2 Materials and Methods

2.1 Materials

Chemicals/ enzymes/ equipment	Company
Dietylpyrocarbonate (DEPC)	Aldrich
dNTPs (2'-Deoxynucleoside 5'-triphosphates)	Roche
Ethidiumbromide	Serva, Heidelberg
Ficoll 400	Pharmacia
SDS (sodium dodecyl sulfate)	Serva
Agarose	Gibco BRL
NaCl	Merck
KCl	Merck
LiCl	Merck
$NaH_2PO_4*2H_2O$	Merck
Formamide	Roth
Xylol	Roth
DPX Mountant for histology	Fluka
sodium pyruvate	Merck
Methotrexate	Sigma
Paraffin-paraplasts	Sherwood Medical Co, USA
Tris-HCl	Merck
EDTA (Ethylendiamintetraacetic acid)	Merck
Glutaraldehyde	Sigma
TEA, triethanolamine	Merck
[P^{33}]-UTP (radioactive Uridintriphosphat)	Amersham
CTP, GTP, ATP, UTP	Roche

RNAse inhibitor	Roche
DNAse (deoxyribonuclease), RNAse free	Roche
RNAse A	Roche
T7 RNA-polymerase	Roche
T3 RNA-polymerase	Roche
Sp6 RNA-polymerase	Roche
Proteinase K	Roche
Acetic anhydride	Sigma
Acetic acid	Merck
Glycerol	Merck
Ethanol	Roth
Glycogen	Roche
Dextran Sulfat	Sigma
PFA (Paraformaldehyde)	Merck
All used restriction endonucleases (see table 2 in 2.1.10)	NEB, Roche
Taq-DNA polymerase	Eppendorf,
HEPES	Calbiochem, USA
fatfree milk (Instant-Magermilchpulver)	Neuform, Germany
Agar	Difco
Yeast extract	Difco
Trypton	Difco
FBS (Fetal Bovine Sera)	Gibco
Ampicillin	Sigma
Centricon Plus-20 filter	Amicon, USA
Cellulose Acetate filter 0.2mm	Millipore
Hybond ECL Nitrocellulose membrane	Amersham
Millipore AA filter, 25 mm	Millipore

Phenol	Roth
Chloroform	Roth
Toluidine Blue O	Sigma
coverslips for *in situ* hybridization	Roth
culture grids	Wire Mesh Corporation, USA
Tissue culture dish 60x15 mm	Falcon
Forceps 5, 55	Dumont, Switzerland
Glass coverslips (Deckglaeser)	Menzel-Glaeser, Germany
Superfrost plus slides	Menzel-Glaeser, Germany
(3-aminopropyl)triethoxysilane	Sigma

2.1.1 Bacterial media

Medium name	**Ingredients**
LB medium (Luria Bertrani):	10g/L trypton
	5g/L yeast extract
	10g/L NaCl
LB agar:	15g agar per 1L LB-medium

2.1.2 Limb-culture media

Media/ Buffer	**Ingredients**
PBS (Phosphat Buffered Saline):	1.5 mM KH_2PO_4
	140 mM NaCl
	3 mM Kcl; pH 7.4
BSA (Bovine Serum Albumin)	Gibco BRL
100x antibiotic-antimycotic	Gibco BRL
BGJ-b medium	Gibco BRL

Limb culture medium	BGJ-b medium
	0.1% BSA
	1x antibiotic-antimycotic
Paraformaldehyde	4% (w/v) in PBS

2.1.3 Reagents and buffers for *in situ* hybridization

Reagent/ Buffer	Ingredients
10x transcription buffer	Roche
Proteinase K:	resuspended in 50 mM Tris-HCl, pH 8,0; 1 mM $CaCl_2$, stored as 20 ng/ml aliquots at -20°C
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)
50x Denhardt's reagent:	50g Ficoll
	5g polyvinylpyrrolidone
	5g BSA
	H_2O to 500 ml
RNAse free water (DEPC water)	0,1% DEPC (Diethylpyrocarbonat)
10xWash buffer:	4 M NaCl
	0.1M Tris-HCl,
	0,05 M EDTA; pH 7.5

20x SSC (standard saline citrate):	300 mM Sodiumcitrate 3 M NaCl; pH 7.0
Photoemulsion (autoradiography emulsion type NTB2)	Kodak, USA
Kodak-developer D-19	Kodak, USA
Kodak-fixer	Kodak, USA
Kodak Scientific Imaging Film	Kodak, USA
DPX Mountant for histology	Fluka

2.1.4 Buffer for mice genotyping

Reagent/ Buffer	Ingredients
10x PCR-buffer (for Polimerase Chain Reaction)	Eppendorf
Tail lysis buffer:	50 mM Tris-HCl, pH 8.0 100 mM EDTA 1% SDS 100 mM NaCl

2.1.5 Reagents and buffers for protein purification

Reagent/ Buffer	Company, Ingredients
Noggin-medium	α-MEM without nucleosides 5% dialysed FBS 80% µl Methotrexate (for first 2 passages, see method 2.2.11) 1mM Sodium Pyruvate 0.1mM non-essential amino acids

Medium for CHO dhfr$^-$ cells	α-MEM with nucleosides 10% dialysed FBS
α-MEM without nucleosides	Gibco
α-MEM with nucleosides	Gibco
SP-Sepharose Fast Flow	Amersham, Sweden
Mark12 MW Standard (4x)	Novex, USA
Chemotripsinogen α	Sigma
4-12% Bis-Tris-Gel	Novex, USA
NuPage Antioxidant	Novex, USA
NuPage Sample buffer (4x)	Novex, USA
NuPage Sample Reducing Agent (10x)	Novex, USA
NuPage MES SDS Running Buffer (20x)	Novex, USA
Buffer A: Low Salt Buffer	0,4 M NaCl 10 mM HEPES 1 mM EDTA 10% glycerol pH 7.6
Buffer B: High Salt Buffer	1 M NaCl 10 mM HEPES 1 mM EDTA 10% glycerol pH 7.6
TBST (1 liter)	5 ml 1 M Tris pH 7.6 30 ml 5 M NaCl 2 ml 25% Tween-20 (v/v, filter sterilized)

2.1.6 Kits

Kit	Company
Plasmid Midiprep Kit	Qiagen, Germany
QIAprep Plasmid Miniprep kit	Qiagen, Germany
BrdU labeling and detection kit II	Roche, Germany
ABC kit	Vectastain, USA
DAB substrate kit for peroxidase	Vectastain, USA
SilverXpress, silver staining kit	Novex, USA
Detection kit II for BrdU	Roche, Germany

2.1.7 Proteins

Protein	Company
mouse 1A4 anti Noggin IgG	kindly provided by R. Harland
biotinylated anti-mouse-IgG (H+L)	Vector, USA
Recombinant murine Shh-N	Ontogeny, Cambridge, MA
Pthlh	human 1-34 PTHrP, Peninsula, USA
recombinant human BMP2	Genetic Institute, Boston, USA
FGF2	FGFb, Sigma
alkaloid cyclopamine	kindly provided by William Gaffield (Incardona et al., 1998)
recombinant Xenopus Noggin protein	purified from stably transfected CHO cells, kindly provided by Richard Harland (Lamb et al., 1993)

2.1.8 Table 1. Protein names and their synonyms

Protein name from a standardized Genetic Nomenclature	used in literature synonyms
BMPR-II, bone morphogenetic protein receptor type II	BMPR2
BMPR-IA, bone morphogenetic protein receptor type 1A	ALK3, activin receptor-like kinase 3
BMPR-IB, bone morphogenetic protein receptor type 1B	ALK6, activin receptor- like kinase 6
BMP2, bone morphogenetic protein 2	BMP2a, bone morphogenetic protein 2a
FGF2, fibroblast growth factor 2	bFGF/ Fgf-2/ Fgfb
Pthlh, parathyroid hormone-like peptide	Pthrp; PTH-like; parathyroid hormone-like hormone (Pthlh); parathyroid hormone-related protein; parathyroid hormone-related peptide; PTH-related peptide
Pthr, parathyroid hormone receptor	PPR/PTH-related peptide receptor; PTH/PTHrPR
Spp1, secreted phosphoprotein 1	osteopontin; bone sialoprotein; 44 kDa bone phosphoprotein; OPN; minopontin; BNSP; BSPI; ETA-1; Apl-1; Eta; Opnl; Opn; Spp-1; Ric
Osteocalcin	mOC-A/ Bglap/ OG1/ bone Gla protein

2.1.9 Table 2. Chicken and mouse DNA probes for *in situ* transcription

Probe	Vector	Insert size (b.p.)	DNA Endonuclease (for antisense)	RNA-polymerase (for antisense)	Reference
cIhh	pBSK	560	*Eco*R I	T3	(Vortkamp et al., 1996)
cPthlh	pBSK	600	*Xba* I	T7	(Thiede and Rutledge, 1990)
rCol II	pGEM-3Z	550	*Hind* III	T7	(Kohno et al., 1984)
rPthlh	pBSK+	500	*Bam*H I	T7	(Karaplis et al., 1990)
rPthr	pcDNAI	500	*Bam*H I	SP6	(Abou-Samra et al., 1994)
mCol X	pBSK+	400	*Cla* I	T3	(Jacenko et al., 1993)
mSpp1	pGEM-T	440	*Not* I	T7	(Kim et al., 1999)
mBmp3	pGEM-3	391	*Hind* III	T7	(Bitgood and McMahon, 1995)
mBmp4	pSP72	1000	*Eco*R I	SP6	(Bitgood and McMahon, 1995)
mBmp7	pBSK-	900	*Hind* III	T3	(Bitgood and McMahon, 1995)
FGF18	pBSK	900	*Pst* I	T3	(Liu et al., 2002)
MMP13	pCR-21	1000	*Hind* III	T7	(Yamagiwa et al., 1999)
OC	pBSK	500	*Xba* I	T3	(Desbois et al., 1994)
mIhh	pBSK	1800	*Xba* I	T7	(Bitgood and McMahon, 1995)

2.1.10 Cell lines

CHO dhfr- (Chinese hamster ovary cells, dihydrofolate reductase-deficient), CHO B3.A4 (Noggin producing CHO cells). Both cell lines were kindly provided by R.Harland, USA (Lamb et al., 1993).

2.1.11 Chicken and mouse lines

Wild type mice (NMRI) and pathogen free white leghorn chicken eggs were derived from Charles River (Sulzfeld, Germany). *Col-II-Gal4* and *UAS-Ihh* transgenic mice were kindly provided by A. McMahon (Long et al., 2001). FGFach mice were kindly provided by D. Ornitz (Naski et al., 1998). Transgenic mice were identified by PCR of tail DNA (see genotyping in methods)**.**

2.1.12 Bacterial strain

Escherichia coli DH5a™	Gibco BRL, Karlsruhe
	Genotype: F⁻f80dlacZDM15 D(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 1-*thi*-1 *gyr*A96 *rel*A1

2.2 Methods

2.2.1 Genomic DNA preparation from mouse tail

Piece of mouse tail was placed in 500 µl of tail lysis buffer with 20 µl of 20 mg/ml proteinase K in 1.5 ml tube and incubated at 55°C overnight. The mix was cooled until room temperature (RT) and 200 µl of 6 M NaCl was added. Mix was shacked vigorously for 1 minute and incubated for 10 minutes on ice. Mix was centrifuged at 14,000 rpm for 8 minutes. Supernatant was transferred to the clean tube and 1 ml of 100% ethanol (RT) was added. Precipitate was centrifuged at 14,000 rpm for 15 minutes at RT. DNA pellet was resuspended in 100 µl TE at 65°C for 20 minutes.

2.2.2 Genotyping of mice

Genotyping of mice was performed by PCR. PCR mixture contained 100 ng genomic DNA, 0.02 µM of appropriate primers, 0.25 mM dNTPs, 0.5 U Taq-polymerase and 1x PCR-buffer.

Primers for *ColII-Gal4* mice:

forward primer 5'-CTT CTA TCG AAC AAG CAT GCG -3'

reverse primer 5'-GCC AAT CTA TCT GTG ACG GC-3'

PCR conditions for genotyping of *ColII-Gal4* mice were: 94ºC for 5 minutes; 40 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 30 seconds; and 72ºC for 5 minutes.

The resulting fragment of 322bp indicated a transgenic mouse.

Primers for *UAS-Ihh* mice:

forward primer 5'-GGG CGG GCG CTG GCG ACG CTG-3'

reverse primer 5'-CGG GCT GCA CGT GGC TG-3'

PCR conditions for genotyping of *UAS-Ihh* mice were: 94ºC for 10 minutes; 35 cycles of 94ºC for 30 seconds, 72ºC for 30 seconds,72ºC 30 seconds; and 72ºC for 10 minutes.

The resulting fragment of 300 bp indicated a transgenic mouse.

Primers for FGFR3ach mice:

forward primer 5'- AGG TGG CCT TTG ACA CCT ACC AGG-3'

reverse primer 5'-TCT GTT GTG TTT CCT CCC TGT TGG-3'

PCR conditions for genotyping of FGFR3ach 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 850bp fragment indicated a transgenic mouse.

The PCR-products were analyzed in a 1% agarose gel in the TAE-buffer system. DNA was visualized with ethidium bromide staining under ultraviolet light.

2.2.3 Limb culture

Pregnant mice were sacrificed by cervical dislocation. Pups were transferred to PBS containing antibiotic-antimycotic. Forelimbs were dissected from embryo using fine forceps. Skin and muscles were removed from forelimbs under a dissecting microscope. Forelimbs were placed on the Millipore AA filter in the middle of a culture dish so that the palm faced up. 1ml of BGJb medium was added (Fig. 8B). The forelimb were cultured at the air-liquid interface at 37°C, 5% CO_2 under sterile conditions for 2-4 days. The medium was changed daily with/without applying of growth factors. The limb cultures of chicken embryos were performed by the same procedure.

Limb culture dish: An outside volume in the culture dish was filled up with PBS containing antibiotic-antimycotic (Fig. 8A). A culture grid was placed in the middle of culture dish (Fig. 8C) and covered with a piece of Millipore AA filter (Fig. 8E).

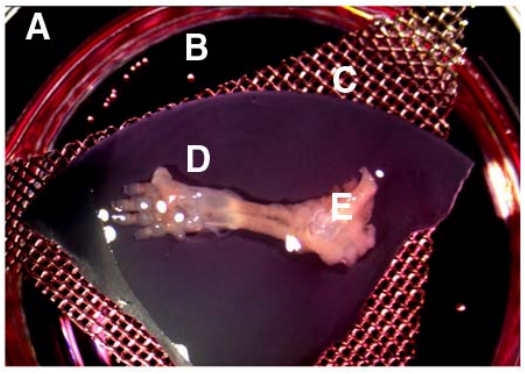

Fig. 8 Limb culture system

Forelimb of E18.5 was photographed in a culture dish (A-E). The outside volume of the culture dish is filled up with the PBS solution containing antibiotic/antimycotic (A). The inside volume is filled up with a culture medium (B). The forelimb (E) is cultured on air-liquid interface on the Millipore AA filter (D), which covers the culture grid (C).

2.2.4 Harvesting and dehydration of limb tissue

Limbs before/after culture were fixed in 0.5 ml per limb of 4% PFA at 4°C overnight. Tissue was rinsed with PBS for 5 minutes three times. Limbs of embryos older than E16.5 were additionally decalcified in 0.5 EDTA during 3-7 days, fixed again in 4% PFA for 2 hours and twice rinsed with PBS. Tissue of E14.5 and E16.5 limbs was dehydrated for 10 minutes in every solution of 30%, 50%, 75%, 80%, 95% and 100% ethanol, 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. For limbs of older embryos the time for all steps before paraffin was increased to 30 minutes. Limbs were embedded into paraffin and sectioned into 5 µm thick slices for 5-6 parallel sections, which were placed on silanized slides.

2.2.5 BrdU labeling of limb explants

For proliferation analysis, limb explants were incubated with 5-Bromo-2-deoxy-uridine (BrdU) in dilution 1:100 for two hours before harvesting. Limbs were embedded in paraffin and sectioned. Proliferating cells were detected by BrdU labeling and detection kit II according to the manufacturer.

2.2.6 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 dried overnight.

2.2.7 Preparation of DNA template for *in vitro* transcription

Plasmid DNA was isolated from *E.coli* DH5α cells using QIAprep Plasmid Miniprep or Plasmid Midiprep Kits according to the manufacturer. 5 µg plasmid DNA was linearized with 5 U of the appropriate restriction endonuclease (see table 2 in 2.1.9) for 1 hour at 37°C. The efficiency of the digestion was analyzed in a 1% agarose gel in the TAE buffer system. Phenol/chloroform extraction was performed twice. The final supernatant was transferred to the clean tube and was precipitated with 2.5 volume of 100% ethanol. The mix was

centrifuged at 14,000 rpm for 15 minutes at RT. The DNA pellet was washed with cold 80% ethanol and resuspended in 10 µl of RNAse free water.

2.2.8 Labeling of antisense riboprobes

Transcription reaction was performed in 20 µl with the following components: 500 ng linearized DNA template for *in vitro* transcription, transcription buffer (in recommended dilution), 0.5 mM NTP mix, 5 U RNAse inhibitor, RNAse free water, 40 U RNA-polymerase and 80 µCi [P^{33}]-UTP. RNA-polymerases T7, Sp6 or T3 were used depending on the insert orientation (see table 2 in 2.1.9). The transcription reaction was carried out for 1 hour at 37°C. To remove the DNA template 10 U DNAse (RNAse-free) was added to the transcription mix and was further incubated for 30 minutes at 37°C. The transcription mix was then diluted with 4 volumes of RNAse free water, containing 20 ng/ml glycogen and 0.5 M LiCl. For precipitation 2.5 volume of 100% ethanol was added. 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. The riboprobe was heated for 5 minutes at 95°C and chilled on ice before use.

2.2.9 *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 paraffin. Then the sections were incubated in each ethanol solution (100%, 95%, 80%, 75%, 50%, 30 %) for 2 minutes and in 0.85% NaCl for 5 minutes, rinsed with PBS for 5 minutes and fixed in 4% PFA for 30 minutes. They were incubated for 5 minutes in each solution: PBS, 0.2 N HCl and RNAse free water. The sections were treated for 5 minutes with 0.02 mg/ml Proteinase K and rinsed with PBS for 5 minutes. The sections were fixed in 4% PFA with 0.2% glutaraldehyde for 10 minutes, rinsed with PBS for 5 minutes. The sections were incubated in 0.2% Triethanolamine with freshly added 0.25% acetic anhydride for 10 minutes, then rinsed with PBS for 5 minutes and additionally incubated in 0.85% NaCl for 5 minutes. Dehydration of the sections was done through the ethanol solutions (30%, 50%, 75%, 80%, 95%, 100%) for 2 minutes in each solution. The sections dried at RT for 15 minutes. A minimal volume of labeled riboprobe (50 µl) was distributed throughout the

sections on one slide. The sections were covered with coverslips to prevent the evaporation. *In situ* hybridization was performed at 70°C overnight.

2.2.10 Washing of slides and dipping in photoemulsion

For all following solutions bidest water was used. The sections were washed in 5xSSC for 30 minutes and the coverslips were removed. The sections were washed in 2xSSC for 30 minutes and treated with 0.02 mg of RNAse A in 1xWash buffer for 30 minutes. The sections were washed with 2xSSC with 50% formamide for 30 minutes and then twice in 2xSSC for 30 minutes. The sections were dehydrated in 0.3M ammonium acetate with different dilutions of ethanol (30%, 50%, 75%, 80%) for 2 minutes in each solution. The sections were incubated in 100% ethanol for 15 minutes, they dried for 10 minutes and were exposed to X-ray film (Kodak Scientific Imaging Film) overnight at RT. 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 cointerstained with 0.2% Toluidin blue O for 5 minutes and dehydrated in ethanol solutions 30%, 50%, 75%, 80%, 95% and 100% for 2 minutes in each solution. The sections were incubated in 100% xylol for 30 minutes and then covered with the glass coverslips using minimal volume of embedding medium (DPX Mountant for histology). The pictures from the sections were taken using dark-field microscopy.

2.2.11 Production and purification of Noggin protein

The Noggin producing CHO B3.A4 cells were grown in 5 ml of Noggin-medium with 80 µM Methotrexate before the cells reached confluent density and covered about 90% of bottom of a culture bottle. The cells were split 1:4 for the next passage. Methotrexate was used for first two passages. One liter of the medium was collected from the fifth passage. To the medium 0.4 M NaCl, 10 mM HEPES, 1 mM EDTA, 10% glycerol were added. This medium was filtered through a cellulose acetate filter (0.2mm). From the medium Noggin was affinity-purified by chromatography at 4°C. The peristaltic pump was used to load the medium at 0.35 ml/minute to the column with 5 ml SP-sepharose Fast Flow, which was equilibrated with buffer A. After loading the column was extensively washed with 30 volumes of buffer A. The Noggin protein was eluated with 40 volumes (200 ml) of

linear gradient from 0.4 M to 1 M NaCl (mixed buffers A and B). Noggin eluated between 0.7 M - 0.8 M NaCl. Fractions were collected and analyzed with dot-blot hybridization (see 2.2.12). The Noggin containing fractions were analyzed in SDS PAAG (polyacrylamid gel) electrophoresis system. The Noggin protein samples were mixed with 0.25 volume of the NuPage Sample buffer (4x) and 0.1 volume of NuPage Sample Reducing Agent. The samples were heated for 3 minutes at 95°C. Noggin samples were analyzed in 4-12% Bis-Tris-Gel in the MES-buffer system with NuPage Antioxidant, according to the manufacturer. The gel was stained with a Silver staining kit, according to the supplemented protocol. The fractions with the highest content of Noggin were combined. To define a concentration of Noggin, different dilutions of Noggin and known concentrations of the standard protein, Chemotripsinogen α, were run in 4-12% Bis-Tris-Gel. The silver stained gel was scanned using ImageQuant software and the concentration of Noggin was defined by quantitative analysis from relation to the concentrations of Chemotripsinogen α. If needed Noggin was concentrated with a Centricon Plus-20 filter according to the supplemented instructions. The medium of the CHO dhfr$^-$ cells (control medium) underwent the same purification procedure. There was no protein in fractions, eluated between 0.7 M-0.8 M NaCl, correponding to Noggin containing fractions. In addition, the fractions from the control medium showed no effect in the limb cultures.

2.2.12 Dot-blot hybridization for Noggin protein

2 µl of every fraction of purified protein and positive control was spotted into the 10x10 cm Hybond ECL Nitrocellulose membrane with the distance between dots not less than 0.5 cm between the dots. As the dots dried, the membrane was incubated for 1 hour in 5 ml of TBST with 1% fat-free milk and diluted 1: 250 mouse 1A4 anti Noggin IgG at RT. The membrane was washed twice with TBST for 5 minutes and incubated for 30 minutes in 5 ml TBST with 1: 250 biotinilated antimouse-IgG at RT. The membrane was washed twice with TBST for 5 minutes and incubated with solutions of ABC kit (avidin and biotinilated horseradish peroxidase), according to the manufacturer. Noggin-containing fractions were visualized using the DAB substrate kit for peroxidase, according to the manufacturer.