2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Standard chemicals and reagents are listed in Table 1 in the alphabetical order.

Table 1 Standard chemicals and reagents

Name	Company
Acetic anhydride	Sigma
Acrylamide mix [Rotiphorese® Gel 40 (29:1)]	Roth
L-Adenine hemisulfate salt	Sigma
Agar	Difco
Agarose	Invitrogen
Ammmonium chloride	Merck
Ammonium persulfate (APS)	Invitrogen
Ampicillin	Sigma
L-Arginine HCl	Sigma
Bacto peptone	Difco
Bacto yeast extract	Difco
Blocking reagent	Boehringer
Bovine serum albumin (BSA)	Sigma
Bradford reagent	Sigma
Bromophenol blue	Fluka
Calcium chloride	Merck
Chloroform	Merck
Citric acid	Merck
Complete mini protease inhibitor cocktail tablets	Roche
4’6-diamino-2-phenyl-indole (DAPI)	Serva
[α-^{32}P]-Dctp	Amersham
Dextran blue	Fluka
Dextran sulfate	Sigma
Diethylpyrocarbonate (DEPC)	Sigma

Name	Company
Dimethyl sulfoxide (DMSO)	Merck
Disodiumhydrogen phosphate	Merck
Dithiothreitol (DTT)	Sigma
dNTPs	MBI Fermentas
DO supplement	BD Biosciences
DPBS (PBS for cell culture)	BioWhittacker
Dulbecco's Modified Eagle's Medium (DMEM) with 4,5 g/l glucose	BioWhittacker
Ethanol	Merck
Ethidium bromide	Serva
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal Bovine Serum (FBS)	Biochrom AG
Fluoromount-G	Science Services
Formamide	Merck
Formaldehyde	Roth
GeneRuler 1 kb and 100 bp DNA ladders	MBI Fermentas
Glacial acetic acid	Merck
Glass beads (425-600 μm).	Sigma
Glucose	Merck
L-Glutamine solution for cell culture	BioWhittacker
Glutaraldehyde	Sigma
Glycerol	Merck
Glycine	Sigma
Heat inactivated sheep serum (HISS)	Gibco
Heparin sodium salt	Sigma
Herring sperm DNA	Roche
Hydrogen peroxide	Merck
L-Histidine HCl monohydrate	Sigma
Hybridime human placental DNA	HT
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium (HEPES)	Sigma
L-Isoleucine	Sigma
Isopropanol	Merck
Kanamycin	Invitrogen
L-Leucine	Sigma

Name	Company
Lithium acetate	Sigma
Lithium chloride	Merck
L-Lysine HCl	Sigma
Magnesium chloride	Merck
Magnesium sulfate	Merck
Maleic acid	Merck
Manganese chloride	Sigma
β-Mercaptoethanol	Merck
Methanol	Merck
L-Methionine	Sigma
3-[N-Morpholino]propanesuflonic acid (MOPS)	Sigma
N,N,N ,N –Tetramethylethylenediamine (TEMED)	Invitrogen
Nonidet P40 (NP40)	Fluka
OPTIMEM with GLUTAMAX	Gibco
Paraformaldehyde (PFA)	Sigma
Pd(N)6 random hexamers	Pharmacia
Penicillin/streptomycin antibiotic solution for cell culture	Invitrogen
Phenol	Roth
Phenol red	Fluka
L-Phenylalanine	Sigma
Polyethylene glycol 3350 (PEG 3350)	Sigma
Polyethylene glycol 6000 (PEG 6000)	Merck
Polyfectamine transfection reagent	Qiagen
Potassium acetate	Merck
Potassium chloride	Merck
Potassium dihydrogen phosphate	Merck
Precision Plus Protein Kaleidoscope Standards	Bio-Rad
L-Proline	Sigma
Protector RNase Inhibitor	Roche
Ribonucleic acid from Baker’s yeast type III (tRNA)	Sigma
Rubidium chloride	Sigma
Salmon sperm DNA	Sigma
Sephadex G-50	Pharmacia

Name	Company
Sodium acetate	Merck
Sodium azide	Sigma
Sodium chloride	Merck
Sodium citrate	Merck
Sodium deoxycholate	Sigma
Sodium dihydrogen phosphate	Merck
Sodium dodecyl sulfate (SDS)	Serva
Sodium hydroxide	Merck
Tetramisole hydrochloride (levamisole)	Sigma
Thiamine hydrochloride	Sigma
L-Threonine	Sigma
Tris (hydroxymethyl)-aminomethane	Merck
Triton X-100	Roth
TRIzol reagent	Invitrogen
L-Tryptophane	Sigma
Trypton	Difco
Tween 20	Sigma
L-Tyrosine	Sigma
L-Uracil	Sigma
L-Valine	Sigma
Yeast nitrogen base without amino acids	Difco

2.1.2 Buffers and solutions

Aqueous solutions were prepared using autoclaved Millipore water. For sterilisation solutions were autoclaved or passed through a 0.45 µm filter (Millipore). Solutions used for Southern blot hybridisation are listed in Table 2. Buffers and solutions used for denaturing RNA gel and Northern hybridisation can be found in Table 3. Solutions used for yeast lysis and β-galactosidase assay are listed in Table 4. Solutions required for SDS polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis and immunofluorescence are listed in Table 5. Solutions used for whole mount and section *in situ* hybridisation can be found in Table 6. Buffers used for preparing chemical competent *E.coli* cells are listed in Table 7.

Table 2 Solutions used for Southern blot hybridisation

Solution	**Components**
Nucleus buffer (pH 7.5)	10 mM Tris-HCl 25 mM EDTA 75 mM NaCl
20 × SSC (pH 7.0)	300 mM Sodium citrate 3 M NaCl
Denaturing solution	0.5 M NaOH 1.5 M NaCl
0.5 M Phosphate buffer (pH 7.4)	77.4 mM Na_2HPO_4 22.6 mM NaH_2PO_4
PEG hybridisation buffer	250 mM NaCl 125 mM Na_2HPO_4 1 mM EDTA 7% (w/v) SDS 10% (w/v) PEG 6000
5 × OLB (-dCTP)	0.1 mM each dATP, dGTP, dTTP 1 M HEPES 0.425 mM pd(N)6 25 mM $MgCl_2$ 250 mM Tris-HCl 0.36% (v/v) β-Mercaptoethanol
Stop solution (pH 7.5)	10 mM Tris-HCl 5 mM EDTA 2% SDS 0.1% Dextran blue 0.1% Phenol red
TES (pH 7.5)	10 mM Tris-HCl 5 mM EDTA 0.2% SDS
Washing buffer	40 mM Phosphate buffer 0.5% (w/v) SDS

Table 3 Solutions for Northern blot hybridisation

Solution	**Components/Company**
25 × MOPS solution	1 M MOPS 0.25 M NaAc 50 mM EDTA (pH 7.0)
Electrophoresis buffer	1 × MOPS solution 0.66 M Formaldehyde
Northern blot loading buffer	50% Formamide 2.2 M Formaldehyde 1 × MOPS solution 40 µg/ml Ethidium bromide
QuikHyb Hybridization Solution	Stratagene
Washing solution	2 × SSC 0.1% (w/v) SDS
High stringency washing solution	0.1 × SSC 0.1% (w/v) SDS

Table 4 Solutions used for yeast lysis and β-galactosidase assay

Solution	**Components**
Yeast lysis buffer	2% Triton X-100 1% SDS 100 mM NaCl 10 mM Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0)
Z-buffer (pH 7.0)	60 mM $Na_2HPO_4 \cdot 2H_2O$ 40 mM $NaH_2PO_4 \cdot H_2O$ 10 mM KCl $MgSO_4 \cdot 7H_2O$
Substrate solution	100 ml Z- buffer 0.27 ml β-Mercaptoethanol 33.4 mg X-Gal

Table 5 Solutions used for SDS-PAGE, Western blot analysis and immunocytochemistry

Solution	**Components**
10 × Protease inhibitor solution	1 tablet of complete mini protease inhibitor cocktail per 1 ml H_2O
Cell lysis buffer	150 mM NaCl 50 mM Tris-HCl (pH 7.5) 1% NP40 1 × Protease inhibitor solution
2 × Sample buffer	125 mM Tris-HCl (pH 6.8) 4% SDS 10% Glycerol 0.006% Bromophenol blue 2% β-Mercaptoethanol
Protein loading buffer	350 mM Tris-HCl (pH 6.8) 10% SDS 30% Glycerol 9,3% DTT 0.012% Bromophenol
1 × Electrophoresis buffer	25 mM Tris-HCl 250 mM Glycine 0.1% SDS
Western blot transferring buffer	192 mM Glycine 25 mM Tris-HCl 20% Methanol
1 × PBS (pH 7.3)	137 mM NaCl 2.7 mM KCl 10.1 mM Na_2HPO4 1.8 mM KH_2PO4
PBST	0.1% Tween 20 in 1 × PBS
Blocking buffer for Western blot	5% (w/v) nonfat dry milk in 1 × PBST
Blocking solution for immunocytochemistry	1 × PBS 10% FBS 0.05% NaN_3

Table 6 Compound solutions used for whole mount and section *in situ* hybridisation

Solution	**Components**
DEPC-H_2O	0.1% (v/v) DEPC in H_2O
DEPC-PBS	0.1% (v/v) DEPC in 1 × PBS
DEPC-PBST	0.1% (v/v) Tween 20 and 0.1% DEPC in 1 × PBS
PBST/Glycine	0.2% (w/v) Glycine in DEPC-PBST
PBST/Tetramisole	0.05 % (w/v) Tetramisole in DEPC-PBST
RIPA buffer	0.05% SDS 150 mM NaCl 1% (v/v) NP40 0.5% (w/v) Sodium deoxycholate 1 mM EDTA 50 mM Tris-HCl in DEPC-H_2O
PFA/Glutaraldehyde	4% PFA/PBS (pH 7.4) 0.2% Glutaraldehyde
20 × DEPC-SSC (pH 7.0)	300 mM Sodium citrate 3 M NaCl 0.1 % DEPC
Heparin	100 mg/ml Heparin in 4 × DEPC-SSC
Hybe buffer	50 % Formamide 0.1 % Tween 20 50 µg/ml Heparin 5 × DEPC-SSC (pH 4.5, adjusted with citric acid) diluted in DEPC-H_2O
SSC/FA/T	2 × SSC 50% Formamide 0.1 % Tween 20
5 × MABT	0.5 M Maleic acid (pH 7.5) 0.75 M NaCl 5 % Tween 20
RNase solution	0.5 M NaCl 10 mM Tris-HCl (pH 7.5) 0.1 % Tween 20
Alkaline phosphatase buffer	100 mM NaCl 50 mM $MgCl_2$ 100 mM Tris-HCl (pH 9.5) 0.05 % (w/v) Tetramisole 0.1 % Tween 20

Solution	Components
10 × Wash buffer	4 M NaCl 1 M Tris-HCl (pH 7.5) 0.5 M EDTA
TNE	10 mM Tris-HCl (pH 7.5) 500 mM NaCl 1 mM EDTA
NTMT (pH 9.5)	100 mM NaCl 100 mM Tris-HCl (pH 9.5) 50 mM $MgCl_2$ 0.1% Tween 20
Hybridisation solution (for paraffin sections)	10 mM Tris-HCl (pH 7.5) 600 mM NaCl 1 mM EDTA 0.25% SDS 10% Dextran sulfate 1 × Denhardt solution 200 µg/ml Ribonucleic acid from Baker's yeast type III 50% Formamide

Table 7 Solutions used for preparing chemical competent *E. coli* cells

Solution	Components
Buffer A	30 mM KAc 10 mM $CaCl_2$ 100 mM RbCl 50 mM $MnCl_2$ 15% Glycerol
Buffer B	10 mM MOPS/NaOH (pH 7.0) 75 mM $CaCl_2$ 10 mM RbCl 15% Glycerol

2.1.3 Media

2.1.3.1 Yeast media

Yeast media used for propagation the wild type strains L40 and AH109, as well as selective media required for the yeast two-hybrid assay are listed in Table 8.

Table 8 Yeast media and dropouts (DO)

Solution	**Components**
YPD medium (pH 5.8)	2% Bacto peptone 1% Bacto yeast extract 1% Glucose
YPD agar (pH 5.8)	20 g Agar per 1 l YPD medium
YPDA medium	2% Bacto peptone 1% Bacto yeast extract 2% Glucose 0.003% Adenine
YPDA agar	20 g Agar per 1 l YPDA medium
10 × DO supplement	0.03% (w/v) L-Isoleucine 0.15% (w/v) L-Valine 0.02% (w/v) L-Adenine hemisulfate salt 0.02% (w/v) L-Arginine HCl 0.02% (w/v) L- Histidine HCl monohydrate 0.1% (w/v) L-Leucine 0.03% (w/v) L-Lysine HCl 0.02% (w/v) L-Methionine 0.05% (w/v) L-Phenylalanine 0.2% (w/v) L-Threonine 0.02% (w/v) L-Tryptophane 0.03% (w/v) L-Tyrosine 0.02% (w/v) L-Uracil
10 × DO –T	10 × DO supplement lacking tryptophane
10 × DO –L	10 × DO supplement lacking leucine
10 × DO –TL	10 × DO supplement lacking tryptophane and leucine
10 × DO –THULL	10 × DO supplement lacking tryptophane, histidine, uracil, lysine and leucine
SD medium	0.67% Yeast nitrogen base without amino acids 2% Glucose 1 × appropriate DO supplement
SD agar	20 g Agar per 1 l SD medium

2.1.3.2 Bacterial media

E.coli strains XL1-Blue, DH5α, GM2163 and STBL4 were cultured in LB medium implemented with appropriate antibiotics, the strain HB101 was cultured in M9 minimal medium. Media with their components are listed in Table 9. Dropout solution (DO) used for preparing M9 minimal medium can be found in the previous section (Table 8).

Table 9 Bacterial media

Medium	**Components**
LB medium	10 g/l Trypton 5 g/l Bacto yeast extract 10 g/l NaCl
LB agar	20 g Agar per 1 l LB medium
M9 minimal medium	6 g/l $Na_2HPO_4 \cdot 2H_2O$ 3 g/l KH_2PO_4 1 g/l NH_4Cl 0.5 g/l NaCl 2 mmol/l $MgSO_4$ 0.1 mmol/l $CaCl_2$ 1 mmol/l Thiamine 1× DO –L 8 g/l Glucose 40 mg/l Proline 50 mg/l Ampicillin
M9 agar	16 g Agar per 1 l M9 minimal medium

2.1.3.3 Cell culture media

List of components for COS1 medium can be found in Table 10.

Table 10 Cell culture media

Solution	**Components**
COS1 medium	DMEM medium with 4,5 g/l glucose 5% FBS 1% L-Glutamine 1% Penicillin/streptomycin antibiotic solution

2.1.4 Enzymes

All restriction endonucleases for cloning and genomic DNA digestion were purchased from New England Biolabs, Invitrogen or MBI Fermentas. Reactions were performed in supplied reaction buffers following the manufacturers' instructions. Additional enzymes used are listed in Table 11.

Table 11 Additional enzymes

Enzyme	**Concentration**	**Company**
Taq DNA polymerase	5 U/µl	Promega
AmpliTaq DNA polymerase	5 U/µl	Perkin Elmer
Pfu DNA polymerase	2.5 U/µl	Stratagene
TaKaRa LA *Taq* polymerase	5 U/µl	TaKaRa
Expand Long Template Enzyme mix	5 U/µl	Roche
Klenow fragment	2 U/µl	Roche
Superscript reverse transcriptase	200 U/µl	Invitrogen
DNase I RNase-free	10 U/µl	Roche
Proteinase K	10 mg/ml	Invitrogen
T3 RNA polymerase	20 U/µl	Roche
T7 RNA polymerase	20 U/µl	Roche
RNase A	10 mg/ml	Roche
T4 DNA ligase	400 U/µl	Promega
Shrimp alkaline phosphatase (SAP)	20 U/µl	Fermentas
E. coli exonuclease I	1 U/µl	Fermentas
Calf intestine alkaline phosphatase	1 U/µl	Fermentas

2.1.5 Kits

All kits used are listed in Table 12.

Table 12 List of commercial kits

Kit	Company
BigDye terminator cycle sequencing ready reaction kit	PE Biosystems
10 × DIG RNA labelling mix	Roche
BM Purple AP Substrate	Roche
QIAprep spin miniprep kit	Qiagen
QIAgen plasmid midi and maxi kits	Qiagen
QIAquick gel extraction kit	Qiagen
Roti™-Lumin Chemiluminescence substrate	Roth

2.1.6 Vectors and universal primers

All vectors used for expression studies as well as those used for intermediate cloning steps are listed in Table 13. Multiple cloning sites and/or maps of modified vectors as well as of the vectors obtained from other labs (marked in the last column of the Table 13) can be found in the appendices 11.1, 11.2, 11.3 and 11.4. Vector-specific primers used for colony PCR and/or sequencing of inserts can be found in Table 14.

Table 13 Vectors

Vector	Resistance gene	Company/Remarks
pGEM®-T Easy Vector System I	Ampicillin	Promega
pCRII®-TOPO®	Ampicillin/ Kanamycin	Invitrogen
pVP16	Ampicillin/ Leucine	constructed by Stan Hollenberg*
pBMT116	Ampicillin/ Tryptophane	constructed by Paul Bartel and Stan Fields*
pGBKT7	Kanamycin/ Tryptophane	BD Biosciences
pcDNA-Flag	Ampicillin	modified pcDNA3.1 vector from Invitrogen*
pTL1-HA2	Ampicillin	modified pSG5 vector from Stratagene**

* A kind gift from the group of Prof. W. Birchmeier, Max-Delbrueck-Center, Berlin.
** A kind gift from the group of Prof. E. Wanker, Max-Delbrueck-Center, Berlin.

Table 14 Vector-specific primers

Vector	Primers pairs	Primer sequences	T_A
pGEM®-T Easy and pCRII®-TOPO®	M13for	GTAAAACGACGGCCAGTG	52°C
	M13rev	GGAAACAGCTATGACCATG	
pVP16	VP16F1	GGATTTACCCCCCACGACTCC	62°C
	VP16R1	AGGGTTTTCCCAGTCACGACGTT	
pBMT116	BTM116F1	TCAGCAGAGCTTCACCATTG	55°C
	BTM116R1	GAGTCACTTTAAAATTTGTATAC	
pGBKT7	Y2H-T7	TAATACGACTCACTATAGGGC	54°C
	Y2H-BD	TTTTCGTTTTAAAACCTAAGAGTC	
pcDNA-Flag	T7	AATACGACTCACTATAGGGAA	54°C
	BGHrev	TAGAAGGCACAGTCGAGG	
pTL1-HA2	pSG fw.	TCTGCTAACCATGTTCATGCC	58°C
	pSG rev.	GGACAAACCACAACTAGAATG	

2.1.7 Antibodies

All primary and secondary antibodies used in this study are listed in Table 15.

Table 15 Antibodies

Antibody	Company
Monoclonal anti-c-myc antibody	Invitrogen
Anti-HA antibody produced in rabbit	Sigma
Monoclonal anti-HA agarose conjugate	Sigma
Monoclonal LexA antibody	Clontech
Anti-Flag® M2 Affinity Gel	Sigma
Monoclonal anti-Flag antibody	Sigma
Polyclonal anti-Flag antibody	Sigma
Alexa Fluor® 546 goat anti-rabbit IgG	Molecular Probes
Alexa Fluor® 488 goat anti-mouse IgG	Molecular Probes
Anti-rabbit IgG (Goat), peroxidase conjugate	Oncogene
Anti-Mouse IgG (Goat), peroxidase conjugate	Oncogene
Anti-digoxigenin-AP	Roche

2.1.8 Primers

All primers used in this study were synthesised by MWG Biotech or by Metabion, Germany.

2.1.9 Genomic clones, ESTs and genomic and cDNA libraries

All human YAC, BAC and cosmid clones, as well as EST clones and the spotted human chromosome 2-specific cosmid library (Livermore) were obtained from the Resource Centre of the German Human Genome Project (RZPD).
Mouse embryonic cDNA library (from pooled stages E9.5 – E10.5) cloned in pVP16 vector was a kind gift from the group of Prof. W. Birchmeier, Max-Delbrueck-Center, Berlin.

2.1.10 Bacteria

For all recombinant DNA techniques, competent *Escherichia coli* strain DH5α or XL1-Blue cells (Stratagene) were used. Isolation of prey plasmids from the yeast two-hybrid screen was performed with the help of the *E. coli* strain HB101 (Promega). Non-methylated plasmids were isolated from the dam^-/dcm^- *E.coli* strain GM2163. Cloning of repetitive-rich sequences was performed with the help of the *E.coli* strain STBL4 (Invitrogen).

2.1.11 Yeast

Yeast strain L40 was obtained from Invitrogen, whereas AH109 strain was purchased from Clontech.

2.1.12 Mammalian cell lines

Transient transfection experiments were performed in COS1 cells (African green monkey kidney cells).

2.1.13 Mouse embryos

Mouse embryos were derived from crosses of wildtype C57Bl and Bl10 mice.

2.2 Methods

2.2.1 DNA isolation

Genomic DNAs from lymphoblastoid or fibroblast cell lines were extracted according to standard protocols (Sambrook et al. 1989). Briefly, approximately 1×10^8 cells were suspended in 10 ml of nucleus buffer (see Table 2), and lysed by addition of SDS to a final concentration of 0.5%. Cell lysates were subjected to overnight proteinase K digestion. DNA was extracted by phenol/chloroform, precipitated with 96% ethanol and washed twice with 70% ethanol.

Plasmid DNAs were isolated using QIAprep miniprep kit or QIAgen plasmid midi and maxi kits according to the manufacturer's instruction.

2.2.2 Fluorescence *in situ* hybridisation (FISH)

Metaphase chromosomes were prepared from the Epstein-Barr virus transformed lymphoblastoid cell line, which was derived from the patient with the translocation t(2;10)(q31.1;q26.3). YAC probes were selected from the Whitehead Institute database, and BAC clones were found with the use of the NIX programme available on the UK HGMP Resource Centre webpage. To select cosmid clones, screening of the spotted human chromosome 2-specific cosmid library (Livermore) was performed. A pool of PCR products selected from the breakpoint region of the patient was labelled with [α^{32}P]-dCTP by random priming and was preannealed with Hybridime at 65°C for 1.5 h. Hybridisation to the library filters was performed at the same temperature overnight. Afterwards, the filters were washed in 40mM phosphate / 0.5% SDS solution. Signals were detected by autoradiography and positive clones were selected for FISH.

DNAs from all clones were isolated according to standard protocols (Sambrook et al. 1989). Purified DNAs were labelled with either digoxigenin-11-dUTP or biotin-16-dUTP by nick translation and were hybridised to metaphase chromosomes of the patients. Signals were detected either by anti-digoxigenin or by FITC- or Cy3- conjugated avidin and were visualised by fluorescence microscopy, as described previously (Wirth et al. 1999).

2.2.3 Amplification of DNA probes for Southern blot hybridisation

DNA probes for Southern blot hybridisation were amplified by PCR with the *Taq* DNA polymerase (Promega) in the supplied buffer (1.5 mM $MgCl_2$ final concentration). PCR reactions were carried out in 50 µl reaction volumes and contained 0.2 µM of each primer pair, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase and approximately 100 ng genomic DNA as template. Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at the specific annealing temperature, 1 minute at 72°C, and a final extension step at 72°C for 7 minutes. The PCR products were separated on a 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's instruction. Selected probes used for Southern blot hybridisation, and the respective primer sequences, together with the annealing temperatures (T_A) for each PCR reaction are given in Table 16.

Table 16 Probes for Southern blot hybridisation

Probe	**Primer name**	**Primer sequence [5' → 3']**	**T_A**
538A12_49750 (694 bp)	538A12_49750for 538A12_49750rev	GCTTCCCATTGCAGGTGTAAA ATTACTGGTCATCAATATCTAGC	56°C
538A12_79400 (558 bp)	538A12_79400for 538A12_79400rev	AACTCAACATAAACTTTTCCAAAG GAATGTAAAATATAGACATTTGACATTG	56°C
300B2_80000 (611 bp)	300B2_80000for 300B2_80000rev	TCCTTTGCACACAGGTCCTTTC TGGTGACCATGGCAGTCATAC	58°C
300B2_84000 (766 bp)	300B2_84000_for 300B2_84000_rev	GAGAAGGACTAGAGAGGATG CACAGGTATTTGATATGTTGTCAGC	58°C
300B2_90000 (991 bp)	300B2_90000_for 300B2_90000_rev	GGAAGATGTTGAACAGGTGAGAG CACAGAAAGCACGTGGCTGC	60°C

2.2.4 Isotope-labelling of probes for Southern blot hybridisation

Gel purified DNA probes were denatured for 10 min at 95°C, chilled on ice and radiolabelled using random hexanucleotide primers in 1 × OLB buffer. Essentially, the reaction was carried out at 37°C for at least 1 hour in 25 µl volume containing 25 ng of DNA probe, 20 µCi [α-^{32}P]-dCTP and 2 U Klenow fragment. The reaction was stopped by adding 50 µl of stop solution. To remove the excess of non-incorporated dNTPs and random hexamers, the

reaction mixture was separated by passing through a Sephadex G-50 column and the labelled probe was eluted with TES buffer. Solutions used in this section are listed in Table 2.

2.2.5 Southern blot hybridisation

Genomic DNAs from the patient with the translocation t(2;10)(q31.1;q26.3) and from controls were digested with appropriate restriction enzymes and separated on 0.7% agarose gels. Subsequently, gels were pre-incubated in denaturing solution twice for 30 minutes each, and the DNAs were capillary transferred to nylon membranes (Roth) using 10 × SSC. After an overnight transfer, membranes were incubated for 10 minutes in 50 µM phosphate buffer and the DNAs were fixed by UV crosslinking at 254 nm for 2 min. For hybridisation, membranes were pre-hybridised in PEG hybridisation buffer supplemented with 0.1 mg/ml denatured herring sperm DNA as blocking reagent for at least 2 hours at 65°C. Isotope-labelled DNA probes were denatured for 10 min at 95°C, chilled on ice and blocked with human placental DNA Hybridime in PEG hybridisation buffer for 1 hour at 65°C before added to the pre-hybridisation solution. Membranes were hybridised with isotope-labelled probes at 65°C overnight, washed with washing buffer and exposed to Kodak X-Omat AR film at –80°C for 1-7 days. Solutions used in this section are listed in Table 2.

2.2.6 Genomic walking

For breakpoint cloning, genomic walking was performed, as described elsewhere (Siebert et al. 1995). Genomic DNAs from the patient and a control were digested with appropriate restriction enzymes, phenol/chloroform extracted, and ethanol precipitated. Approximately 1 µg of each digested DNA was ligated to pre-annealed adaptor oligos using T4 DNA ligase (Promega) in supplied buffer. After overnight ligation at 15°C, the reaction was stopped by heating at 70°C for 10 min. 1 µl of each ligation mixture was used as a template in the first round of nested PCR. All PCR reactions were carried out with TakaRa LA *Taq* polymerase (TaKaRa) in 50 µl volume of 1× PCR buffer provided by the manufacturer. The first round PCR contained 30 µM of adaptor primer AP1 and one of the sequence-specific primers: 84364for [for der(10)] or 85206rev1 [for der(2)]. Cycling condition comprised an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles of 40 seconds at 94°C, 40 seconds at 60°C, 3 minutes at 72°C, and a final extension at 72°C for 15 minutes. 1 µl of

each first round PCR product was used as a template in the second round of PCR reaction, which contained 30 µM each of nested adaptor primer AP2 and one of sequence-specific primers 84715for [for der(10)] or 85163rev2 [for der(2)]. Cycling condition for the second round PCR was essentially the same as the first round, except for the annealing temperature, which was adjusted to 58°C. Primers and adaptor sequences are listed in Table 17.

Table 17 Primer/Adaptor sequences for genomic walking

Name	**Sequence [5' → 3']**
Adaptor-long	CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
Adaptor-short*	AATTACCTGCCCGG
AP1	GGATCCTAATACGACTCACTATAGGGC
AP2	TATAGGGCTCGAGCGGC
84364for	CAGATTGTGATTAGATCAGGAG
84715for	GACTTAAAATTGCAGCGTGTGTTTC
85206rev1	GTGTATCTATCTGAGCTCCATG
85163rev2	TTCAGCCTTAAGTCAAAATGTTGG

* 5' phosphate modification

Amplified fragments were isolated from 1% agarose gels, purified with Qiagen columns, subcloned into pGEM-T Easy vector and sequenced using M13 universal primers.

2.2.7 Screening for mutations in the *HOXD13* gene in the translocation patient

In order to screen the *HOXD13* gene in the translocation patient, several PCR reactions were performed using patient genomic DNA as template. First, a 172 bp long fragment encoding the alanine stretch was amplified using HsHOXD13for_1 and HsHOXD13rev_1 primers. Amplification reaction contained 1× PCR Buffer II (Perkin Elmer), 0.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer and 2.0 units of AmpliTaq DNA polymerase (Perkin Elmer) in a final volume of 50 µl. Initial denaturation was at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. The product was purified with the QIAQuick Gel Extraction Kit (Qiagen) and sequenced with HsHOXD13for_1 and HsHOXD13rev_1 primers. Later,

additional PCR reactions were performed in order to screen the whole coding sequence and the splice site acceptor of the *HOXD13* gene. The very 5' end of the coding sequence was amplified with HsHOXD13for_b and HsHOXD13_rev_a primers using patient genomic DNA as template. The reaction contained 10 µl FailSafe PCR Premix J (Epicentre), 2.5 mM of each primer and 0.3 µl Expand Long Template Enzyme mix (Roche) in a final volume of 20 µl. Cycling conditions included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 61°C, 2 minutes at 68°C, and a final extension step at 68°C for 10 minutes. The 3' end of exon 1, the part of the intron containing the splice site acceptor, and the whole exon 2 of *HOXD13* gene were amplified in three PCR reactions using the following primer pairs: HsHOXD13_for_a and HsHOXD13rev, HsHOXD13for2 and HsHOXD13rev2, HsHOXD13for3 and HsHOXD13rev3, respectively. PCR reactions contained 1 × PCR buffer (Promega), 0.4 µM of each primer, 0.2 mM dNTP, 8% DMSO and 1 unit *Taq* DNA polymerase (Promega) in a total volume of 50 µl. Initial denaturation was at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at the appropriate temperature (T_A) for 30 seconds, and extension at 72°C for 45 seconds.

All PCR products were separated on the 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's instruction, subcloned into pGEM-T Easy vector and sequenced using M13 universal primers. Primer sequences used for *HOXD13* amplification are listed in Table 18.

Table 18 Primers used for amplification the *HOXD13* gene

Primer name	**Primer sequence [5' → 3']**	**PCR product***	**T_A**
HsHOXD13for_b HsHOXD13_rev_a	GGAGCTGGGACATGGACG AATGCGTCCC GGCGAACAC	8881-9027 bp (147 bp long)	61°C
HsHOXD13for_1 HsHOXD13rev_1	CAGTGCCGCGGCTTTCTCTC CTACAACGGCAGAAGAGGAC	8982-9153 (172 bp long)	60°C
HOXD13_for_a HsHOXD13rev	CTCGTCGTCGTCCTCTTCTG GACATACGGCAGCTGTAGTAGC	9125-9289 bp (165 bp long)	62°C
HsHOXD13for2 HsHOXD13rev2**	CTACTACAGCTGCCGTATGTCG TCGGTCCCTTTTCTCCATC	9269-9675 bp (407 bp long)	58°C
HsHOXD13for3** HsHOXD13rev3	AGCTAGGTGCTCCGAATATCC AAGCTGTCTGTGGCCAACC	10405-10739 bp (335 bp long)	58°C

* Positions corresponding to the *HOXD13* gene sequence, GenBank entry AB032481.

** Primers are lying in the intron of the *HOXD13* gene.

2.2.8 RNA isolation

Total RNAs from lymphoblastoid or fibroblast cell lines, as well as the RNA from mouse embryos were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol.

2.2.9 Analysis of *MGMT* expression by Northern blot

In order to analyse *MGMT* expression in lymphoblastoid cell lines total RNAs from the translocation patient and from control cell lines were isolated. Approximately 10 µg of each RNA was mixed with 2.7 volumes of Northern blot loading buffer and denatured for 10 minutes at 65°C. Afterwards, samples were chilled on ice, loaded onto a denaturing 1% agarose gel containing 2.2 M formaldehyde and 1 × MOPS and separated at 28 V for 16–24 hours. After electrophoresis the gel was washed 3 times with DEPC-H_2O in order to remove formaldehyde, and the RNA was capillary transferred to the nylon membrane Hybond™-XL (Amersham Pharmacia Biotech) using 10 × SSC. After an overnight transfer, the RNA was fixed in UV Stratalinker 1800 (Stratagene) for 2 minutes. In order to remove the rests of formaldehyde the membrane was subsequently baked for 2 hours at 80°C.

The *MGMT* and *G3PDH* probes were amplified from total RNA isolated from control cell lines. About 2.5 µg of the RNA was used for the first strand synthesis with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Probes were PCR-amplified using specific primers (see table Table 19) and 1µl of the prepared cDNA as a template. Cycling conditions and purification of PCR products were basically the same as described in section 2.2.3. Labelling and purification of radioactive probes were performed as described in section 2.2.4.

Table 19 Probes used for Northern hybridisation

Probe	Primer name	Primer sequence [5' → 3']	T_A
MGMT (403 bp)	MGMT_ex.2_for MGMT_ex.4_rev	GGACA AGGATTGTGA AATGAAACG TCTCATTGCT CCTCCCACTG	58°C
G3PDH (454 bp)	G3PDH_for G3PDH_rev	GACCACAGTCCATGCCATCACT GTCCACCACCCTGTTGCTGTAG	58°C

Prehybridisation and hybridisation of radioactive probes was performed in QuikHyb® Hybridization Solution (Stratagene) at 68°C for 30 minutes and 3 hours, respectively. Washing was performed according to the manufacturer's protocol. Signals were detected on the phosphoimager. Intensity of the *MGMT* signals was compared to the intensity of the *G3PDH* (housekeeping gene) signals using ImageQuant software (Amersham Biosciences).
MGMT expression in different human tissues was examined by hybridisation of the *MGMT* probe to the human multiple tissue Northern blot (Clontech) and human fetal multiple tissue Northern blot (Clontech). Hybridisation and washing were performed as described above.

2.2.10 RT-PCR analysis

Total RNAs from cell lines, human brain or mouse embryos (E16.5) were digested with the RNase-free DNase I (Roche) in the supplied buffer, according to the manufacturer's protocol. Reverse transcription (RT) reactions were performed according to the manufacturer's instruction using the Superscript reverse transcriptase (Invitrogen) or water (for RT negative control). Efficiency of the first strand synthesis was subsequently controlled by amplification of the housekeeping gene *G3PDH* on cDNA templates. High quality cDNAs were used as templates for expression studies of homologous regions identified in the vicinity of the *HOXD* cluster, as well as for analysis of human ESTs found close to the chromosome 2 breakpoint region in the translocation patient. All RT-PCR reactions were carried out in 50 µl reaction volumes and contained 1 × reaction buffer (1.5 mM $MgCl_2$ final concentration), 0.2 µM of each primer pair, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase and 2 µl of proper cDNA as template. Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at the specific annealing temperature, 1 minute at 72°C, and a final extension step at 72°C for 7 minutes. After the first PCR reaction, 2 µl of each product was used for the second round of PCR with the same primers and reaction conditions. In parallel, reactions with the RT negative control were performed in order to make sure that PCR products did not derive from genomic contamination of the cDNA. PCR products were analysed on 1 % agarose gels.
In general, analyses of conserved elements on chromosome 2 were performed using RNA from mouse embryos, whereas human ESTs were amplified either on reported source tissue or on cell lines, in case when the source tissue was not accessible. Normally, primers were designed according to both ends of tested sequences. In case amplification with such primer

pairs did not result in a specific product, inner primers (which did not allow amplification of the whole sequence of interest) were ordered and used for RT-PCR experiments.

Primers used for human EST expression analysis can be found in Table 20. Sequences of primers used for analysis of conserved regions together with the expected length of PCR products and annealing temperatures are listed in Table 21.

Table 20 Primers used for expression analysis of EST sequences on chromosome 2q31

EST	**Primer name**	**Primer sequence [5' → 3']**	**T_A**
AI075926	Hs_EST8_for Hs_EST8_rev1	GTAGTGATACTCAGAAACTGACAC AGAGGGATCATTTACAGCAGG	56°C
AW850653	Hs_EST10_for1 Hs_EST10_rev1	ATTGCTAACCTAATGTGTGAGC GTGGTATGGACCAAAAACCAG	58°C
BG980989	Hs_EST5_for1 Hs_EST5_rev1	AAGTCACATTTAATGGGAGGATC CAGGACATTCTGATGCTAAACG	58°C
BG980131	Hs_EST3_for Hs_EST3_rev	TCGTTGGGAAGATCAAATGAG GGTTCCCATGGTCTGTTTC	56°C
BG979719 and BG979037	Hs_EST6/16_for Hs_EST6/16_rev	AATGCTCCATGCATACCCAAC CAGGGCCACTATAATGTCTC	58°C
BF815673	Hs_EST4_for1 Hs_EST4_rev1	CTTCTATATTCCAGTTATGATTTCAAGG ACACATCTGGAATGAAAACATAAACC	54°C
AW858552 and AW858470	Hs_EST2_for Hs_EST15_rev	CAGGACAATTAGTTCTAGAAGG CTCCTATTTAAATTGCTCACACTC	56°C
AW167235	Hs_EST17_for Hs_EST17_rev	TGACTATCTCTGTTGGGTAGAG GTGGGTTTTCAGAAATCTGAGC	
BE064736 and BE064727 and BE065063 and BE064976	4ESTfor 4ESTrev	TAACCCTATGTAGCTGGTGC CACACAATGCTCTCTCATTGG	58°C
BG952464	Hs_78_for Hs_78_rev2	CACATTCCTCCTCCTTCATTC GATGTGCACTGTCCATTTTAGATC	56°C
AW937867 and AW937872	Hs_EST9/14for Hs_EST9/14rev	GCAGAAATTCTTTGTGAAAGGAG ACTGTACACACAGGATTGTGC	58°C

Table 21 Primers used for expression analysis of high homology regions (HHR)

HHR	Primer name	Primer sequence [5' → 3']	PCR product	T_A
1.	Mm_46_for Mm_46_rev	GTACTTGGTAGCCCTTCAAG CTGAAAAATGAACTGCTTCTAGC	185 bp	56°C
2.	Mm_47_for rej57rev	CTCTCCCTAGCCCACTTAG CTATTACCCTGGCGAAACC	390 bp	56°C
3.	Mm_48_for1 Mm_48_rev1	CATGCTAACAACGCCCCTAG AGCCCCACTTTGCCTTCC	224 bp	56°C
4.	Mm_48_for2_new Mm_48_rev2_new	GGAATTGCTCATTAAATTGCCTAC GAATTTGACTTGGGGTGGACT	98 bp	56°C
5.	Mm_361_for1 Mm_361_rev1	TTTGGAAGATGTATAATGCAATATAAAC GAGCAATTGAAAGTAATATGGGCA	244 bp	56°C
6.	Mm_50_for1 Mm_50_rev1	CAATATATTTTTCAATCCTGACTGTTGG TGAGGCAGTGGCACTAAATG	85 bp	60°C
7.	Mm_50_for2 Mm_50_rev2_new	ATAGAGAAAATGCGTAGATGTGCC TTCAGCTACAGAGAGCTCCCCCA	94 bp	60°C
8.	Mm_51_for1 Mm_51_rev1	GTTTTACACTCTAAATGAAAGCCAC TACCTTACTTTAGCAGCGTGG	92 bp	56°C
9.	Mm_365_for1 Mm_365_rev1	CCATTGTCATGCAAGCACAG GGGATTTGTCCTTTTTATCTAGTC	341 bp	60°C
10.	Mm_54_for rej67rev	CATGGCGAATTCAGTATGAAGG GCAGCTATTTAGCTCGAATTGG	275 bp	58°C
11.	Mm_65_for1_new1 Mm_65_rev1_new1	ATGTGTCTAGGAAGGACATGC AGAGTTCAGCGACATTTGCCTC	299 bp	58°C
12.	Mm_65_for2 Mm_65_rev2	GACAACAATGCCTCGGAAG GACTTCTGAGCTTTCAGAGTG	133 bp	56°C
13.	Mm_68_for1 Mm_68_rev1	CAGCTGTACCCATGAGCATC GGGAGACAATGAGAACGTTTG	130 bp	56°C
14.	Mm_68_for2 Mm_68_rev2	TCATGCACTTCGTACACCTG TTCCTTGACCTAGAAATACGATAC	106 bp	56°C
15.	Mm_70_for Mm_70_rev	GAGTCAGAAAATTGCGATTCATTCC CTTGGCTCCAACAGAGTAGC	196 bp	56°C
16.	Mm_73_for Mm_73_rev	GGGACATGCCATATATTAGCAG GATACAGGAATCGAAGAAAACAGG	446 bp	56°C
17.	Mm_77_for1 Mm_77_rev1	CCTTGTTTTCTTCTGGCCATTTC TTACCAGTGTGACAGTATTAGAAAG	95 bp	56°C

HHR	Primer name	Primer sequence [5' → 3']	PCR product	T_A
18.	Mm_77_for2 Mm_77_rev2	CCTGTCTCCAGAGATGGATC GTCTCTGATGTGTAAGATCAAGC	143 bp	56°C
19.	Mm_78_for Mm_78_rev	GAAGGCACCTCTCACATAAG GTGCTCTATAATTCTACGTGAAG	532 bp	56°C
20.	Mm_79_for Mm_79_rev	CAGAGATCACCCTCTTTCAG TACATAGAATTGTCTTCTGGACC	106 bp	56°C
21.	Mm_80_for Mm_80_rev	GTAAAAGCAGCACACAGTAGTC CTTAGATCTTGATTCTACTCCAACC	221 bp	56°C
22.	Mm_81_for_new Mm_81_rev	GTAGTTTAATGCCCAGCGG GTGTAAAGCAGTTGCTAGAAATC	178 bp	58°C
23.	Mm_86_for Mm_86_rev	TTCTCCGTGAAAGGAGGAG GTGCTGTCACTGAATTCCTTG	158 bp	56°C
24.	Mm_89_for Mm_89_rev	TCTGTCCCTTCTCAAATGGAAG GTGGAGTTAAAGTAGACATATGAGC	86 bp	56°C
25.	Mm_90_for_new2 Mm_90_rev	ATCCATTCTCTGCCCCACTC ATCAGAGGTATTATGGGTGAGC	89 bp	58°C
26.	Mm_95_for Mm_95_rev	CTTGTGAGGCATTAAGATGTTCTC GGTGCTGCCGTGTTAGTATG	116 bp	56°C
27.	Mm_97_for Mm_97_rev	CCCAGTGGCCTTTCTAGTC GGTATGTAGGGCAGGAATATG	118 bp	56°C
28.	Mm_98_for Mm_98_rev	CCTTATTCTAGAATGGCCCAG CTTTGTAAGAGCCCAGAAATGG	122 bp	56°C
29.	Mm_100_for1 Mm_100_rev1	GAGCTAAATTTCCTAGATGGTTATG CTTGGAAAGGTTCTAGGTGTC	127 bp	56°C
30.	Mm_100_for2 Mm_100_rev2	CATAAAACCGGGGCTCCCA CTTATGGGGACTAATGACTAATTCC	226 bp	56°C
31.	Mm_105_for Mm_105_rev_new1	CCCTGCAAATTATAAGCAGCTC CTAATGAAAAGCAGAGGCAAATGAG	126 bp	58°C
32.	Mm_105_for_new1 Mm_105_rev	CACATTTCCTATCAGCCCCT TGCAGTTTGTGACTCCCAAAG	470 bp	56°C
33.	Mm_106_for1 Mm_106_rev1	CTGATCTTTTCCTCTAGCCAG CCTGAGCCCAAGTATTCAC	190 bp	56°C
34.	Mm_106_for2 Mm_106_rev2	TCTTCCCAGGAATGCATCTG CCCTTTTCCTGTTGCTATTTCC	356 bp	56°C
35.	Mm_106_for3 Mm_106_rev3	ACGGACGGTTGTTACACTAG TTTCTGAATCATGCTGACGACG	394 bp	56°C

HHR	Primer name	Primer sequence [5' → 3']	PCR product	T_A
36.	Mm_107_for Mm_107_rev	TGATCAACTTTACTCCTGTTGCTT CTTTATGATATCAGTCACACAG	125 bp	52°C
37.	Mm_108_for Mm_108_rev	TGTCAGGAGTACTAGGAAATGG AATGGATCCTCTCTAGGGGTGT	147 bp	58°C
38.	Mm_111_for Mm_111_rev_new	CATGTTTTGAGAGGTCAACAATGTC CTTCACAAGGAGCCTCAGATG	110 bp	58°C
39.	Mm_113_for2 Mm_113_rev	TCTAAGTAAGAGAACAGATGTGG GTACTTCCTTAATGTAAAGCTCG	92 bp	56°C

2.2.11 Construction of yeast and mammalian expression vectors

DNA manipulations were carried out using standard techniques (Sambrook et al. 1989). In order to facilitate cloning, appropriate restriction sites were incorporated into gene-specific primers used for PCR amplification. Amplified DNA fragments were cloned via A overhangs into the pCRII-TOPO vector as intermediate products or digested with relevant restriction enzymes and cloned directly into proper vectors. In all cases a *Taq*/*Pfu* polymerase mix (*Taq*:*Pfu* in proportion 36:1) was used for amplification in order to improve the fidelity. Sequence identity of each construct was verified by sequencing.

2.2.11.1 Hoxd13 cloning

The yeast two-hybrid bait construct LexA_Hoxd13-HD was cloned in two steps. First, the Hoxd13 fragment lacking the homeodomain (*Hoxd13-HD*) was amplified using primers listed in Table 22 on the cDNA template derived from mouse embryonic stage E14.5. Afterwards, the PCR product and the pBTM16 vector carrying the LexA DNA binding domain were digested with *Eco*RI and *Sal*I restriction nucleases, ligated using T4 DNA ligase (Promega) according to the manufacturer's protocol and transformed into *E. coli*. Similarly, for construction of the vector GAL_Hoxd13-HD, the *Hoxd13-HD* fragment was amplified using specific primers (see Table 22) and cloned into the pGBKT7 vector carrying the GAL DNA binding domain via *Eco*RI and *Sal*I sites.

In order to clone *Hoxd13-HD* into the pTL1-HA2 vector, an *Eco*RI-site-containing 5' primer and a stop codon- and the *Xho*I-site-containing 3' primer were used for amplification of the insert (Table 22). Following restriction digestion with *Eco*RI and *Xho*I enzymes, the PCR product was ligated with the vector, giving rise to the Hoxd13-HD-pTL1-HA2 construct.

All PCR reactions were performed in 1 × PCR buffer (Fermentas) and contained 1.5 mM $MgCl_2$, 1 µM each primer, 8% DMSO and 0.1 mM dNTPs. Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at the specific annealing temperature, 1 minute at 72°C, and a final extension step at 72°C for 10 minutes.

pTL1-HA2 constructs carrying the wild type *Hoxd13* gene (wtHoxd13-pTL1-HA2), as well as the *Hoxd13* gene with the expanded stretch for additional 14 Ala residues (Hoxd13+14Ala-pTL1-HA2) or with the alanine stretch reduced to two residues only (Hoxd13_2Ala-pTL1-HA2) were kindly provided by A. Albrecht (MPI for Molecular Genetics, FG Mundlos).

Table 22 Primers for amplification and cloning of *Hoxd13*

Cloned fragment	**Primer**	**Sequence [5' → 3']***	**PCR product****	**Cloning vector**
Hoxd13-HD	EcoRI-HoxD13-For	GAGAATTCGGAATGAGCCGCTCGGGGACT	Hoxd13 (1-816 bp)	pBTM116
	SalI-HoxD13-Rev	CTGTCGACTCCCCGTCGGTAGACGCA		
Hoxd13-HD	EcoRI-HoxD13-For	GAGAATTCGGAATGAGCCGCTCGGGGACT	Hoxd13 (1-816 bp)	pGBKT7
	Hoxd13-SalI-rev-sto	CTGTCGACTTATCCCCGTCGGTAGACGCA		
Hoxd13-HD	EcoRI-HoxD13-For	GAGAATTCGGAATGAGCCGCTCGGGGACT	Hoxd13 (1-816 bp)	pTL1-HA2
	Hoxd13-XhoI-rev-sto	CTCTCGAGTTATCCCCGTCGGTAGACGCA		

* Recognition sites for restriction endonucleases are underlined within primer sequences.
** Positions correspond to the *Hoxd13* mRNA sequence NM_008275.

2.2.11.2 Peg10 cloning

At the beginning, *Peg10* fragments found in the yeast two-hybrid screen were cloned into the mammalian expression vector pcDNA-Flag, later however the full ORF2 of *Peg10* was amplified and cloned into the same vector.

The yeast two-hybrid inserts were cut out of the respective prey plasmids using *Not*I restriction endonuclease and the inserts were cloned directly into the *Not*I-digested and dephosphorylated pcDNA-Flag vector.

For cloning ORF2 of *Peg10*, inserts were amplified on mouse cDNAs from embryonic stages E9.5, E11.5, E12.5 and E13.5 using specific primers with incorporated recognition sites for restriction nucleases *Not*I or *Xba*I. Following digestion with *Not*I and *Xba*I, PCR products were ligated into the pcDNA-Flag vector. Primer sequences, annealing temperature (T_A) and length of amplicon can be found in Table 23.

Table 23 Primers used for *Peg10* amplification and cloning

Primer	**Sequence [5' → 3']***	**T_A**	**PCR product****
Peg10_Not_ORF2_for	TTGCGGCCGCTTATGCTCCA GATTCATATGCCGG	60°C	Peg10-ORF2 (1502-3172 bp)
Peg10_Xba_ORF2_R	GGTCTAGACTACGCAGCAC TGCAGGATG		

* Recognition sites for restriction endonucleases used for cloning the PCR products are underlined within primer sequences.
** Positions correspond to the *Peg10* mRNA sequence AB091827.

PCR products as well as plasmids carrying ORF2 inserts were sequenced with gene-specific primers (see Table 24). Additionally, recombined plasmids were sequenced with vector-specific primers (see Table 14) in order to confirm the cloning boundaries.

Table 24 *Peg10*-specific primers used for sequencing

Primer	**Sequence [5' → 3']**	**Primer binding site***	**T_A**
Peg10_seq4_for	CAATTGCCTCGGGCCCAATC	1665–1684 bp	60°C
Peg10_seq5_for	CCGCATCAGTATCCGCATCC	2021-2040 bp	60°C
Peg10_seq6_for	CATATGAATCCGGATCCACATCAC	2276–2299 bp	60°C
Peg10_seq7_for	TTGACCCTAACATTGAGATGATTCC	2619–2643 bp	60°C
Peg10_seq8_rev	GTCCACGAAATTTCGCAGAGC	2702–2722 bp	60°C
Peg10_seq9_rev	GACAAATTCACCATAGCTTGCCAG	2900–2923 bp	60°C

* Positions correspond to the *Peg10* mRNA sequence AB091827.

2.2.11.3 Cloning of other candidate genes identified in the yeast two-hybrid screen

Fragments of *Dlxin-1*, *Wtip*, *Limk1*, *Limd1* and *Cnot3* found in the yeast two-hybrid screen, were subcloned into the pcDNA-Flag vector basically as described for *Peg10* yeast two-hybrid fragments. The inserts were cut out of the respective prey plasmids using *Not*I restriction endonuclease and cloned directly into the *Not*I-digested and dephosphorylated pcDNA-Flag vector. All fragments were cloned in frame and corresponded to the following positions, for *Dlxin-1*: 1252–1600 bp (GenBank entry AB029448), for *Wtip*: 706–1177 bp (GenBank entry NM_207212), for *Limk1*: 380–803 bp (GenBank entry NM010717), for *Limd1*: 1974–2287 bp (GenBank entry NM_013860), for *Cnot3*: 639–941 bp (GenBank entry NM_146176).

The full length *Wtip* sequence cloned into the pcDNA-Flag vector (Wtip-pcDNA-Flag) was a kind gift of N. Verhey van Wijk (MPI for Molecular Genetics, FG Mundlos).

2.2.12 Small scale yeast transformation

LexA_Hoxd13-HD and the empty pBTM116 vector were transformed to the yeast cells L40 based on the protocol from the Yeast Protocols Handbook (Clontech). Briefly, a single colony of the wild type L40 yeast strain was used to inoculate 40 ml of YPD liquid medium supplemented with 50µg/ml ampicillin. Flasks were incubated overnight at 30°C with vigorous shaking. On the next day approximately 10-20 ml of the overnight culture was diluted in 300 ml of the fresh YPD medium, so that the OD_{600} of the new culture was between 0.2 and 0.25. Yeast cells were cultivated at 30°C for 3-5 hours until the OD_{600} reached 0.5-0.7. Furthermore, the cells were pelleted by centrifugation for 5 minutes at 1000 g at room temperature, washed with 40 ml sterile water and centrifuged again under the same conditions. The pellet was resuspended in 1.5 ml sterile 100 mM LiAc. Competent cells prepared in this way were used for heat shock-based transformation. Shortly, 50µl of competent yeast cells were mixed together with 0.2 µg of the appropriate plasmid, 50 µg of the denatured carrier DNA (salmon sperm DNA) and 300 µl sterile 40% PEG/100mM LiAc solution. All components were vortexed and incubated for 30 minutes at 30°C. Afterwards 35 µl DMSO were added and the tubes were incubated for 15 minutes at 42°C, followed by a short incubation on ice (heat shock). Yeast cells were shortly centrifuged, the pellet was resuspended in 100 µl sterile water and the suspension was streaked on SD agar plates lacking tryptophane (SD –T).

The construct GAL_Hoxd13-HD and the empty vector pGBKT7 were transformed into *ADE2*-deficient AH109 yeast. In order to culture the AH109 strain, YPDA medium was used. Preparation of yeast competent cells as well as transformation were performed according to the protocol described above.

2.2.13 Preparation of yeast protein extracts

A 10 ml liquid culture was prepared from each transformed yeast strain and was incubated in appropriate medium at 30°C overnight. On the next day, OD_{600} was measured and the amount of yeast culture corresponding to $OD_{600} = 1$ was centrifuged for 30 seconds at 8000 rpm in a table centrifuge. The supernatant was discarded and the pellet was resuspended in 100 µl 2 × Sample buffer (see Table 5). Samples were boiled for 5 minutes, afterwards cooled on ice and stored at –20°C.

2.2.14 Yeast two-hybrid screen

The yeast two-hybrid library screen was carried out using the LexA system. The yeast L40 strain carrying the vector LexA_Hoxd13-HD was transformed with the mouse cDNA library from pooled embryonic stages E9.5 – E10.5 constructed in the pVP16 vector carrying the VP16 activation domain. The screening protocol resembles the small-scale yeast transformation protocol. In short, one colony of the LexA_Hoxd13-HD positive yeast was cultured overnight in 5 ml of the SD –T medium at 30°C. The primary culture was subsequently transferred into a bigger flask containing 100 ml SD –T medium and was incubated overnight under the same conditions. On the third day, the culture was diluted in 300 ml YPD so that the OD_{600} reached 0.2 – 0.25. Yeast cells were grown approximately 5 hours until the culture reached $OD_{600} = 0.5$. Afterwards, cells were centrifuged for 5 minutes at 1000 g, washed with 10 ml 100 mM LiAc, pelleted again and resuspended in 2 ml of 100 mM LiAc. 50 µl of yeast competent cells were used for a mini transformation with the empty pVP16 vector (negative control) according to the protocol described in section 2.2.12. After transformation yeast suspension was in parallel streaked on SD plates lacking tryptophane and leucine (SD –TL), and on SD agar plates lacking tryptophane, histidine, uracil, lysine and leucine (SD –THULL).

At the same time 2 ml of yeast competent cells were mixed with 200 µg of the mouse cDNA library and with 3 mg of the denatured carrier DNA (salmon sperm DNA), and kept at the

room temperature for 3 minutes. After adding 20 ml of 40% PEG/100 mM LiAc solution yeast cells were incubated for 30 minutes at 30°C with shaking (200 rpm). Subsequently 2 ml DMSO was added and cells were delicately shaked for 15 minutes at 42°C in order to keep the whole volume equally warm. Later, cells were cooled for 1 minute on ice (heat shock) and 400 ml of warm YPD medium supplemented with 50 µg/ml ampicillin was added. Following incubation for 1h at 30°C, cells were pelleted at 1000 g for 5 minutes, washed in 40 ml sterile H_2O, pelleted once more and resuspended in 5 ml H_2O. 5 µl of transformed cells were used to prepare serial solutions 1:10, 1:100, 1:1000, 1:10000 and 1:100000 which were plated on 12 × 12 cm plates containing SD medium lacking tryptophane and leucine (SD –TL) (control of transformation efficiency). The rest of the cells was plated on SD medium lacking tryptophane, histidine, uracil, lysine and leucine (SD –THULL) in order to identify protein-protein interactions. The clones surviving from the nutrition selection (HIS3-positive colonies) were re-streaked three times on the same SD –THULL medium in order to reduce the number of false positives, and later they were tested for expression of the second reporter gene (see section 2.2.15). Different media used during the yeast two-hybrid screen are listed in Table 8.

2.2.15 LacZ colony filter assay

In order to screen HIS3-positive yeast colonies for the expression of *LacZ* (the second marker gene indicating interaction between proteins) a colony filter assay has been performed. Briefly, 12 × 12 cm filters were cut out of Whatman paper 3MM and were put onto SD –TL plates on which positive yeast colonies and a negative control were growing (yeast transformed with the LexA_Hoxd13-HD construct and the empty pVP16 vector). Yeast cells that attached to the filters were permeabilised by repeated freeze/thaw procedure (three times freezing in liquid nitrogen for ~10 seconds each, and then thawing at room temperature). Fresh Whatman filters were put into clean 12 × 12 cm plates and were soaked with 7 ml of the substrate solution containig X-Gal. Filters with yeast colonies were placed on the pre-soaked filters in the plates and incubated at 37°C. Filters were monitored for the appearance of blue colour once per hour. The reaction was stopped when the negative control (yeast clone transformed with the bait and the empty prey vector) started to get the blue staining. Only colonies showing very intensive blue colour were considered as true positives. Solutions used in this section can be found in Table 4.

2.2.16 Analysis of prey inserts in double positive yeast clones

For all positive yeast clones that survived from nutrition selection and turned blue in the presence of the X-Gal, inserts of prey plasmids were amplified by colony PCR. In short, fresh yeast colonies were picked and dispersed in 50 µl H_2O by vigorous vortexing. Cell suspensions were subjected to three times freeze/thaw treatment (frozen for ~10 seconds in liquid nitrogen, and thawed at room temperature). Five µl of the supernatant containing the released plasmids were used as template for PCR. PCR reactions were carried out with VP16F1 and VP16R1 primers. Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 62°C, 1 minute at 72°C, and a final extension step at 72°C for 7 minutes. Positive PCR products were enzymatically purified using shrimp alkaline phosphatase (SAP) and *E. coli* exonuclease I in the supplied buffer according to the manufacturer's protocol. After purification, PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Samples were analysed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. Sequences were analysed by BLAST searches against the non-redundant nucleotide database at the National Center for Biotechnology Information (NCBI).

2.2.17 Preparation of chemical competent *E. coli* cells

A single colony of *E. coli* HB101, DH5α, XL1-Blue, GM2163 or STBL4 cells was used for inoculation of 5ml LB medium without antibiotics. Cells were grown overnight at 37°C with vigorous shaking. On the next day the overnight culture was used to inoculate 500 ml of the fresh LB medium and afterwards bacteria were cultured under the same conditions until they reached OD_{600} = 0.9 – 1.0. Subsequently the culture was centrifuged for 15 minutes in a GSA rotor at 8000 rpm at 4°C. The medium was discarded and the bacterial pellet was resuspended in 125 ml of buffer A and incubated 30-60 minutes on ice. Next, the centrifugation step was repeated and the pellet was resuspended in 20 ml of buffer B. Bacteria were aliquoted, frozen in liquid nitrogen and stored at -80°C. Media and solution used for preparing competent cells can be found in Table 7 and Table 9.

2.2.18 Isolation of prey plasmids from double positive yeast colonies

Isolation of prey plasmids from HIS3- and LacZ-positive yeast clones was performed in three steps, which included isolation of total yeast DNA, separation of the plasmid DNA from yeast genomic contamination in HB101 *E. coli* strain, and finally plasmid purification from bacterial cells.

Each HIS3/LacZ double positive yeast colony was cultured overnight at 30°C in SD medium lacking leucine. The next day, cells were harvested by centrifugation for 10 seconds at 13000 rpm. The supernatant was discarded and the cell pellet was resuspended in 200 µl of yeast lysis buffer. Subsequently, 200 µl of phenol/chloroform and approximately 0.3 g of glass beads were added to the buffer. The yeast cell suspension was vortexed for 2 minutes in order to destroy cell walls, and centrifuged at room temperature for 5 minutes at 13000 rpm. Yeast total DNA was precipitated by adding 1/10 volume of 3 M NaAc and 2.5 volumes of 96% ethanol to the supernatant, and by centrifugation for 15 minutes at 13000 rpm at 4°C. The DNA pellet was washed twice with 70% ethanol, dried and suspended in 15 µl sterile water.

Approximately 10–15 µl of yeast total DNA was used to transform chemical competent HB101 *E. coli* cells with the "heat shock" method according to standard procedures (Sambrook et al. 1989). Before plating, cells were harvested at 2500 rpm for 5 minutes. The pellet was washed twice with M9 minimal medium, resuspended in 80 µl of M9 minimal medium, plated on M9 agar and incubated at 37°C. Colony growth was observed 36-48 hours after transfection. Plasmid DNAs were isolated from HB101 colonies using the QIAprep spin miniprep kit according to the manufacturer's protocol.

All solutions, kits and media used in this section are listed in Table 4, Table 8 and Table 9.

2.2.19 Confirmation of protein-protein interactions in yeast

Each positive prey plasmid was separately transformed into the L40 yeast strain carrying the LexA_Hoxd13 construct according to the small scale yeast transformation protocol described in section 2.2.12. After transformation, cells were plated on SD –THULL agar. Interactions between GAL_Hoxd13-HD construct and the candidate genes were confirmed in yeast strain AH109 basically in the same way, however in order to detect protein-protein interactions yeast cells were plated on a slightly different medium (SD agar lacking adenine, histidine, leucine and tryptophane).

2.2.20 Cell culture and DNA transfection

COS1 cells were cultured in the appropriate medium at 37°C in 5% CO_2. For immunocytochemistry studies, cells were grown on glass coverslips in 12-well plates. Transient transfection was performed using Polyfect transfection reagent (Qiagen), according to the manufacturer's protocol. Typically, 4×10^4 cells per well were seeded 24 hours before the experiment, and transfected with 0.6 µg plasmid DNA and 4 µl Polyfect Transfection Reagent.

For Western blot and coimmunoprecipitation analysis transfection was performed in 6-well plates and the amount of plasmid DNA and transfection reagent was adjusted proportionally to the surface of wells.

2.2.21 Immunocytochemistry

Immunofluorescence experiments were performed 48 hours after transfection. All steps were performed at room temperature. First, medium was sucked off and the cells on coverslips were rinsed with PBS and fixed in 4% paraformaldehyde/PBS for 15 minutes. Later, cells were washed one time with PBS to remove excess of paraformaldehyde, and permeabilised in PBS buffer containing 0.2% Triton X-100 for 15 minutes. After washing three times in PBS, cells were blocked for 45 minutes in blocking solution and incubated for 1 hour with primary antibodies diluted 1:250 in blocking solution. After washing three times with PBS, cells were incubated for another hour with the appropriate Alexa Fluor-conjugated secondary antibodies diluted 1:500 in blocking solution. Subsequently, cells were washed 3 times with PBS, incubated in DAPI/PBS (1:2000) solution for 10 minutes in order to stain nuclei, and washed twice in PBS to remove excess of DAPI. Coverslips were fixed on slides with Fluoromount-G slide mounting medium and cells were visualised using 63 × oil-immersion lens on an Axiovert 200 M fluorescence microscope (Zeiss) equipped with filters for excitation of green, red and blue. Digital images were captured using an AxioCam MRm camera and the AxioVision 4.2 fluorescence image analysis software.

For the detection of different Hoxd13 proteins anti-HA antibodies produced in rabbit and Alexa Fluor® 546 goat anti-rabbit immunoglobulins were used. Detection of Peg10, Dlxin-1, Wtip, Limk1, Limd1 and Cnot3 proteins was performed with the use of monoclonal anti-Flag antibodies and Alexa Fluor® 488 goat anti-mouse immunoglobulins.

Antibodies and solutions used in this section are listed in Table 5 and Table 15.

2.2.22 Coimmunoprecipitation

Coimmunoprecipitation experiments were performed 48 hours after transfection. All steps were done at 4°C in order to avoid protein degradation. COS1 cells were shortly washed with ice cold 1 × PBS and cell-containing plates were immediately transferred onto ice. 400 µl of cold lysis buffer containing protease inhibitors was added to every well and cells were scraped off the bottom of the wells with plastic scrapers (Biochrom). Afterwards, COS1 cells were incubated for 1 hour with very delicate shaking. Cell lysates were collected in eppendorf tubes and centrifuged for 15 minutes at 14000 rpm in a table centrifuge. Lysates were transferred into fresh tubes and pellets were discarded. Total protein concentration in lysates was measured according to the standard Bradford method (Bradford 1976) using Anthos Reader 2020 (Anthos Labtec Instruments, Austria).

For one immunoprecipitation reaction 30 µl of anti-Flag gel or anti-HA agarose conjugate (both called beads for short) were used. Appropriate beads were washed 3 times with 800 µl of the lysis buffer, which was followed by addition of cell lysates containing 0.8 –1.0 mg of total protein. Bead suspensions were incubated for 2 hours with delicate shaking, pelleted and washed 4 times with 1 ml of lysis buffer. In the end, 45µl of protein loading buffer were added, samples were denatured for 5 minutes at 95°C and stored at –20°C.

2.2.23 SDS-PAGE and Western blot analysis

Polyacrylamide gels were prepared according to standard protocols (Sambrook et al. 1989). Protein samples were heated at 95°C in protein loading buffer for 5 min and separated by 10-15% SDS-PAGE. For Western blot analysis, proteins were transferred from gels to microporous polyvinylidene difluoride (PVDF) membrane Immobilon-P (Millipore) using a mini tank transfer unit TE22 (Amersham Bioscience) according to the manufacturer's instructions. After transfer, blots were incubated with Western blot blocking buffer for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Blots were washed three times in PBST, and incubated for 1 hour at room temperature with peroxidase-conjugated anti- rabbit IgG or anti-mouse IgG. Following 5 times washes in PBST, blots were incubated with Roti-Lumin chemiluminescence substrate (Roth) according to the manufacturer's protocol and exposed to Fuji X-ray film. Anti-HA and anti-Flag antibodies were used at 1:2000 dilution. Anti- c-myc monoclonal antibodies were used at 1:500 dilutions. Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were

used at 1:2000 dilution. Solutions and antibodies used in this section are listed in Table 5 and Table 15.

2.2.24 Synthesis of RNA probes for *in situ* hybridisation

The part of the *Peg10* coding sequence corresponding to 2075–2348 bp according to GenBank entry AB091827 (yeast two-hybrid positive prey number 37) was cloned into the pcDNA-Flag vector, as described in section 2.2.11.2. One of the positive plasmids containing the insert cloned in the antisense orientation was used to prepare the probe for RNA *in situ* hybridisation experiments. For negative control, the vector pcDNA-Flag_37, which carried the same *Peg10* insert in a sense orientation, was used. Similarly, the *Limk1*, *Limd1* and *Cnot3* yeast two-hybrid clones were subcloned into pcDNA-Flag vector (described in the section 2.2.11.3). Plasmids carrying inserts in frame were used for synthesis of negative controls, whereas vectors with inserts cloned in the antisense orientation were used to synthesise *in situ* probes. In short, 30 µg of each plasmid was linearised with 3 units of *Bcl*I restriction endonuclease in a total reaction volume of 100 µl. After 3 hours of digestion at 37°C, 20 µg proteinase K was added to the reaction in order to remove ribonucleases. Starting from this step, all used solutions were treated with DEPC or they were taken from fresh aliquots in order to ensure that they were RNase-free. After 1 hour incubation at 37°C the reaction was stopped and 400 µl DEPC-H_2O was added, followed by phenol/chloroform extraction of the plasmid DNA, according to standard procedures (Sambrook et al. 1989). Precipitated vector was resuspended in 25 µl DEPC-H_2O and used as a template for *in vitro* transcription. The reaction was performed in a total volume of 20 µl and contained 1 µg of vector, 1 × DIG labelling mix, 40 units of RNase inhibitor, 1 × transcription buffer (Roche), and 40 units of T7 RNA polymerase. All components were incubated for 2 hours at 37°C, afterwards 20 units of RNase-free DNase I were added in order to remove the plasmid DNA template and incubation was proceeded. After 15 minutes, the reaction was stopped by adding 2 µl of 0.2 M EDTA, synthesised RNA was precipitated using 1/10 volume of 4 M LiCl and 2.5 volumes of ethanol, and diluted in 100 µl DEPC-H_2O. The quality of the synthesised RNA probe was checked on 1% agarose gel.

2.2.25 Whole mount *in situ* hybridisation

Isolation of mouse embryos was performed in DEPC-PBS. Clean embryos were fixed overnight in 4% PFA/PBS at 4°C. On the next day embryos were washed at room temperature two times for 5 minutes in DEPC-PBST, two times for 5 minutes in 50% methanol/DEPC-PBST, and finally one time for 5 minutes in 100% methanol. In the end, embryos were soaked in fresh 100% methanol and stored at –20°C.

Hybridisation with DIG-labelled probes was performed essentially as described elsewhere (Albrecht et al. 2002). In short, embryos were rehydrated at 4°C in 75%, 50%, 25% methanol/DEPC-PBST (10 minutes per each solution) and washed 2 times in ice-cold DEPC-PBST. Afterwards, embryos were bleached with 6% hydrogen peroxide for 1 hour at 4°C, followed by 3 washes in DEPC-PBST (10 minutes each). Younger embryos (E10.5 and E11.5) were digested for 3 minutes with 10 µg/µl proteinase K in proteinase K buffer at room temperature. For older embryos (E12.5), proteinase K concentration was raised to 20 µg/µl and the reaction time was increased up to 5 minutes. After digestion, embryos where washed 2 times in PBST/Glycine, 2 times in DEPC-PBST, 3 times in RIPA buffer, again 3 times in DEPC-PBST and fixed for 20 minutes in PFA/Glutaraldehyde. Subsequently, embryos were washed 3 times in DEPC-PBST and incubated in hybe buffer:DEPC-PBST (1:1 dilution) for 10 minutes, followed by a single washing step in hybe buffer at room temperature. Prehybridisation of embryos was performed in hybe buffer at 65°C. Prior to hybridisation, DIG-labelled RNA probes were diluted 1:100 and denatured for 5 minutes at 80°C, and added together with tRNA (100 µg/ml) to the prehybridisation solution. Hybridisation was performed at 65°C overnight. In order to remove unbound probe, embryos were washed 2 times for 30 minutes in hybe buffer at 65°C, followed by a single washing step in 50% RNase solution/50% hybe buffer at room temperature and by digestion with RNase A (100 µg/ml in RNase solution) for 1 hour at 37°C. Subsequently, embryos were incubated for 5 minutes in 50% RNase solution/50% SSC/FA/T at room temperature, and washed with SSC/FA/T at 65°C (2 times for 5 minutes, 3 times for 10 minutes and 6 times for 30 minutes). After cooling down to room temperature embryos were washed for 10 minutes with (1:1) SSC/FA/T/1 × MABT and subsequently 2 times for 10 minutes with 1 × MABT. Prior to the incubation with antibodies, embryos were blocked with 10% Blocking reagent (Boehringer) diluted in 1 × MABT for 1 hour at the room temperature. At the same time 1 × MABT solution containing 1% Blocking reagent and alkaline phosphatase-conjugated anti-DIG antibodies (diluted 1:5000) was prepared and incubated for 1 hour at 4°C. After the

blocking step, embryos were incubated overnight at 4°C in anti-DIG-antibody-containing solution and subsequently transferred to room temperature and washed 8 times for 1 hour in fresh PBST/Tetramisole solution in order to remove unbound antibodies. Staining of the embryos was based on the enzymatic reaction performed by alkaline phosphatase (AP) in AP buffer using the BM Purple AP Substrate (Roche) according to the manufacturer's protocol. After the reaction, embryos were washed in alkaline phosphatase buffer, fixed in PFA/Glutaraldehyde and stored at 4°C. Images of the stained embryos were captured using the Leica MZ 12.5 stereomicroscope (Leica) coupled to the AxioCam HRc camera and the AxioVision 4.2 image analysis software.
Solutions and antibodies used in this section are listed in Table 6 and Table 15.

2.2.26 Section *in situ* hybridisation

Mouse embryos were isolated and fixed as described in the previous section. On the second day the embryos were washed twice for 15 minutes in fresh DEPC-PBS, followed by incubation for 1 hour in 70% ethanol. After changing the ethanol, embryos were incubated in the tissue processor Leica TP 1020 (Leica) according to the manufacturer's protocol, embedded in paraffin and sectioned at 7 µm with the Reichert Jung 2050 microtome (Reichert Jung). Sections were attached to glass slides, baked for 1 hour at 60°C, dewaxed in xylene and rehydrated using 100%, 75%, 50% and 25% ethanol concentrations. At the end, slides were washed 2 times in DEPC-PBS, followed by a fixation step in 4% PFA/PBS for 10 minutes at room temperature. After 2 washes in fresh DEPC-PBST, mouse sections were digested with proteinase K (1.5 µg/ml) for 10 minutes and washed again 2 times in DEPC-PBST, followed by a second fixation step in 4% PFA/PBS for 5 minutes. Afterwards, sections were acetylated for 10 minutes with 0.25% acetic anhydride, washed 2 times in DEPC-PBST and prehybridised for 1–4 hours in hybridisation solution (for paraffin sections) in a humified slide box. Two µl of the specific probe was denatured in 100 µl of hybridisation solution (for paraffin sections) and hybridised to the slides at 65°C overnight. On the next day slides were rinsed with 5 × SSC, washed with 1 × SSC/50% formamide for 30 minutes at 65°C and in TNE for 10 minutes at 37°C, followed by RNase digestion (20 µg/ml diluted in wash buffer). Later, slides were washed in TNE for 10 minutes at 37°C, followed by a single wash in 2 × SSC for 20 minutes at 65°C and 2 washes in 0.2 × SSC in the same conditions. For detection of DIG-labelled probes, slides were washed 2 times with 1 × MABT at room

temperature and blocked in 1 × MABT containing 20% heat inactivated sheep serum (HISS). 1:2500 diluted alkaline phosphatase-conjugated anti-digoxygenin antibodies were pre-incubated for 2 hours at 4°C in 5% HISS/1 × MABT and pipetted onto the slides. After an overnight incubation at 4°C, slides were washed 3 times in 1 × MABT, incubated for 10 minutes in NTMT and developed with BM Purple AP Substrate (Roche), similarly as described for whole mount *in situ* hybridisation. After the reaction, slides were rinsed with NTMT, washed 2 times for 5 minutes in PBS, fixed in 4% PFA/PBS and embedded with the help of Tissue-TEK (A. Hartenstein).

For cryo-sections, freshly collected embryos were placed into a chamber filled with OCT cryomount medium (Sakura) and frozen in dry ice/ethanol. The resulting frozen blocks were stored at –80°C. Prior to sectioning, blocks were equilibrated for 24–72 hours at –20°C and sectioned at 10 µm with the HM 560 Cryo-Star cryostat (MICROM International GmbH). Hybridisation with DIG-labelled probes and signal detection was performed with the use of the Genesis RSP 150 automation system supplied with the Gemini pipetting software (Tecan Group Ltd.) as described elsewhere (Carson et al. 2005).

Images were captured using the Leica DMR light microscope (Leica) coupled to the AxioCam HRc camera and the AxioVision 4.2 image analysis software.