3* mice:
XtJ580-rev 5'-AAACCCGTGGCTCAGCACAAG-3'
XtJ580-for 5'-TACCCCAGCAGGAGACTCAGATTAG-3'
C3-back 5'-GTTGGCTGCTGCATGAAGACTGAC-3'
C3-Rev 5'-GGCCCAAACATCTACCAACACATA-3'

PCR conditions for genotyping of *Gli3* mice were: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and 72°C for 7 minutes. The resulting 600 bp fragment indicated the transgenic allel and a 200 bp fragment indicates the wild-type allel.

Primers for *Ihh* deficient mice:
Neo: 5´-TACCGGTGGATGTGGAATGTGTGCG-3´
Ihh-fw : 5´-AGGAGGCAGGGACATGGATAGGGTG-3´
mIhh ex2 rv2 *:* 5'-TGTTCTCCTCGTCCTTGAAGA-3'

PCR conditions for the wild-type allel were: 94°C for 5 minutes; 32 cycles of 94°C for 45 seconds, 59°C for 45 seconds, 72°C for 60 seconds; and 72°C for 7 minutes. The resulting 160 bp fragment indicated the wild-type allel. For genotyping of the transgenic allel the following PCR conditions were used to amplify a 300 bp fragment: 94°C for 5

minutes; 30 cycles of 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute and 72°C for 7 minutes.

Primers for *Fgfr3*$^{Ach/+}$ mice :
forward primer 5'- AGGTGGCCTTTGACACCTACCAGG-3'
reverse primer 5'-TCTGTTGTGTTTCCTCCCTGTTGG-3'

PCR conditions for genotyping of *Fgfr3*$^{Ach/+}$ mice were: 94°C for 7 minutes; 27 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds; and 72°C for 7 minutes. The resulting 850 bp fragment indicated a transgenic mouse.

Primers for *ColII-Gal4* mice :
forward primer 5'-CTTCTATCGAACAAGCATGCG -3'
reverse primer 5'-GCCAATCTATCTGTGACGGC-3'

PCR conditions for genotyping of *ColII-Gal4* mice were: 94°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; and 72°C for 5 minutes. The resulting fragment of 322 bp indicated a transgenic mouse.

Primers for *UAS-Ihh* mice :
forward primer 5'-GGGCGGGCGCTGGCGACGCTG-3'
reverse primer 5'-CGGGCTGCACGTGGCTG-3'

PCR conditions for genotyping of *UAS-Ihh* mice were: 94°C for 10 minutes; 35 cycles of 94°C for 30 seconds, 72°C for 30 seconds,72°C 30 seconds; and 72°C for 10 minutes. The resulting fragment of 300 bp indicated a transgenic mouse.

4.2.12.2 Inverse PCR

The gene trap vector (Leighton et al., 2001) inserted into the first intron of the *Ext1* gene in the *Ext1*$^{Gt/Gt}$ mice. The exact insertion site in this 274 kb long first intron had to be determined. The recognition sequences of two restriction enzymes, *BamHI* and *XbaI*, lay close to the linearization side of the gene trap vector (Fig. 8). These two restriction enzymes were chosen to digest genomic DNA, which was isolated from liver of *Ext1*$^{Gt/+}$ mice. Subsequently the genomic DNA fragments were circularized with T4 DNA ligase. The circularized DNA was used as a template for the first round of PCR. The two rounds of PCR were performed with nested primer pairs located in the *pGT2TMpfs* vector (Fig. 8, red and green arrows):

(1) Inv-fw1: 5'-TGCTTCTGATGAGGTGGTCC-3'

(1) Inv-rv1: 5'-TACATAGTTGGCAGTGTTTGGG-3';

(2) Inv-fw2: 5'-GCCAGAGACTCAGTGAAGCCT-3'

(2) Inv-rv2: 5'-GGGTCTCAAAGTCAGGGTCAC-3'

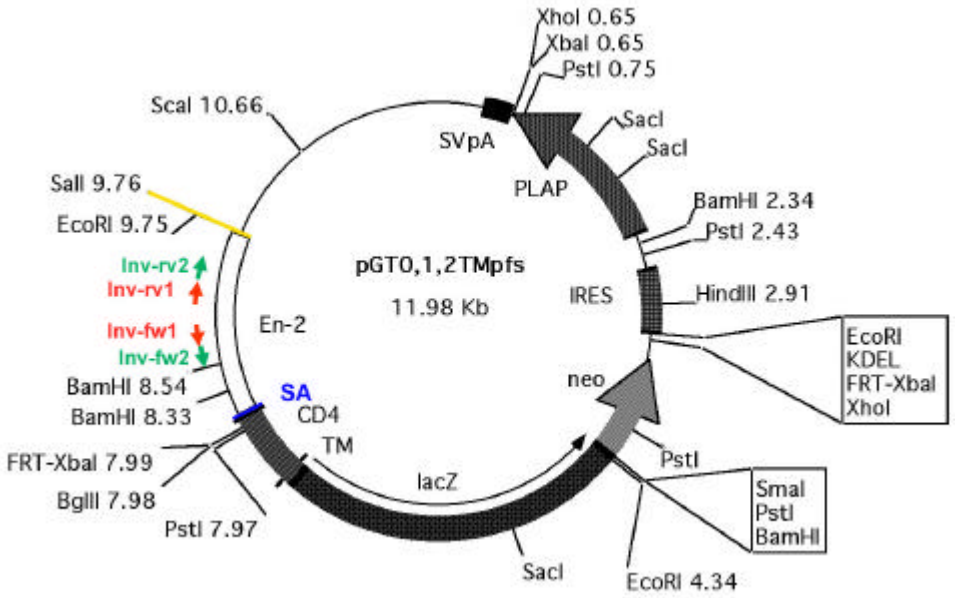

Fig. 8. Map of the *pGT2TMpfs* vector. The construct was linearized with *SalI* (yellow line) before injecting it into embryonic stem cells (Leighton et al., 2001). The green and red arrows demarcates the position of primers used for inverse PCR.

The PCR reaction mixture contained 1 M aqueous betaine, which improves amplification of DNA by reducing the formation of secondary structures caused by GC-rich regions. The first PCR run (primer pair Inv-fw1; Inv-rv1) was carried out with a low annealing temperature: 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 55°C for 30 seconds and 68°C for 5 minutes, 72°C for 7 minutes. 1 µl of the first run was used as a template for the second round of PCR. The second run (primer pair Inv-fw2; Inv-rv2) was carried out with a higher annealing temperatur, to gain specificity of the amplification products: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 58°C for 30 seconds and 68°C for 3 minutes, 72°C for 7 minutes. The PCR fragments of the second round were analysed by gel-electrophoresis. PCR-probes showing a distinct band on the gel were purificated with a PCR purification kit (Qiagen), cloned into the TOPO vector and transformed in E. coli. Clones were analysed by colony PCR, positive clones were picked,

grown in LB-media and processed for plasmid preparation. DNA of the plasmids was sequenced, to identify the unknown genomic region of the insertion side.

4.2.12.3 RT-PCR

The reverse transcription polymerase chain reaction is the most sensitive method for the detection of low-abundance mRNA. Total RNA was isolated from limbs of E14.5 *Ext1*$^{Gt/Gt}$, *Ext1*$^{Gt/+}$ and wild-type embryos. The cDNA was generated by reverse transcription using random primers. For PCR the following primers were used:

Ext1-fw4 (fw-exon6) 5'-TGTGACTCCCCTGGTCTCTC-3'
Ext1-fw3 (fw-exon7) 5'-GTGCCTGTCATCGTCATTGAAG-3'
Ext1-rv2 (rv-exon8/9) 5'-GAAGGCAAAATCCACTTCCG-3'
Ext1-rv4 (rv-exon9) 5'-CACCATGGAGTAGTCATTCGTC-3'

4.2.12.4 Real Time PCR

Direct detection of the PCR product can be monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double stranded DNA. Total RNA was isolated from limbs of E14.5 *Ext1*$^{Gt/Gt}$, *Ext1*$^{Gt/+}$ and wild-type embryos and the cDNA was generated by reverse transcription using random primers. Quantification of *Ext1* mRNA expression was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green I. Primers were selected with Primer Express Software (Applied Biosystems) as follows:

exon1-fw-2: 5'-CTGGCAAAAGCACAAGGATTC-3' and
exon2-rv: 5'-TTGTGCAGCATTTCCCGATA-3';
exon1-fw-3: 5'-TGGCAAAGACTGGCAAAAGC-3' and
gene trap3Gtrv: 5'-GTTTTCGGGACCTGGGACTT-3';
exon4-fw: 5'-GCCGGTTTCTGCCCTATGA-3' and
exon4-rv: 5'-TACGGTGAAGGCAAAATCCA-3'.
cDNAs were normalized against transcript levels of *Hprt*:
Hprt-fw: 5'-GCTCGAGATGTCATGAAGGAGAT-3' and
Hprt-rv 5'-AAAGAACTTATAGCCCCCCTTGA-3'

One PCR reaction mixture consisted of: 10 µl cDNA (diluted 1/10 V), 5 µl primer (final concentration 7.5 pM in 5 µl) and 15 µl SYBR Green Master Mix. PCR conditions

were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. After the last PCR cycle the melting curves for the amplification products were measured from 60°C to 95°C. The results were analysed with the SDS software (Applied Biosystems). The quantification of the PCR product is based on the calculation of the fluorescence treshold value (C_T-value). The C_T-value is that PCR-cycle in which the reporter-fluorescence exceeds the backround-fluorescence. After the C_T-value is reached the kinetics of the amplification reaction should be exponential. The C_T-values of different templates can be compared with each other (relative quantification) or to a C_T-value of a known template concentration using a standard curve (absolute quantification).

4.2.13 Organ cultures of embryonic limb explants

Pregnant mice were sacrificed by cervical dislocation. Pups were transferred to PBS containing antibiotic-antimycotic. Forelimbs were dissected from the embryo using fine forceps. Skin and muscles were removed from forelimbs under a dissecting microscope. Culture dishes were prepared as follows: The outside chamber was filled with PBS containing antibiotic-antimycotic. The middle chamber was filled with 1 ml of BGJb medium. A culture grid was placed in the middle of the culture dish and covered with a Millipore AA filter. Forelimbs were placed on the Millipore AA filter in the middle of the culture dish with the palm faced up. Forelimbs were cultured at the air-liquid interface at 37°C, 5% CO_2 under sterile conditions for 2 days. The medium was changed daily with/without applying of growth factors. Forelimbs of E15.5 and E16.5 mouse embryos were supplemented with 10 µM cyclopamine (Incardona et al., 1998), 250 ng/ml Fgf2, 100 ng /ml Tgfb1, 1 µg/ml, 10 µg/ml, or 100 µg/ml HS sodium salt; 1 µg/ml, 10 µg/ml, or 100 µg/ml heparin sodium salt; or 10 µg/ml or 100 µg/ml CS sodium salt. The limbs were cultured for 2 days. Each treatment was repeated at least five times.

4.2.14 Harvesting and dehydration of limb tissue

Limbs were fixed in 4% PFA at 4°C overnight. Tissue was rinsed three times with PBS for 5 minutes. Tissue of E14.5 and E16.5 limbs was dehydrated for 10 minutes in 30%, 50%, 75%, 80%, 95% and 100% ethanol and subsequently incubated twice in 100% xylol. The next steps were done under vacuum in xylol/paraffin (1:1) for 30 minutes and twice in 100% paraffin for 2 hours. Limbs were embedded into paraffin and sectioned into 5 µm slices for 4-5 parallel sections, which were placed on silanized slides.

4.2.15 Silanization of slides

Superfrost plus slides were used for silanization. The slides were incubated for 1 minute in each solution: 2N HCl, RNAse free water, 100% acetone, 100% acetone with 1% 3aminopropyltrioxysilane, 100% acetone, 95% ethanol, 50% ethanol, 30% ethanol, RNAse free water twice. The slides were dried overnight.

4.2.16 Preparation of a murine *Pthlh*-probe for in situ hybridisation

A 423 bp mouse *Pthlh*-fragment was amplified by RT-PCR from embryonic limbs using primers:

fw5'-GCTGCCGCCAAGACTAATTAG-3'
rv5'-GTAGACTAGCGCCTCTAGGTG-3'

Standard PCR conditions were used. The fragment was ligated into the TOPO vector (Invitrogen) and transformed in E. coli.

4.2.17 Preparation of DNA template for in vitro transcription

Plasmid DNA was isolated from *E.coli* DH5 cells using QIAprep Plasmid Miniprep or Midiprep Kits according to the manufacturer instructions. 5 µg plasmid DNA was linearized with 5 U of the appropriate restriction endonuclease for 2 hours at 37°C. Efficiency of the digestion was analyzed on a 1% agarose gel. For purification phenol/chloroform extraction was performed twice. The final supernatant was transferred to a clean tube and was precipitated with 2.5V of 100% ethanol. The mixture was centrifuged at 14.000 rpm for 15 minutes at room temperature (RT). The DNA pellet was washed with cold 80% ethanol and resuspended in 10 µl of RNAse free water.

4.2.18 Labeling of antisense riboprobes

Transcription reaction was performed in 20 µl with the following components: 500 ng linearized DNA template for *in vitro* transcription, 1x transcription buffer, 0.5 mM NTP mix, 5 U RNAse inhibitor, RNAse free water, 40 U RNA-polymerase and 80 µCi [P_{33}]-UTP. The transcription reaction was carried out for 1.5 hours at 37°C. To remove the DNA template, 10 U DNAse (RNAse-free) were added to the transcription mixture and incubated for 30 minutes at 37°C. The transcription mixture was then diluted with 4 V of RNAse free water, containing 20 ng/ml glycogen and 0.5 M LiCl, and precipitated with 2.5 V of 100% ethanol. Probes were incubated at -20°C for 30 min and then centrifuged at 14.000 rpm for

15 minutes at 4°C. The RNA pellet was washed twice with cold 80% ethanol, resuspended in 50 µl RNAse free water, and diluted 1: 20 with hybridization buffer. Before use the riboprobe was heated for 5 minutes at 95°C and chilled on ice.

4.2.19 Prehybridization and in situ hybridization

All solutions for the prehybridization procedure were prepared with RNAse free water. The sections were incubated in xylol for 30 minutes to remove the paraffin. To rehydrate the tissue, the sections were incubated in a descending ethanol series (100%, 95%, 80%, 75%, 50%, 30%) each for 2 minutes, followed by 0.85% NaCl for 5 minutes and PBS for 5 minutes. After rehydration the tissue was fixed in 4% PFA for 30 minutes. The sections were then incubated for 5 minutes in following solutions: PBS, 0.2N HCl and RNAse free water. The tissue was digested for 5 minutes with 0.02 mg/ml Proteinase K and rinsed with PBS for 5 minutes. Subsequently the sections were refixed in 4% PFA with 0.2% glutaraldehyde for 10 minutes and rinsed with PBS for 5 minutes. The sections were acetylated by 0.25% acetic anhydride (freshly added) to 0.2% Triethanolamine for 10 minutes, then rinsed with PBS for 5 minutes and additionally incubated in 0.85% NaCl for 5 minutes. Sections were dehydrated with the following ethanol solutions: 30%, 50%, 75%, 100%. The sections dried at RT for 15 minutes. A minimal volume of labeled riboprobe (50 µl) was distributed evenly on the slide and the sections were covered with plastic coverslips to prevent evaporation. Hybridization was performed at 70°C overnight.

4.2.20 Washing of slides and dipping in photoemulsion

After hybridization the sections were washed in 5xSSC for 30 minutes and coverslips were removed. The sections were washed in 2xSSC for 30 minutes and treated with 0.02 mg of RNAse A in 1x Washing buffer for 30 minutes. Stringend washing took place in 2xSSC with 50% formamide for 30 minutes and then twice in 2xSSC for 30 minutes. The sections were dehydrated in an ascending ethanol series (30%, 50%, 75%, 80%) containing 0.3M ammonium acetate. Finally the sections were incubated in 100% ethanol for 15 minutes and dried for 10 minutes at RT. The slides were exposed to a X-ray film (Kodak Scientific Imaging Film) overnight at RT to estimate the intensity and strenght of each probe. The sections were dipped in photoemulsion NTB2 at 40°C in the darkness. Dried sections were stored at 4°C in darkness for the time predicted from the developed X-ray film. The dipped sections were developed in Kodak-developer for 5 minutes at 15°C, rinsed

in water and fixed in Kodak-fixer for 15 minutes at RT. The sections were counterstained with 0.2% Toluidin blue O for 5 minutes and dehydrated in ethanol solutions 30%, 50%, 75%, 80%, 95% and 100%. The sections were incubated in 100% xylol and then covered with glass coverslips using minimal volume of mounting medium (DPX mountant for histology). The sections were analysed by dark-field microscopy.

4.2.21 BrdU Labeling

Mice were sacrificed 2 hr after receiving an intraperitoneal injection of 31 µg/g body weight 5-bromo-2-deoxy-uridine (BrdU). Cultured limb explants were treated with 31 µg/ml for 2 hr before harvesting. Limbs were fixed in 4% paraformaldehyde at 4°C and embedded in paraffin. Proliferating cells on 6 µm sections were detected by antibody staining according to the manufacturer. BrdU-positive and -negative nuclei were counted in periarticular and columnar chondrocytes in zones of equal size (n = 4, eight sections each).

4.2.22 Immunohistochemistry

Immunohistochemistry with ShhAb80 (Bumcrot et al., 1995) and 3G10 (biotinylated antibody) (David et al., 1992) was performed as described in (Gritli-Linde et al., 2001). The sections were deparaffinized and rehydrated as follows: 2 times for 5 minutes in xylol, 2 times for 5 minutes in 100% ethanol, 2 times for 5 minutes in 95% ethanol, 2 minutes in 50% ethanol and for 5 minutes in PBS. Endogenous peroxidase was blocked for 10 minutes in the dark and at RT in 3% H_2O_2 in methanol. The tissue was washed 3 times for 5 minutes in PBS. Unspecific binding was blocked by incubating the slides in PBS containing 5% normal serum, 0.2% BSA and 0.1% Triton-X100 for 40 minutes at RT in a humidified chamber. For 3G10 antibody immunohistochemistry limb sections were overlayed with 250 mU heparinase III in digestion buffer for 4 hr prior to the incubation with the primary antibody. The primary antibody ShhAb80 was diluted 1:500, whereas 3G10 was diluted 1:100 in PBS containing 0.2% BSA and 0.1% Triton-X100. The slides were incubated overnight at 4°C in a humidified chamber. After primary antibody incubation, slides were washed 3 times for 5 minutes in TNT. For Shh Ab80 immunohistochemistry the sections were incubated at RT for 45 min in the dark with biotinylated goat anti-rabbit diluted 1: 300 in TNT containing 2% non-fat dry milk and slides were washed again 3 times for 5 minutes in TNT. Sections were blocked in TNB for 30 minutes at RT in the dark and then incubated for 30 minutes with Strepavidin-HRP from

the NEN TSA kit, was freshly diluted 1:100 with TNB in the dark. Slides were washed 3 times for 5 minutes in TNT an incubated for 9 minutes in Biotinyl Tyramide diluted 1:50 in NEN amplification buffer. Slides were washed 3 times for 5 minutes and incubated again in Streptavidin-HRP. Slides were washed 3 times for 5 minutes in TNT and then incubated with diaminobenzidine (DAB) in stable peroxide solution usually from 3-10 minutes. Staining was monitored under a microscope with weak light.

4.2.23 Hematoxylin/Eosin staining

This histological staining method stains nuclei blue (Hematoxylin) and the cytoplasm red (Eosin). Sections were deparaffinized, rehydrated and subsequently incubated for 45 seconds in Hematoxylin, rinsed shortly with tab water and then incubated for 2 to 3 minutes in Eosin. Finally slides were rinsed with tab water, dehydrated by an increasing ethanol series, transferred into Xylol and embedded in DPX mounting medium.

4.2.24 Safranin Weigert staining

This method is used for the detection of cartilage. Cartilage is stained red, nuclei are stained black and background is stained green. Sections were deparaffinized, rehydrated and subsequently incubated in the following solutions (rinsed with tab water in between): 2 to 3 minutes in Weigerts solution, 2 minutes in Scott's buffer and 2 minutes in fast green. After staining with fast green the slides are rinsed in 1% glacial acid, rinsed with tab water and incubated in Safranin for 5 minutes. Finally slides are rinsed with tab water, dehydrated by an increasing ethanol series, transferred into xylol and embedded in DPX mounting medium.

4.2.25 Skeletal staining with alcian blue

Alcian blue stains mucopolysaccharides in the matrix of cartilage. Whole embryos were dehydrated in 100% ethanol for 3 days and than incubated in alcian blue staining solution for 24 h at 37°C. Excess color was removed by incubating the embryos for 12 h in 100% ethanol. Then the stained embryos were cleared by incubating them in 1% KOH for 1 to 3 days. The skeletal preparation were transferred through an ascending glycerol/PBS series and stored in 100% glycerol.