2. Material 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
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
ES cell	embryonic stem cell
et al.	*et altera*
EDTA	ethylene-diaminetetraacetic acid
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
i.s.h	*in-situ hybridization*
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, antibodies and oligonucleotides, unless otherwise indicated, were provided by the following companies: 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 DY380	(Yu et al., 2000)
Escherichia coli DH10B	Invitrogen
Escherichia coli EL350	(Lee et al., 2001)
BL21 (DE3)pLysS	(Stratagene)

2.2.3 Genomic libraries

BAC clones containing mouse genomic inserts were provided by C.H.O.R.I. BACPAC resources; the BAC clones used in this work belong to the Library RPCI 22, generated from females from the 129/SvEvTac-strain.

2.2.4 Cell lines

Embryonic stem (ES) cells from the line E14.1, derived from the 129/Ola mouse strain (Hooper et al. 1987; Kühn et al. 1991) and from the R1/RV strain (Rossant and Nagy, 1995) were used for introducing targeted mutations into the mouse.

2.2.5 Vectors

Name	**Insert**	**Source/Reference**
pBluescript II SK+	-	Sorge, 1988
pGEM-Teasy	-	Promega
pZERO™-2	-	Invitrogen
pET14b	-	Novagen
pKS-DTA	*DTA-cassette*	M. Treier, EMBL, Heidelberg
pBS-GAD43-GFP	membrane GFP	PAdlox-EGP
LoxP_FRT_Dualneo-FRT1	flox-FRT-Neo-FRT	H. Wende, MDC, Berlin

2.2.6 Antibodies

a) Primary

Antigen	**Host Animal**	**Dilution**	**Source**
BrdU	rat	1:200	Abcam
GFP	sheep	1:2000	Biogenesis
Anti-Digoxygenin	rabbit	1:2000	Roche
Tshz1	rabbit	1:5000	Produced in this work
Tshz2	rabbit	1:5000	Produced in this work
Pax6	rabbit	1:5000	Chemicon
Mash1	rabbit	1:3000	Cemines
Dcx	goat	1:500	Sigma
NeuN	mouse IgG	1:1000	Chemicon
Islet1	guinea pig	1:5000	T. Jessell, Columbia Univ.
Er81	rabbit	1:3000	Sigma
GABA	rabbit	1:5000	Sigma
TH	rabbit	1:5000	Pel-Freeze
Calretinin	goat	1:1000	Swant

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 of 1:250 for Cy2 and 1:500 for Cy3 and Cy5 were routinely used

2.2.7 cDNA probes for *in situ hybridization*

GAD67	T.Müller, MDC, Berlin
GAD65	T.Müller, MDC, Berlin
Reelin	T.Müller, MDC, Berlin
Sema3c	Cloned in this work
Cypin	RZPD clone p981B1043D

2.2.8 Oligonucleotides

All the oligonucleotides used in this work were ordered from MWG-Biotech (Ebersberg) or BIOTEZ (Berlin). All the primers are indicated in the direction 5´-3´.

a) genotyping primers

Tshz1_sense	GTTGAGGTGGCCTTGTAAGC
Tshz1-anti-WT	AAGTCGTGCTGCTTCATGTG
Tshz1_anti_mut	GTCCAGATAGGCAGGTCAGC
Tshz2_sense	CCCCCATTACAGGAATATCAGAGTG
Tshz2_anti_WT	GCTGGCCTCAAACTTGCTATGTAGC
Tshz2_anti_mut	GGACTTGAAGAAGTCGTGCTGCTTC
NRG-Ig-flox_up	GTAACCGTTCGTACGAGAATGGA
Tsh1flox_Typ_anti	GTCTCTTCATTGCACGCAAAT
Tsh1flox_Typ_sense	AAGCCAGCCTGGGCTACT

b) Primers for cloning of cDNA probes

Sem_sense	GCAGGAAAAGCAGAAACAGGAGGAA
Sem_anti	ACTGATAGATCTCCCATCAAAGGCC

c) Primers for the establishment of Tshz1^{GFP} allele

Gap_Tsh1_anti	CAGGGTGAGGAAGGGGACAATGGACCACCATGAAAGCTGG GCCCTTGGTGCCAAGGCTTTTAATTAATTCGCCCTATAGTG AGTCGT
Gap_Tsh1_sense	GCTGCTCAAAGGAAAGCAATAGATGTATAAAAGAGATACC AAGCGACCTACCTCTTACTTTCCTACAGATGCTTCGGCTCT
Tsh1-5´ha_sense	ACGGGTACCGCCAAGTTAGAAAGGCTCTGTG
Tsh1-5´ha_anti	ACGCCATGGTGGCCTCGGGAACGTAAGCTACGA

Tsh1-3´ha_sense	ACGTCTAGAGAACCAAGTTCCTTAAGAAC
Tsh1-3´ha_anti	ACGGAGCTCCATTGGAATGTGGAGCCCAG

d) Primers for establishment of Tshz2^{GFP} allele

Gap_Tsh2_anti	CCTGGAAAGTTGTAATTCTATCGATATTAATCATGTAGATT CCATGTCAATCTAGGAGGCCCGTTTAAACTTCGCCCTATAG TGAGTCGT
Gap_Tsh2_sense	CAAAGATCTTCACCACTAACAGGGGAACAATAATAAGATC TAGTTTGTAAGGTGGTCAGGCCTACAGATGCTTCGGCTCT
Tsh2-5´ha_sense	ACGTGGTACCGAATTTTGCCACTGCCCCCA
Tsh2-5´ha_anti	ACGCCATGGTGGCCTCCTGCCTCTACTTCCTCA
Tsh2-3´ha_sense	GAATTCGGATCCCATTGCTGATATGGTCAAGGTCCTC
Tsh2-3´ha_anti	GAGCTCCTGGTATTTGACGTTAGCCAGCCAG

e) Primers for establishment of Tshz1 conditional allele

Left.ha.ls.sense	ACCGGATCCAAGAATTCAGTTCAGTCCTTCCGTGGTG
Left.ha.ls.anti	ACCCTCGAGTCATGTGCCTTGGAAATGAC
Right.ha.ls.sense	ACCGCGGCCGCAAGAATTCGGACTCCACCCCAAACAACC
Right.ha.ls.anti	ACCTCTAGAAAGGATCCCCCTCACCTTTAGAGCAAGC
Left.ha.ss.sense	GGACTAGTTTACTGCTTCCCATGAAGGAC
Left.ha.ss.anti	CCGCTCGAGCAGGCTGGCTTTGAACTCTC
Right.ha.ss.sense	CGCGGATCCGGCTACTTGAGACTGTAGGA
Right.ha.ss.anti	ATAAGAAtGCGGCCGCAAACTAGTAGCTTACAAGGCCACCT CAA

2.2.9 Mouse strains

For the establishment of the mutant mouse strains, chimeras and F1 were crossed with C57BL/6J and CD1 backgrounds.

2.2.10 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

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. Beside that, detailed descriptions for other specific techniques are provided in the following section.

2.3.1. Extraction and purification of nucleic acids

2.3.1.1 Isolation of plasmid DNA, BAC DNA and DNA fragments

E.coli cells containing plasmid and BAC 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 and BAC 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.

The isolation of DNA fragments from agarose gel was performed with Quiaquick Gel Extraction Kit (Quiagen) or with NucleoSpin-Extract II-Kit (Machery-Nagel). The DNA was eluted with 10-25 µl TE and stored at -20°C.

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^{0}C 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^{0}C 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^{0}C 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^{0}C 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 Isolation and restriction hydrolysis of genomic DNA from ES-cells

Genomic DNA was isolated from the ES-cells that were cultivated in gelatinized 96- or6-well plates. For that, the cells in 96-well-plates were washed twice with PBS and then incubated over night in 50μl ES-lysis buffer per well at 60^{0}C. The DNA was then precipitated 30 min with 100μl Ethanol-Natriumacetate mix per well at room temperature, washed three times with 70% Ethanol and air dried 15-20 min. The final restriction hydrolysis of the ES-cell-DNA was made with 20 U of the appropriate restriction enzyme per well in a 35μl solution. This was incubated over night at 37^{0}C and the digested DNA was then separated by electrophoresis and used for Southern-Blot-Analysis.

The cells in 6-well-plates were washed with PBS, 0.5 ml of ES-cell lysis buffer was added pro well and left to incubate over night at 55^{0}C. The DNA was then extracted with 25:24:1 Phenol:Chloroform:Isoamylalcohol (PCI), washed with Ethanol and dissolved in an appropriate volume (0.2-1 ml) of TE. Restriction digest for Southern Blot analysis followed, with about 5-10µg per digestion.

2.3.2 Polymerase chain reaction (PCR)

PCR was routinely used to genotype mice and embryos, and to amplify DNA fragment for cloning steps. Optimal PCR conditions were established according to general rules (Innis et al. 1989). Primers were purchased from Biotez. The primers used are listed in 2.1.8. For genotyping PCRs, $MgCl_2$ 1,5 mM was used. Each 30µl reaction contained: 2µl of inactivated lysate, 3µl of 10x buffer (Invitrogen), 1.8µl $MgCl_2$, 1.5µl dNTP (2.5µM every nucleotide ; Invitek, Berlin), 1.5µl primer 1, 1.5µl primer 2, 0.2µl Taq polimerase (Invitrogen) and MilliQ water. The cycling conditions were : 4 min 94^0C, 35 times the cycle 45 sec 94^0C, 45 sec 63^0C (for Tshz1 and Tshz2 reporter PCR) or 62^0C (for Tshz1 flox PCR), 1 min 72^0C, followed by 5 min 72^0C and finally 4^0C.

2.3.3. Southern blot analysis

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 $Tshz1^{GFP}$ mice to confirm germline transmission.

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 0.2 M HCl for 15 min, shortly rinsed in distilled water and denatured by two 25 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 and 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, the 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 70°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 two hours (Southern of BAC clones) or over night (Southern of genomic DNA).

2.3.4 Generation, in vitro transcription and DIG-labeling of probes for in situ hybridization

A specific probe for mouse *Sema3C* was amplified from total mouse cDNA using PCR. The reaction product was then cloned into the pGEM-Teasy vector and used for *in vitro* transcription to generate RNA probes. Primers are listed in 2.1.8 and PCR conditions are the same as those used for Tshz genotyping.

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 to 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.

2.3.5 Microarray expression analysis

2.3.5.1 RNA isolation and purification

The Microarray expression analysis for the identification of genes differentially expressed was performed in RNA extracted from E15.5 dorsal spinal cord and P0 olfactory bulbs. Dorsal spinal cords and olfactory bulbs from WT and Tshz1$^{GFP/GFP}$ mice were dissected in cold PBS and incubated over night at 4^{0}C in RNAlater solution. Then four independent samples for WT and four for mutant were created. For each sample, four dorsal spinal cords (or four pairs of olfactory bulbs) were collected together and homogenized in 500µl Trizol. Every suspension was filled to 1600µl with Trizol. After adding 320µl chloroform, the suspension was centrifuged at 1000 x g in a cold centrifuge (Universal 32R; Hettich, Tuttlingen); the upper phase was separated and 2µl polyacrilcarrier (MRC; Fermentas, St.Leon-Rot) were added together with 0.83 volumes isopropanol. The solution was then incubated 10 min at RT and the RNA was precipitated with 10 min centrifugation at 12000 x g in a cold centrifuge. The pellet was washed with 1,5 ml of 75% ethanol, centrifuged 2 min at 14000 rpm in a RT centrifuge (Centrifuge 5417; Eppendorf), air dried and dissolved in 100µl H_2O_{DEPC} for the column purification. The RNA was purified with columns using the kit Rneasy MinElute Cleanup (Qiagen); the RNA was eluted twice with 11µl H_2O_{DEPC} and the concentration was spetrophotometrically measured with 1µl RNA solution in 80µl H_2O_{DEPC}.The RNA was now used for the cDNA synthesis or stored at -70°C.

2.3.5.2 cDNA-synthesis

In order to generate biotin-labeled cRNA probes for hybridization on Affymetrix chips, cDNA was synthesized from total RNA with the SuperScript Double-Stranded cDNA Synthesis Kit. 8μl of total RNA collected from column purification were mixed with 1μl of T7-$(dT)_{24}$primer (5`-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGG-CGG -$(dT)_{24}$-3`) (100μM) and denatured for 10 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 III 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 1x2nd strand synthesis buffer (Superscript 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 12 μl of H_20_{DEPC}. This cDNA was then used for the synthesis of Biotin-marked cRNA.

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

To generate biotin-labeled cRNA probes, cDNA was transcribed using the IVT Labeling kit (Affymetrix, High Wycombe, UK). A reaction tube containing 6μl cDNA-solution, 4μl MilliQ water, 2μl IVT labeling buffer, 6μl IVT labeling NTP mix, 2μl IVT enzyme mix was incubated for about 16 hours at 37^{0}C. The biotin-marked cRNA was then cleaned up with the GeneChip Sample Cleanup Module (Affymetrix) and eluted twice with 25 μl MilliQ water. The concentration and purity of the RNA were assessed by UV-spectrophotometry and elettrophoresis.

The biotin-marked cRNA was hybridized on Affymetrix MOE430 2.0 Microarrays. Per each genotype, three Microarrays were hybridized with 10μl cRNA. The hybridization as well as scanning of hybridized chips was performed at the microarray unit of the Max-Delbrueck Center (Berlin) by the group of Prof. Huebner. Signal intensity values obtained after hybridization of 3 independent cRNA probes for both *Tshz1*$^{GFP/+}$ and *Tshz1*$^{GFP/GFP}$ were statistically analyzed using the software Bioconductor (Gentleman et al., 2004). The quality of the Microarray hybridization was evaluated with the Biconductor-modul AffyPLM and the data were then normalized with the GCRMA-module (Zhijin et al., 2004). The probes with smaller variance of expression filtered from all the microarrays and the differentially expressed genes were identified with the limma-module (Smyth, 2005). This module implements the empiric Bayes-Method and offers the error correction from all the

false positives. Genes were considered differentially expressed when p value for the difference of expression levels was smaller than 0.05.

2.3.6 Establishment of anti-Tshz1 and anti-Tshz2 antibodies

With the help of the software MegAlign the aminoacid sequences of the three murine Teashirt proteins were compared and for Tshz1 and Tshz2 sequences a 100 aminoacid region was selected, where the divergence from the other two sequences is maximum. Each region was amplified and cloned into the bacterial expression vector pET14b, which codes also for the the sequence of the His_6-tag. The His_6-tag domain is necessary for the purification of the proteins. The vectors containing the inserts were transferred into the bacterial strain BL21 (DE3)pLysS and the protein production was induced through incubation with IPTG. The proteins were purified with TALLON-Metall-Granulate and the concentration was determined with the Bradford-Method (Biorad), according to manifacturer´s instructions. The antibody generation was performed in rabbit by Charles River Laboratories (Sulzfeld).

2.3.7 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	⎬ 30x
72°C 1 min	⎭
95°C 3 min	

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).

2.3.8. Cell culture

Feeder cells as well as ES 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 plated on the cell culture dish.

2.3.8.1 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.

2.3.8.2 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.

2.3.8.3 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 (300 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, 75µl of ice-cold 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 para film and gradually frozen to –80^{0}C 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% gelatine (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. ES clones were screened by Southern analysis as described above.

2.3.9 Establishment of Tshz1 and Tshz2 mutant strains

Superovulation and isolation of blastocysts, as well as the injection of embryonic stem cells in blastocysts (Bradley and Robertson, 1986) and the final uterus transfer of the blastocysts were performed by the Transgenic Core Facilities of the Max Delbrück Center for Molecular Medicine (Berlin). The germline transmission of the mutated allele was checked with Southern blot analysis and PCR.

2.3.10 Staining procedures

2.3.10.1 Preparation of frozen sections

Immunohistochemical analysis and *in situ* hybridization 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 E12.0, head walls were opened to expose the tissue for better solution penetration. Embryo tissue was fixed with freshly prepared 4% PFA/PBS at 4^{0}C for 2 h (for immunohistochemistry). Afterwards, the tissue was washed extensively at 4^{0}C 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). For in situ hybridization, the tissue was dissected in cold PBS and immediately embedded in "Tissue-Tek". 12 μm sections were cut on a cryostat (Microm HM560, Walldorf). Sections were collected onto slides (Histobond, Marienfeld) and dried at 37^{0}C for 2 h. Slides as well as "TissueTek" blocks can be stored for 6 months at –70^{0}C.

2.3.10.2 In situ hybridization on frozen sections

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

1st day

Slides with frozen sections were thawed at RT for at least 2 h, and post fixed 10-30 min with 4% PFA. DIG-labeled RNA probes were prepared as described above (§2.3.4). RNA probes were denatured for 5 min at 80^{0}C, 0.5 μg RNA/slide in hybridization buffer was directly applied on sections, cover-slipped and hybridized ON at 65^{0}C 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^{0}C 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^{0}C.

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).

2.3.10.3 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. 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).

2.3.10.4 Whole-mount in situ hybridization

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

Tissue preparation

Embryos were harvested from uterus, 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, optic 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 taking 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 months.

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.

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 for 10-15 min. Proteinase K activity was then blocked by 2 min incubation with 2 mg/ml of glycin/PBT (Sigma). Afterwards embryos 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 the RNA probes, 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}	

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 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
Formamide	50%	50%
SSC, pH 5.0	2x	1.3x
EDTA, pH 8.0	5 mM	5 mM
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 had 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.

2.3.10.5 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. Gelatine 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 'ImmunoMount' (Shandon, Frankfurt).

2.3.11 Birth dating of neuron progenitors

To detect birth date of olfactory bulb neuronal precursors, BrdU (5-Bromo-2'-deoxy-uridine) was injected into pregnant females at different embryonic stages 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. At E18.5, 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. After extensive washing, the sections were blocked and immunohistochemistry was performed as described above.

2.3.12 Analysis of data

Documentation of histological data

The histological data were documented through ligh- and fluorescence microscope. The light microscopy of sections was done at an upright microscope (Axiophot; Zeiss, Oberkochen) equipped with camera (Axiocam Hrc; Zeiss) and Axiovision AC software Version (Vers.) 4.5 (Zeiss). The fluorescence microscopy was done with an inverse laser Scanning Microscope (LM 5 Pascal; Zeiss) with the LSM 5 Pascal Software Vers. 3.2 (Zeiss). The generated pictures were edited with Photoshop Vers. 8 (Adobe).

Statistic analysis of data

Cell counts and proportions were statistically evaluated and diagramed with Excel Vers. X (Microsoft). For the comparison of different groups of data a t-test (Excel) was used. The differences were considered statistically significant when $p<0.05$. The average values were generally given +/- standard deviation.