2. Materials and Methods

2.1. Abbreviations

bp	base pair(s)
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BrdU	5-bromo-2'deoxyuridine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
ES cell	embryonic stem cell
et al.	*et altera*
EDTA	ethylene-diaminetetraacetic acid
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
g	gram
G418	geneticin
GFP	green fluorescent protein
HEPES	4-(2-Hydroxyethyl)-piperazin-1-ethansulfonic acid
h	hour
HRP	horseradish peroxidase
HS	horse serum
kb	kilobase pairs
l	liter
LIF	leukemia inhibitory factor
M	molar
min	minute
ml	milliliter

mM	millimolar
µ	micro
NBT	nitroblue tetrazolium salt
NEB	New England Biolabs
nm	nanometer
neo^R	neomycin resistance gene
ON	over night
PBS	phosphate-buffered saline
PBT	PBS containing 0.15% Tween-20
PBX	PBS containing 0.1% Triton X-100
PCR	polymerase chain reaction
PFA	paraformaldehyde
pH	*potentium hydrogenii*
PI	propidium iodide
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rotations per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
Tris	Tris-(hydroxymethyl)aminoethane
U	unit (enzymatic activity)
UV	ultraviolet (light)
UTR	untranslated region

2.2. Materials

2.2.1. Chemicals and enzymes

Chemicals, enzymes and kits for molecular biology, oligonucleotides, or antibodies were purchased from the following companies, unless indicated otherwise: Ambion (Huntingdon, UK); Amersham-Pharmacia (Freiburg); Affymetrix (Santa Clara, USA); Becton-Dickinson (Erembodegem, Belgium); Biotez (Berlin); Biozym (Hess. Oldendorf); Boehringer Ingelheim GmbH (Ingelheim); Cell Signaling, Dianova (Hamburg); Gibco BRL (Karlsruhe); Charles River (Sulzfeld); Clontech (Heidelberg); Enzo (New York, USA); Jackson Laboratories (Baltimore, USA); Invitrogen (Groningen, Netherlands); Molecular Probes (Eugene, USA); MWG-Biotech (Ebersberg); NEB (Frankfurt); PerkinElmer Life Siences (Boston, USA); Pharmacia Biotec (Freiburg, Germany); Promega (Mannheim); Stratagene (Amsterdam, Netherlands); Qiagen (Hilden); Roche (Mannheim); Roth (Karlsruhe); Santa Cruz (Heidelberg); Shandon (Frankfurt); Sigma (Deisenhofen).

2.2.2. Bacterial strains

Escherichia coli XL1-Blue MRF '	(Jerpseth et al. 1992)
Escherichia coli GM2163 (*Dam*$^-$, *Dcm*$^-$)	NEB

2.2.3. Vectors/plasmids

pBluescript II SK+	Sorge, 1988
pGEM-Teasy	Promega
pTV-flox constructed by	(backbone: pIC19R,
D. Riethmacher and M. Sachs	(Marsh et al. 1984)
pAdlox-EGFP (contains *GAP43-EGFP* cassette)	U. Mueller laboratory, Basel
pIC (Cre expression plasmid)	H. Gu/K. Rajewsky laboratory
pEGFP-C1 (contains *EGFP* cassette)	Clontech
pcDNA3.1(-)	Invitrogen

2.2.4. Cell lines

ES cell line:

Embryonic stem (ES) cells from the line E14.1, derived from the 129/Ola mouse strain (Hooper et al. 1987; Kühn et al. 1991) were used for introducing targeted mutations into the mouse.

COS-1 cell line:	(Gluzman 1981);
(green monkey kidney cells)	Europian collection of animal Cell Cultures, Porton Down, Salisbury, United Kingdom

2.2.5. Antibodies

a) Primary:

Antigen	**Host animal**	**Dilution**	**Source**
BrdU	mouse IgG	1:200	Sigma
caspase-3-cleaved (Asp175)	rabbit	1:500	Cell Signaling
CXCR4	rabbit	1:100	S.Schulz, Magdeburg
GFP	rabbit	1:1000	Molecular Probes
Lbx1	guinea pig	1:20000	T. Muller, Berlin
MyoD	rabbit	1:100	Santa Cruz
Myosin heavy chain	mouse IgG	1:200	Sigma
Pax3	rat	1:400	M. Goulding, USA

b) **Secondary**:

The secondary antibodies coupled with Cy2, Cy3 or Cy5 fluorescent reagent were purchased from Dianova and dissolved in sterile 50% glycerin to a final concentration 0.5 mg/ml. Dilutions 1:250 for Cy2 and 1:500 for Cy3 and Cy5 were routinely used.

2.2.6. cDNA probes for *in situ* hybridization

mouse CXCR4	945 bp, complete cDNA*
mouse Epnb2	D. Wilkinson laboratory
mouse EphA4	RZPD clone IMAGp998A1714407Q3

mouse FoxM1	RZPD clone IRAKp961J2210Q2
mouse Fhl1	RZPD clone IMAGp998M0710985
mouse Pcdh17	441 bp, complete cDNA*
mouse SDF1	660 bp, encompasses 3' coding sequence and 3' UTR of mouse *SDF1**
chicken CXCR4	(Stebler et al. 2004)
chicken MyoD	(Lin et al. 1989)
chicken Myf5	(Dechesne et al. 1994)
chicken Pax3	(Goulding et al. 1994)
chicken SDF1	(Stebler et al. 2004)

*cDNA probe was generated using PCR; size in base pairs and relative position of probe sequence in respect to the coding sequences of the gene is indicated.

2.2.7. Mouse strains:

C57Bl/6J:	Charles River
CXCR4:	Jackson Laboratories
Gab1:	M. Sachs/W. Birchmeier laboratory

2.2.8. Chicken eggs:

Fertilized White Leghorn chicken eggs were purchased from Charles River.

2.2.9. Bacterial and cell culture media

Fibroblast Medium:

500 ml Dulbecco's MEM (DMEM) with Glutamax-I, 4500 mg/l Glucose, with Pyridoxin,

Natriumpyruvat (Gibco BRL)

60 ml FCS (Heat inactivated at 55°C for 30 min, Sigma)

5.7 ml 100x non-essential aminoacids (Gibco BRL)

5.7 ml Penicillin/Streptomycin-solution (10000 U/ml Penicillin G/10000μg/ml Streptomycin; Gibco BRL)

1.2 ml 50 mM ß-Mercaptoethanol (Gibco BRL)

ES Cell Medium:

500 ml DMEM/Glutamax (see above, Gibco BRL)

90 ml FCS (Heat inactivated at 55°C for 30 min, Sigma)

6 ml 100x non-essential aminoacids (Gibco BRL)

6 ml Penicillin/Streptomycin-solution (Gibco BRL)

1.2 ml 50 mM ß-Mercaptoethanol (Gibco BRL)

60 µl LIF (500-1000 U/ml)

LIF-containing supernatant was routinely prepared from COS cells stably transfected with a LIF-expression plasmid (Genetics Institute Inc., Cambridge, MA, USA).

LB Medium:

10 g/l bacto-tryptone

5 g/l yeast extract

10 g/l NaCl

2.3. Methods

Besides the techniques detailed in the following section, standard protocols for various procedures in molecular biology, like molecular cloning, sequencing and targeting vector construction were performed according to "Molecular Cloning" (Sambrook and Russell 2001) or manufacturers' instructions in the case of kits.

2.3.1. Extraction and purification of nucleic acids

2.3.1.1. Isolation of plasmid DNA

E.coli cells containing plasmid DNA were cultured in sterilized LB-medium with an appropriate antibiotic, ampicillin (100 μg/ml) or kanamycin (30 μg/ml), ON at 37°C. Small-scale preparations (3 ml culture) of plasmid DNA were performed by the alkaline lysis method (Birnboim and Doly 1979). Large-scale (200 ml culture) preparations of plasmid DNA were performed using the Plasmid Maxi-Kit from Qiagen. All procedures for this were carried out according to the manufactures protocol. The concentration and purity of the DNA were determined by UV-spectrophotometry.

2.3.1.2. Isolation of genomic DNA from ES cells

ES cells were screened for homologous recombination events by Southern-hybridization of genomic DNA. For the genomic DNA isolation, ES cells were grown in gelatinized 96 well plates up to a confluent layer. Cells were rinsed twice with PBS and lysed in 50μl ES cell lysis buffer per well (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% N-Lauroylsarcosin, 200 μg/ml proteinase K) at 60^0C in a humid chamber ON. The DNA was precipitated for 30 min at RT with 100 μl of 100% ethanol containing 0.15 M sodium acetate, pH 5.2. Following precipitation, DNA was washed twice with 70% ethanol, air-dried for 20 min and directly digested with the corresponding restriction enzyme. Restriction digests were performed at 37^0C ON in 50 μl reaction mix (1x restriction buffer, 100 μg/ml BSA, 50 μg/ml RNAse, 10-15 U of restriction enzyme).

2.3.1.3. Isolation of genomic DNA from mouse tissue

To genotype adult mice or embryos by PCR, DNA was isolated from ear holes or yolksac, respectively. The tissue was lysed for 2-10 h at 55^0C in 50 μl of lysis buffer containing proteinase K (1 mg/ml). After the tissue lysis was completed, proteinase K was inactivated by incubation at 95^0C for 10 min. Lysates were diluted with distilled water to a final volume of 300μl and were directly used for PCR.
If the genomic DNA was to be used for Southern blot analysis, the preparation was performed differently. A piece of embryonic tissue (usually head) was lysed in 100 μl of lysis buffer ON at 55^0C and then the DNA was purified by phenol/chloroform extraction. DNA was precipitated from the supernatant with 2 volumes of ice-cold 100% ethanol. The sample tube was centrifuged (14000 rpm, 10 min), and the pellet was washed twice with 70% ethanol and air-dried. The DNA was dissolved in 5 mM Tris-HCl at a final concentration of 1 mg/ml.

2.3.1.4. Extraction of RNA from embryonic tissue

RNA was isolated from whole mouse embryos (E10.5) or from FACS sorted *Lbx1*$^{GFP/+}$ cells and used to generate cDNA or biotin-labeled cRNA probes, respectively. For this, embryonic tissue or sorted cells were lysed in 500 μl Trizol reagent (Invitrogen) for 5 min at RT. Trizol lysate could be stored at -70^0C for up to 6 month, if necessary. RNA was extracted from the Trizol lysate with 100 μl of chloroform and was then precipitated with 250 μl isopropanol. The samples were centrifuged (10000 rpm, 10 min), washed twice with 70% ethanol and dissolved in 3-30 μl DEPC-treated water (H_20_{DEPC}).

2.3.2 Polymerase chain reaction (PCR)

2.3.2.1. Genotyping PCRs

Mice and embryos were routinely genotyped by PCR. Optimal PCR conditions were established according to general rules (Innis et al. 1989). Primers were purchased from Biotez. A list of all reaction conditions used for each genotyping PCR is presented below.

a) Lbx1^{GFP}

Wild-type PCR, 1.5 mM $MgCl_2$, 10% DMSO

Primers: HB24: 5'- CCGTACGCCGTTCAGCATCGAGGACATC -3'

HB27: 5'- GAGGCAGGGGGTACGAAGGGCAGGACAC -3'

Mutant PCR, 1.5 mM $MgCl_2$, 10% DMSO

Primers: gfp5: 5'- CAAAGGAGGCCGCCATGGTGTGCTGT -3'

gfp4: 5'- GATGCCCTTCAGCTCGATGCGGTTCAC -3'

Cycling conditions:

95°C	3 min	
95°C	30 sec	⎫
62°C	30 sec	⎬ 40x
72°C	40 sec	⎭
4°C	∞	

Amplicon length: wild-type: 301 bp;

Lbx1^{GFP}: 464 bp.

b) Met

Wild-type PCR, 3 mM $MgCl_2$

Primers: Wmet8S: 5'-CTTTTTCAATAGGGCATTTTGGCTGTG-3'

Wmet10: 5'-GTACACTGGCTTGTACAATGTACAGTTG-3'

Mutant PCR, 4 mM $MgCl_2$

Primers: Wmet5: 5'-CACTGAGCCCAGAAGAGCTAGTGG-3'

neo1L: 5'-CCTGCGTGCAATCCATCTTGTTCAATG-3'

Cycling conditions:

94°C	2 min	
94°C	45 s	⎫
70°C	30 s	⎬ Mutant PCR 35x
72°C	30 s+1 s/cycle	⎭ Wild-type PCR 40x
4°C	∞	

Amplicon length: wild-type: 520 bp;

mutant: 310 bp.

c) CXCR4

Wild-type PCR, 1.5 mM $MgCl_2$

Primers: EngS: 5'- AACGTCCATTTCAATAGGATCT -3'

EngAS: 5'- TTAGCTGGAATGAAAACTGGAGGA -3'

Mutant PCR, 1.5 mM $MgCl_2$

Primers: CK4: 5'- CCATCCACAGGCTATCGGGGTAA -3'

Neo4: 5'- GCAGCGCATCGCCTTCTATC -3'

Cycling conditions:

95°C	2 min	
95°C	40 s	⎫
60°C	40 s	⎬ 40x
72°C	1 min	⎭
4°C	∞	

Amplicon length: wild-type: 950 bp;

mutant: 450 bp.

d) Gab1

Wild-type PCR, 1.5 mM $MgCl_2$

Primers: Gab1-wt1: 5'- CCCTTTGTGGATGGCTTCTTTGT-3'

Gab1-wt2: 5'- TTCTTGGCATGATCGTTTTTGTAA -3'

Mutant PCR, 1.5 mM $MgCl_2$

Primers: Gab1-wt1

NLSas2: 5'- TTGTTTTTCGAGCTTCAAGGTTCAT -3'

Cycling conditions:

95°C	2 min 30 s	
95°C	30 s	⎫
65°C	30 s	⎬ 36x
72°C	40 s	⎭
4°C	∞	

Amplicon length: wild-type: 336 bp;

mutant: 280 bp.

2.3.2.2. Generation of probes for *in situ* hybridization

Probes specific for mouse *SDF1*, *CXCR4* and *Pcdh17* were amplified from total mouse cDNA using PCR. The reaction product for both *SDF1* and *CXCR4* were then cloned into the pGEM-Teasy vector and used for *in vitro* transcription to generate RNA probes. Primers and optimal PCR conditions are presented below.

a) SDF1_*in situ* probe

Primers: sdf_upper: 5'- TGCACGGCTGAAGAACAACAAC -3'

sdf_lower: 5'- TCCCGACCCTGGCACTGAACTG -3'

Amplicon length: 660 bp.

Cycling conditions and reaction buffer are the same as was used for CXCR4 specific PCR.

b) CXCR4_*in situ* probe

Primers: cxcr_upper: 5'- TTGCCATGGAACCGATCAGTGTG -3'

cxcr_lower: 5'- CCCCGAGGAAGGCATAGAGG -3'

Amplicon length: 945 bp.

Cycling conditions and reaction buffer are the same as was used for CXCR4 specific PCR.

c) Pcdh17_*in situ* probe

Primers: pcdh_upper: 5'- GGGGGAAGGGCAAGAAGAAGAAAAT -3'

pcdh_lower: 5'- CACTGTCCGCCTGATCGCTGTCC-3'

Amplicon length: 441 bp.

Cycling conditions and reaction buffer are the same as was used for Gab1 specific PCR.

2.3.2.3. DNA sequencing

DNA sequences were determined using the dideoxy-chain-termination reaction protocol (Sanger et al. 1977) modified by Tabor and colleagues (Tabor and

Richardson 1987) and using the non-radioactive 'Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing'-Kit (Amersham-Pharmacia). Fluorescently labeled primers were obtained from MWG-Biotech. Cycle sequencing was performed using the following cycling parameters.

95°C	3 min
95°C	35 s
65°C	35 s
72°C	1 min
95°C	3 min

30x

Reaction products were resolved on 6% Sequagel XR polyacrylamide gels (Biozym) in 1xTBE running buffer and analyzed using a Li-Cor-Sequencing device (Model 4000L or 4200, MWG-Biotech).

2.3.3. Cell culture

All cell culture procedures were based on protocols according to "Gene Targeting: A Practical Approach" (Joyner 1999). Feeder cells as well as ES and COS-1 cells were stored in liquid nitrogen. For thawing vials with cells were quickly warmed up at 37°C and then the cells were transferred to 10 ml of warm medium. After centrifugation (1100 rpm, 3 min) the cells were resuspended in fresh medium and platted on the cell culture dish. Fibroblast medium was generally used for COS-1 and feeder cells, ES cell medium was used for culturing the ES cells.

2.3.3.1. Primary fibroblast cell culture

Primary fibroblast cells (feeder cells) were routinely prepared from embryos derived from mouse strains homozygous for a transgene containing a neomycin resistance cassette (*neoR*). *neoR* feeder cells survive during positive selection of ES cells with G418. Stocks of primary neomycin-resistant feeder cells were prepared from E14-16 embryos, passaged up to three times, and treated with mitomycin C for growth arrest prior to culture with ES cells.

2.3.3.2. Growth-arrest of embryonic fibroblast by mitomycin C treatment

A confluent plate of embryonic fibroblasts was washed with PBS and incubated for 2 h with 100 μl of mitomycin C stock solution (1 mg/ml in PBS, 5% DMSO, Sigma) in 10 ml of feeder medium. Then the cells were washed two times with PBS, incubated with 3 ml of 1x trypsin/EDTA at 37°C for 5 min, resuspended in feeder medium and centrifuged. The cell pellet was brought to a concentration of 2-3x10^5 cells/ml of feeder medium and plated on gelatinized plates.

2.3.3.3. ES cell culture, electroporation and neomycin-resistance selection

To maintain the pluripotency, ES cells were cultured in the presence of leukemia-inhibitory factor (LIF) on a layer of growth-arrested feeder cells. LIF-containing supernatant was routinely prepared from COS cells stably transfected with a LIF-expression plasmid (Genetics Institute Inc., Cambridge, MA, USA). Frozen ES cells were thawed rapidly and the DMSO-containing medium was immediately replaced with warm (37°C) ES medium. As a standard procedure, 10^7 ES cells were electroporated with 20 μg of linearized targeting vector in 0.8 ml PBS (240 V, 500 μF, BioRad Gene Pulser). The transfected cells were then plated on growth-arrested feeder cells (see chapter 2.3.3.2.) at a density of 2.5x10^6 cells per 100 mm dish and cultured in ES cell medium. Selection with 400 μg/ml G418 (Geneticin) was started 48 h later. Fresh selection medium was added daily to the ES cells. After additional 5-7 days culture with selective medium single, undifferentiated ES cell colonies were picked and cultured for additional 1-2 days in 96-well plates on feeder cells. Then the ES cell colonies were trypsinised and split into two 96-well plates; one plate without feeder cells for screening (replica plate – see below) and one plate with feeder cells for freezing. To freeze ES cells down, 1 volume of ice-cold 2X freezing medium (ES medium plus 13.3% DMSO) was added to confluent, trypsinised 96 well plates that had 1volume of trypsin in them. The plates were carefully wrapped in paraffilm and gradually frozen to –80^0C in styrofoam boxes. To screen the cells for homologous recombination by Southern analysis, a replica plate was made of each 96-well plate. The replica plate was coated with 0.1% gelatin (Sigma) before seeding of ES cells. These plates were grown to confluence and used to extract DNA to screen for targeted clones as described above.

The *neo*^{R} gene was removed from targeted ES cell clones by transient expression of Cre recombinase in ES cell (Gu et al. 1994; Kuhn and Torres 2002). The ES cell were electroporated with the pIC-cre vector as described above except that 10 μg of vector was used. The ES cell suspension was diluted with ES cell medium, and up to 1x10^{3} cells were plated on one 100 mm cell culture dish. ES cell colonies were picked after 8-9 days in culture and processed as above. ES clones were screened by Southern analysis as described above.

2.3.4. Generation of *Lbx1*^{GFP} mice

2.3.4.1. Generation of *Lbx1*^{GFP} targeting vector

The *Lbx1* targeting vector was assembled using the pTV-flox vector backbone (Riethmacher et al. 1995), which contains a *neomycin* gene flanked by loxP sites as well as a *thymidine kinase* gene to allow positive and negative selection of ES clones, respectively. *Lbx1* genomic DNA was isolated from a genomic library of 129Sv mouse DNA. A Cla I restriction fragment, which encompasses the first exon of *Lbx1* gene and 3 kb of upstream sequences and a Sse8387 I - Nhe I fragment that containes the second exon of the *Lbx1* gene and 4.8 kb of downstream sequence were subcloned separately into pBluescript II SK+ vector. The Cla I fragment was modified as follows: the *GAP43-GFP* cassette (from pAdlox-EGP) was fused to the start codon of *Lbx1*, sequences of the first exon of *Lbx1* were excised; in addition, a short double-stranded oligonucleotide containing a recognition site of the Pme I restriction enzyme was fused with the 5'-end of the Cla I fragment. An oligonucleotide that contains stop codon sequences was cloned into Bgl II site of the Sse8387 I - Nhe I fragment (which corresponds to the second exon of *Lbx1*); this is expected also to cause a frame-shift mutation. Finally, the targeting vector was assembled by cloning the modified 3.9 kb Cla I and the 6.1 kb Sse8387 I - Nhe I fragments into the pTV-flox vector upstream and downstream of the loxP-*neomycin*-loxP cassette, respectively. Prior to electroporation into ES cells, the targeting vector was linearized with Pme I and ethanol precipitated. The sample was centrifuged (10000 rpm, 10 min) and DNA pellet was washed twice with 70%

ethanol, air-dried and diluted in sterile-filtered water to a final concentration of 1 mg/ml.

2.3.4.2. Southern blotting

The Southern blot analysis (Southern 1975; Sambrook and Russell 2001) was used to screen ES cell clones for homologous recombination events, as well as during the establishment of the F1 generation of $Lbx1^{GFP}$ mice (to confirm germline transmission) and to verify efficiency of cre-mediated recombination.

Genomic DNA (10 μg) was digested ON with 20 U of restriction enzyme. The digested DNA was resolved on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. The DNA in the gel was depurinated and denatured. For this, the gel was incubated in 0.25 M HCl for 15 min, shortly rinsed in distilled water and denatured by two 30 min incubations with gentle shaking in a solution of 1.5 M NaCl and 0.5 M NaOH. Finally the gel was rinsed in 10X SSC and blotted ON using 20X SSC, in order to transfer the DNA onto a nylon membrane (Hybond N+, Amersham-Pharmacia) as described by (Southern 1975; Sambrook and Russell 2001). After the transfer, the membrane was air-dried and exposed to UV-light (120 mJ/cm^2) to prevent the loss of DNA from the membrane during hybridization. Subsequently the membrane was hybridized with specific radioactive probes. DNA probes (20-50 ng) were radioactively labeled with 50 μCi γ^{32}P-dCTP (Amersham-Pharmacia) using the 'Prime-It RmT Random-Primed Labeling Kit' (Stratagene). The labeled probes were purified from unincorporated nucleotides over Sephadex-G50 spin columns (Probe Quant G50, Amersham-Pharmacia). Prior to the hybridization probes were denatured by boiling for 5 min.

The hybridization protocol was adapted from Denhardt (1966). In order to prevent unspecific DNA binding, the membranes were prehybridized in 20-25 ml hybridization solution (6x SSC, 5x Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA) at 65^{0}C for at least 2 h in the hybridization oven (Biometra). The denatured probes were then added to the tubes containing the membranes in prehybridization buffer. The hybridization was carried out at 65^{0}C ON. In order to remove non-specifically bound probe from the membrane, the following washing steps were carried out in a shaking water bath at 65°C: 2x 15 min in 2x SSC,

0.1% SDS, 1x 30 min in 0.1x SSC, 0.1% SDS. The membranes were then sealed in plastic bags and exposed to a Phosphoimager (Fujix, BAS 2000) for several hours.

2.3.4.3. Microinjection into the blastocysts and establishment of the *Lbx1*$^{GFP/+}$ mutant strain

Two independent heterozygous *Lbx1*$^{GFP/+}$ ES cell clones were injected to establish mouse strains that carry a mutation in the *Lbx1* gene (Hogan et al. 1994). C57Bl/6J females (20-23 days old) were injected interperitoneally with 100 μl 50 U/ml PMS in PBS („Pregnant Mare's Serum“ = Intergonan, Intervet GmbH, Tönisvorst). This serum contains Follicle-Stimulating hormone (FSH). Two days later females were injected with 100 μl 50 U/ml hCG in PBS (human Chorionic-Gonadotropin, Ovogest, Intervet GmbH, Tönisvorst) und then mated with C57Bl/6J males. Blastocysts were harvested on day 3.5 post-coitum by flushing the uterus with blastocyst medium (Fibroblasts-medium with 30 mM HEPES, pH 7.2). For microinjection, 10-15 *Lbx1*$^{GFP/+}$ ES cells were injected into each blastocysts and approximately 16 of such injected blastocysts were implanted into the uterine horns of time-matched pseudo-pregnant CB6F1 foster mice (day 2.5 post-coitum). The resulting chimeric male offspring were crossed with C57Bl/6J females, and the offsprings with brown coat color were analyzed for their genotype by PCR.

2.3.5. Affymetrix analysis

2.3.5.1. FACS sorting

The forelimbs of *Lbx1*$^{GFP/+}$ or *Lbx1*$^{GFP/GFP}$ embryos were dissected; cells were dissociated by treatment with 0.02% Trypsin (PAN^{TM} Biotech GmbH) for 10 min at 37°C with mild agitation. Tissue debris and non-dissociated cells were excluded from the cell suspension by passing the cells through a 70 μm cell strainer. Prior to the sorting procedure, cell suspension was stained with propidium iodide (PI). PI-negative, GFP-positive cells were isolated using fluorescence-activated cell sorting (FACS). Cell sorting was performed on a FACSVantage™ SE (Becton-Dickinson) at the FACS unit of the Rheumaforschungszentrum, Berlin. 488 nm- and 635 nm-lasers were used for excitation. GFP fluorescent cells were routinely collected at 540 nm.

The sorted cells were lysed in Trizol (Gibco BRL) for 5 min at RT and stored at -70°C.

2.3.5.2. Synthesis of cDNA

In order to generate specific probes for *in situ* hybridization or biotin-labeled cRNA probes for hybridization on Affymetrix chips, cDNA was synthesized from total RNA as follows. Total RNA isolated from FACS sorted cells was mixed with 1 μl (10 mM) of T7-$(dT)_{24}$ primer (5`-GGCCAGTGAATTGTAATACGACTCACTATAGGGA GGCGG -$(dT)_{24}$-3`) and denatured for 5 min at 70°C, followed by annealing for 2 min at RT. For the first strand cDNA synthesis the RNA- T7-$(dT)_{24}$ primer cocktail was combined with 1st strand synthesis mixture (10 mM DTT, 20 U RNAse Inhibitor (Gibco BRL), 1.25 mM dNTPs, 1 μl Superscript II RT Polymerase in 1x1st strand synthesis buffer (SupesScript II kit, Invitrogen)) and incubated for 1 h at 42°C. The reaction was cooled on ice for 5 min and then the second strand synthesis reaction mixture (0.33 mM dNTPs, 40 U E.coli DNA Polymerase I (NEB), 5 U E.coli DNA Ligase (NEB), 0.7 U RNAse H (Roche) in 1x2st strand synthesis buffer (SupesScript II kit, Invitrogen)) was added. The second strand synthesis was performed for 2 h at 16°C. The resulting double stranded cDNA was cleaned up by extraction with neutral phenol/chloroform (Ambion) and precipitation with 100% ethanol in the presence of 0.1 M ammonium acetate. After a centrifugation (14000 rpm, 10 min), the DNA-precipitate was washed twice with 70% ethanol, air-dried and dissolved in 4 μl of H_20_{DEPC}. The synthesis of cDNA from whole embryo RNA was carried out similarly and the cDNA-pellet was dissolved in 50 μl of H_20.

2.3.5.3. Preparation of biotin-labeled cRNA probes and hybridization on Affymetrix GeneChips

RNA obtained after FACS sorting of limb cells from approximately 15 embryos (E10.5) was extracted, purified and cDNA synthesis was performed as was described above (see chapters 2.3.1.4. and 2.3.5.2.). Hybridization of one Affymetrix GeneChip requires 10 μg of cRNA probe. To obtain the required amount of probe, cDNA was subjected to an amplification step. For this, the cDNA, that contains sequences, complementary to those of the T7 promoter, was annealed with T7-$(dT)_{24}$ primer.

Antisense RNA was transcribed from the T7 promoter using the T7 Megascribe kit (Ambion) and following the manufacture's protocol. Antisense RNA was cleaned up using acidic phenol/chloroform (Ambion) extraction and was precipitated with 100% ethanol in the presence of 0.1 M ammonium acetate. The mixture was centrifuged (14000 rpm, 10 min), washed twice with 70% ethanol, air-dried and dissolved in 5 μl of H_20_{DEPC}. The second round of cDNA synthesis was performed as follows. Antisense RNA was annealed with 1 μg (0.5 μg/μl) of random hexamer oligonucleotides (Pharmacia Biotec), combined with 12 μl of the 1st strand synthesis mixture and incubated for 1 h at 42^0C. Following the 1st strand synthesis, RNA was cleaved by treatment with RNAse H (1 U; Roche) for 20 min at 37^0C and the reaction mixture was then denatured (2 min at 95^0C followed by 5 min on ice) to dissociate the 1st DNA strand from RNA fragments. After this, the 2nd strand of DNA was synthesizes. For this, the 1st strand of DNA was annealed with 10 μg of T7-$(dT)_{24}$ primer DNA and then combined with 130 μl of the 2nd strand synthesis mixture. The sample was incubated for 2 h at 16^0C, then 5 U of T4 DNA polymerase was added and incubation was continued for another 5 min. The resulting cDNA was cleaned using double-stranded DNA cleanup columns (Affymetrix). To generate biotin-labeled cRNA probes, cDNA was transcribed using the Bioarray High Yield RNA Labeling Kit (Enzo) according to manufacturer's instructions and purified by phenol/chlorophorm (Ambion; pH 5.0) extraction. The concentration and purity of the RNA were assessed by UV-spectrophotometry. 10 μg of cRNA were fragmented in 1x fragmentation buffer (Affymetrix) for 35 min at 94^0C. Hybridization of Affymetrix GeneChips MGU74v2 (Affymetrix) with 10 μg of cRNA probe per chip as well as scanning of hybridized chips was performed at the microarray unit of the Max-Delbrueck Center (Berlin). Signal intensity values obtained after hybridization of 3 independent cRNA probes for both $Lbx1^{GFP/+}$ and $Lbx1^{GFP/GFP}$ were statistically analyzed using the Microarray Suite 5.0 software.

2.3.6. COS-1 cell transfection and implantation *in ovo*

The coding sequences of chicken *SDF1* (Stebler et al. 2004) or *EGFP* (Clontech) were cloned into the pcDNA3.1(-) expression vector (Invitrogen). Before transfection, 10^6 COS-1 cells were platted on 100 mm dish and incubated ON. COS-1 cells grown to near confluence were transfected with 10 µg of pcDNA-SDF1 and/or

pcDNA-EGFP, using the Lipofectamine2000 kit (Gibco BRL) and following manufacturer's instructions. Transfection efficiency was estimated 36 h later by expression of EGFP that was visualized under UV-light. Fertilized White Leghorn chicken eggs (Charles River) were incubated at 38°C in a humidified incubator until stages HH19–20 (Hamburger and Hamilton 1992). Following the removal of 1 ml of albumin, eggs were windowed and COS-1 cell aggregates that express SDF1/EGFP or EGFP alone were implanted into the right limb of chick embryos with the help of a thin glass capillary. Chick embryos (HH24-25), in which I detected EGFP expressing cells in the limb, were harvested and analyzed.

2.3.7. Phenotype analysis: histology and staining procedures

2.3.7.1. Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was used to detect the mRNA expression in entire mouse and chick embryos and optimal hybridization conditions were established based on the protocol developed by D. Henrique and D. Ish-Horowicz (Oxford, UK). Mouse and chick embryos were processed similarly unless noted otherwise.

Tissue preparation

Embryos were harvested from eggs (in the case of chick) or from uterus (of mouse), extraembrionic membranes were dissected in ice-cold PBS, collected and used to determine the genotype of mouse embryos. To avoid probe trapping, all embryonic cavities (forebrain, otic vesicles, eyes, heart) were opened by puncturing with a tungsten needle. All procedures for tissue preparation were performed at 4^0C with mild agitation. Embryo tissue was fixed in 3 ml of 4% PFA/PBS ON. After the fixation step, embryos were washed three times in PBT (PBS, 0.15% Tween-20) for 10 min each and dehydrated by proceeding the tissue through a gradient of methanol for 15 min each step: 25%, 50%, 75%, 100%, 100%. To discolor the embryos, tissue was treated with 3% H_2O_2 in methanol for 1 h at -20^0C. Finally embryos were washed with 100% methanol three times 10 min each and stored in 100% methanol at -20^0C for up to 6 month.

1st day

Procedures at the 1st day of *in situ* hybridization were performed at 4^0C with mild agitation; rinsing steps were immediate, washing steps lasted for 10 min, unless indicated otherwise. During the first day of *in situ* hybridization special attention should be paid to the quality of solutions: buffers should be mixed from stock-solutions on the day of use, all solutions must be RNAse-free. Mouse and chick embryos were processed similarly at the first day of whole-mount *in situ* hybridization with small variations, which are indicated separately.

Embryos were rehydrated in 75%, 50%, 25% methanol/PBT gradient (10 min each step) and afterwards washed twice with PBT. To improve the permeability, the tissue was treated with 10 μg/ml proteinase K in PBT at 37^0C: E10-11 mouse embryos were incubated for 10-15 min, while proteinase K treatment of HH22-25 chick embryos was prolonged up to 30 min due to the larger size of these embryos. Proteinase K activity was then blocked by 2 min incubation with 2 mg/ml of glycin/PBT (Sigma). Embryos afterwards were rinsed twice with PBT and post-fixed for 20 min in freshly prepared 4% paraformaldehyde + 0.2% Glutaraldehyde in PBT. Finally embryos were washed three times with PBT, equilibrated in hybridization buffer and transferred into screw cap 4 ml hybridization tubes. To prevent unspecific binding of RNA probe, embryos were incubated for 2 h in 2 ml of hybridization buffer at 70^0C. After the prehybridization, the solution in the tubes was replaced with hybridization buffer containing 1 μg/ml denatured (80^0C, 5 min) DIG-labeled RNA probe and incubation was continued at 70^0C for at least 16 h.

Hybridization buffer:	Final concentration
Formamide (Gibco BRL Ultrapure)	50%
SSC (pH5.0)	1.3xSSC
EDTA (pH8.0)	5 mM
Yeast RNA (Sigma)	50 μg/ml
Tween-20 (Sigma)	0.2%
CHAPS (Sigma)	0.5%
Heparin (Fisher)	100 μg/ml
H_2O_{DEPC}	

Preparation of DIG-labeled RNA probe

DIG-labeled antisense RNA probes were transcribed from cDNA templates cloned into a vector (usually pBluescript II SK+ or pGEM-Teasy). To terminate *in vitro* transcription, plasmid DNA was linearized at the 5'-end in respect to the sense direction of cDNA using appropriate restriction enzymes. Usually 30 μg of cDNA were digested in 40 μl reaction mixture. *In vitro* transcription reaction was initiated from T7, T3 or Sp6 promoters and carried out using the 'DIG-RNA Labeling-Kit' (Roche) according manufacturer's instructions. After the reaction was completed, RNA was cleaned through RNeasy mini spin columns (RNeasy Mini kit (250), Qiagen). RNA probes were stored in 50% formamide at -70^{0}C.

2nd day

In order to remove unbound RNA probe, following washes were performed with 3 ml of preheated solutions per hybridization tube: embryos were rinsed 2 times with Solution 1 (see below) at 70^{0}C followed by a 30 min wash in Solution 1 at 70^{0}C; 2 times 30 min each at 65^{0}C in Solution 2; 20 min at 65^{0}C in Solution 2/MABT (1:1). Solution 1 and Solution 2 are distinct for mouse and chick embryos (see below). Then the embryos were washed 3 times in MABT for 30 min each at RT and incubated in 2% Boehringer Blocking Reagent/20% sheep serum/MABT for 2 h at 4^{0}C. In meantime, an antibody mixture was prepared. To pre-absorb the antibody, embryo powder was used, which was prepared as follows: embryos at E18 were collected, homogenized in ice-cold acetone and air-dried. A small amount of embryo powder was heat inactivated in 1 ml of MABT at 55^{0}C, shortly centrifuged (1000 rpm, 1 min) and resuspended in 5% sheep serum/MABT. Alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Ingelheim GmbH) was incubated in embryo powder/5% sheep serum/MABT suspension for 2 h at 4^{0}C. The embryo powder was removed from the antibody containing solution by short centrifugation (1000 rpm, 1 min), supernatant was added to 2% Boehringer Blocking Reagent/20% sheep serum/MABT and embryos were incubated ON at 4^{0}C in 2 ml of this final antibody mixture.

	Solution1:		Solution2	
	Chick	Mouse	Chick	Mouse
Formamide	50%	50%	50%	50%
SSC, pH 5.0	2x	2x	1.3x	1.3x
EDTA, pH 8.0	5 mM	5 mM	5 mM	5 mM
SDS	1%	-	1%	-
Tween-20	-	0.2%	-	0.2%

MABT:
100 mM maleic acid, 250 mM NaCl, 0.15% Tween-20; pH is adjusted with NaOH up to 7.5.

3rd day

Unbound antibody was removed by extensive washes (at least 10 times, 30 min each) at RT. Afterwards embryos were equilibrated in freshly prepared NTMT buffer (100 mM NaCl; 100 mM Tris-HCl, pH 9.5; 50 mM $MgCl_2$; 0.15% Tween-20) and alkaline phosphatase activity was visualized by adding the NBT/BCIP substrates to the NTMT buffer (3.5 μl NBT (Roche) + 3.5 μl BCIP (Roche) in 1 ml of NTMT). Staining was carried out at RT. When color has developed to the desired extent, NBT/BCIP was removed from the staining solution by several washes with NTMT buffer. Embryos were then refixed in 4% PFA/PBS in order to inactivate alkaline phosphatase activity, washed for several times in PBS and stored at 4^0C.

2.3.7.2. Preparation of vibratome sections

In order to analyze the histology of embryos after whole-mount *in situ* hybridization, 35 μm vibratome sections were performed using a Vibratome (Leica VT1000S, Bensheim). For this, embryos were placed into preheated (55^0C) 20% gelatin/PBS and embedded at 4^0C. Gelatin molds were fixed ON in 4% PFA/PBS at 4^0C and were washed several times with PBS to remove PFA. Sectioning was carried out in PBS, sections were placed on to glass slides, dried at RT and finally covered with 'Immu-Mount' (Shandon, Frankfurt).

2.3.7.3. Preparation of frozen sections

Immunohistochemical analysis and *in situ* hybridization (in some cases) were performed on frozen sections. Mouse embryos were dissected from the uterus in PBS; embryonic membranes were striped and used to determine the genotype of the embryo by PCR. If prepared embryos were older then E11.0, body walls were opened and internal organs were removed for better solution penetration. Embryo tissue was fixed with freshly prepared 4% PFA/PBS at 4^0C for 2 h (for immunohistochemistry) or ON (for *in situ* hybridization). Afterwards, the tissue was washed extensively at 4^0C in PBS for several hours and cryo-protected by incubation in 20% sucrose/PBS ON. At the next day embryos were embedded in "TissueTek" (Sakura, Zoeterwoude, Nederland) and 12 μm sections were cut on a cryostat (Microm HM560, Walldorf). Sections were collected onto slides (Histobond, Marienfeld) and dried at 37^0C for 2 h. Slides as well as "TissueTek" blocks can be stored for 6 month at -70^0C.

2.3.7.4. *In situ* hybridization on frozen sections

The protocol for *in situ* hybridization on frozen section was adapted from N. Pringle (Richardson lab, London).

1st day

Slides with frozen sections were thawed at RT for at least 2 h, no further treatments were performed prior to hybridization. DIG-labeled RNA probes were prepared as was described before (chapter 2.3.7.1. "Whole-mount *in situ* hybridization"). RNA probes were denatured for 5 min at 80^0C, 0.5 μg RNA/slide in hybridization buffer was directly applied on sections, cover-slipped and hybridized ON at 65^0C in a sealed container with Whatman filter paper soaked in 2x SSC plus 50% formamide.

Hybridization buffer:	Final concentration
Formamide (Gibco BRL Ultrapure)	50%
SSC (pH 5.0)	2xSSC
EDTA (pH 8.0)	5 mM
Yeast RNA (Sigma)	0.1 mg/ml
Dextrane sulfate (Sigma)	10%
Denhardt's (Sigma)	1x
H_2O_{DEPC}	

2nd day

After the overnight hybridization, slides were washed with MABT (100 mM maleic acid pH 7.5, 150 mM NaCl, 0.15% Tween-20) at RT to remove the coverslips from the slide, which was followed by 2 washes 30 min each at 65^0C in 1x SSC, 50% formamide, 0.1% Tween-20. The slides were then equilibrated in MABT and incubated in blocking solution (2% Boehringer Blocking Reagent/10% sheep serum/MABT) for 1 h at RT. Alkaline phosphatase anti-DIG antibodies were diluted in blocking buffer (1:2000), 150 μl of antibody solution was applied on each slide and incubated overnight in a humidified chamber at 65^0C.

3rd day

The slides were transferred into Coplin jars and washed 6 x 30 min in MABT, then 2 x 10 min in staining solution (100 mM Tris-HCl pH 9.0, 100 mM NaCl, 50 mM$MgCl_2$, 5% polyvinyl alcohol (av. Mw ~100k)). The colour reaction was performed at RT in a humidified chamber with staining solution containing 3.5 μl/ml NBT (Roche) and 3.5 μl/ml BCIP (Roche). When the signal reached a satisfactory intensity, slides were washed in PBS several times to remove excessive NBT/BCIP and finally covered with Immu-Mount (Shandon).

2.3.7.5. Immunohistochemistry

Tissue sections were thawed at RT for at least 2 h and then washed shortly with PBS to remove the "TissueTek". Unspecific binding of antibodies was blocked by incubation with 2% inactivated horse serum/0.1% Triton X-100/PBS (HS/PBX) for 1-2 h at RT. For immunostaining with anti-myosin antibody, 5% HS/PBX was used for blocking step as well as for further procedures. After the tissue was saturated, slides were incubated with the primary antibody diluted in 2% HS/PBX (ON, 4°C) with rocking or alternatively the incubation was performed at RT for 2 h. Following the incubation with primary antibody, the sections were washed 4 times with PBX for 10 min each to remove unbound antibodies, and then Cy2, Cy3 or Cy5-conjugated secondary antibodies (diluted in 2% HS/PBX) were applied to the sections and the sections were incubated for 1 h at RT. The same washing procedure as above was performed or if necessary washes were repeated more than 4 times. Finally, slides were covered with "Immu-mount" (Shandon).

Immunofluorescence staining with anti-CXCR4 antibody was performed using the 'Cy3-TSA Fluorescence System kit' (PerkinElmer Life Siences). The protocol provided by the manufacturer was slightly modified to obtain better signal intensity. For this, frozen section were thawn, dried at RT and shortly rinsed with PBS to remove the "TissueTek". Unspecific antibody binding was blocked by incubation with TSA-blocking buffer (10% heat inactivated goat serum, 0.5% NEN powder (PerkinElmer Life Siences), 0.1% Triton X-100 in PBS). Afterwards sections were incubated with rabbit anti-CXCR4 primary antibody prediluted in TSA-blocking buffer for 1 h at RT. Sections were washed in PBX four times 10 min every washing step and were incubated for 1 h at RT with biotinilated anti-rabbit secondary antibodies (in TSA-blocking buffer). Then the slides were washed again and the sections were incubated for 1h at RT with HRP-conjugated streptavidin (PerkinElmer Life Siences ; prediluted 1:200 in TSA-blocking buffer). TSA-Cy3 was prediluted 1:50 in Amplification buffer (PerkinElmer Life Siences) and applied on the sections at room temperature for 7 min. Afterwards the sections were extensively washed and slides were covered with "Immu-mount" (Shandon). When Cy3-conjugated tyramide reagent (TSA-Cy3) is cleaved by HRP, it gets covalently bound to the tissue in the vicinity of HRP source thus visualizing its location.

2.3.7.6. Detection of cell proliferation and apoptosis

To detect muscle precursor cells proliferation, BrdU (5-Bromo-2'-deoxy-uridine) was injected into the dams 2 h prior to the dissection of the embryos with 75 μg of BrdU per gram of body weight. BrdU incorporates into DNA only in mitotically active cells and can be detected using anti-BrdU antibodies. After 1 h of chasing time, embryos were harvested and processed as described for preparation of frozen sections. Sections were postfixed in 4% PFA/PBS for 15 min at RT and then washed with PBS three times for 10 min. DNA was denaturated by incubation in 2.4 M HCl for 30 min at 37^0C. Afterwards sections were washed as above and incubated with 20 μg/ml proteinase K in PBS at RT for 10 min to ensure good penetration of the antibody. After extensive washing, the sections were blocked and immunohistochemistry was performed as described above.

Cell apoptosis was detected by terminal deoxynucleotidyle transferase nick-end labeling (TUNEL; (Gavrieli et al. 1992). Slides were immunohistochemically stained with the 1st and 2nd antibodies as described above (see chapter 2.3.7.5.) and afterwards were postfixed in 4% PFA/PBS for 15 min at 4^0C. After that the slides were processed according to manufacturer instructions.

2.3.7.7. Preparation of paraffin sections

In situ hybridization on sectioned tissue was routinely performed using paraffin sections. Embryos were dissected in PBS, fixed with 4% PFA/PBS at 4°C ON and extensively washed with cold PBS. Afterwards embryonic tissue was dehydrated at RT in an ethanol series: 35%, 50%, 80%, 96%, 100% for at least 5 h each step. After dehydration, embryos were incubated in toluol two times for 1 h to facilitate the paraffin penetration into the tissue. Tissue was then incubated in three series of paraffin (Roti-Plast, Roth) for at least 12 h each time at 56^0C. Afterwards the embryos were embedded into cassettes at RT and the resulting solid molds were used for sectioning. The 5-10 μM sections were spread out onto glass slides and dried at 40^0C for 6 h. Paraffin sections can be stored for up to 1 year at 4°C.

2.3.7.8. *In situ* hybridization on paraffin sections

In situ hybridization on paraffin sections was performed similarly to hybridization on frozen sections with alteration introduced only during the 1st day (tissue pretreatment and hybridization).

1st day

All procedures at the 1st day were carried out at RT with mild agitation, unless noted otherwise. Paraffin was removed from tissue by 3x 5 min incubation in xylol and sections were rehydrated at RT in an ethanol series: 100%, 95%, 85%, 70%, 50%, 35% for 2 min each step. Then the slides were washed in PBS_{DEPC} and postfixed in 4% PFA/PBS for 15 min, which were followed with 2x 5 min in PBS. In order to discolor the sections, slides were treated with 6% H_2O_2/PBS for 15 min and again washed 3x 2 min with PBS. Tissue was then permeabilized by proteinase K treatment (10 µl/ml in PBS, 10 min), shortly incubated with 0.2% glycin/PBS (inactivation of

proteinase K), rinsed in PBS and again postfixed in 4% PFA/PBS for 15 min. PFA was removed by 2x 5 min in PBS and 1x 2 min in 100 mM Tris-HCl (pH 7.5). Then the slides were acetylated (10 min in 0.25% acetic anhydride in 100 mM Tris-HCl (pH 7.5)), equilibrated in 2x SSC (pH 7.5) and dehydrated in an increasing gradient of ethanol (2 min each step): 30%, 50%, 70%, 85%, 95%, 100%. Dehydrated slides were dried for 30 min and 100 µl of hybridization solution plus denatured DIG-labeled RNA probe was applied on each slide. Afterwards the slides were covered with Sigmacote-pretreated coverslipes (Sigmacote (Sigma) 5 s; air-dry 20 min; 100% ethanol 5 s; air-dry) and hybridized overnight at 65°C in a chamber with Whatman filter paper soaked in 2x SSC plus 50% formamide.

Hybridization buffer:	Final concentration
Formamide (Gibco BRL Ultrapure)	30%
SSC (pH 5.0)	3xSSC
Boehringer blocking reagent	3%
Dextrane sulfate	7%
EDTA (pH 8.0)	3 mM
Yeast RNA (Sigma)	50 μg/ml
Tween-20 (Sigma)	0.05%
Heparin (Fisher)	100 μg/ml
DIG-labeled RNA probe (denatured 5 min, 95°C)	1 μg
H_2O_{DEPC}	

On the 2nd (post-hybridization washes, antibody staining) and 3rd day (post-antibody washes, color reaction) paraffin sections were processed as described in chapter 2.3.7.3. „*In situ* hybridization on frozen sections“ starting with the 2nd day procedures.