Materials and Methods

Materials

Technical equipement

ADInstruments PowerLab/4s
Aestesiometar, Ugo Basil
Applied Biosystems PRISM 7700 Sequence Detection System
BDK Laminar Flow Hood
Biometra TRIO-Thermoblock PCR machine
BioRad Mini Protean II
BioRad Mini Transblot Apparatur
BioRad PowerPac 300
Contact thermal stimulator Yale University, Instrumentation Repair and Resign Shop
Cryostat
Digitimer Ltd. NeuroLog Amplifier
Eppendorf Thermomixer Compact and 5436
EquiBio Easyject Electroporation Apparatus
EPC-9 amplifier, Heka
Forma Scientific -80°C Freezer
Forma Scientific Steri-Cult 200 Incubator
GeneChip fluidic station 400
GeneChip Hybridisation oven
Gilson Minipuls 3 Peristaltic Pump
Hamamatsu Digital Camera C4742-95
Harnischmacher Labortechnik DNA Electrophoresis Chambers
Heidolph Duomax 1030 and Promax 1020 Shakers
Heraeus Biofuge 13
Heraeus Megafuge 1.0
Hereaus Biofuge 15R
Herolab E.A.S.Y 429K Digital Camera
Herolab UVT 2035 Transilluminator 302nm

HP GeneArray Scanner
Incubator Unitherm Hybridizationsoven or Thermo Hybaid
Ikamag Reo Magnetic Stirrer
Julabo MP and Medingen Waterbaths
Kleindieck Nanomotor
Leica DM 500B with Metamorph software
Leica DM RBE Upright Fluorescence Light Microscope
Leica KL 750 Fiber Optic Light Source
Leica MS5 dissecting microscope
Millipore Multiscreen Resist Vaccum Manifold
Mettler Toledo 320 pH Meter
Mitstbishi Video Copy Processor
MJ Research PTC 200 PCR machine
MoTil System (TSE Systems)
Harmacia Biotech Ultrospec 1000 Spectrophotometer
Plantar test, Ugo Basil
Rotorod Test (TSE Systems)
Sartorius Weigh machine
Scientific Industries Vortex-Genie 2
Stratagene UV Stratalinker 2400
Tektronix TDS 220 Two Channel Digital Real Time Oscilloscope
PerkinElmer Gene Amp PCR system 2400
Uni Equip Unitherm Hybridization oven 6/12
WAS02 automated perfusion system

Analytical Software

AnalySIS 3.2 Software, Soft Imaging System
Chart v5.2 for Windows, ADI instruments
Data mining tool, Affymetrix
Lasergene Software, DNAStar Inc.
MetaVue v6.2, Universal Imaging Corp.
Openlab 3.0.4
Pulse and PulseFit software, HEKA
7000 System Software, Applied Biosystems

Chemicals and reagents

REAGENT AND CHEMICAL	COMPANY
10 x PCR buffer	Invitrogen Life Technologies
100bp and 1kb ladder	Gibco
20x TaqMan Gene Expression Assay	Applied Biosystems
2x TaqMan Universal Master Mix	Applied Biosystems
5x First-strand buffer	Invitrogen Life Technologies
5x Second-strand buffer	Invitrogen Life Technologies
APES	Sigma-Aldrich
Aqua-Polymount	Polyscience Inc.
Bovine Serum Albumin (BSA)	Invitrogen Life Technologies
dNTPs (10mM each)	Invitrogen Life Technologies
DTT	Invitrogen Life Technologies
ECL	Amersham Bioscience
ExpressHyb solution	Clontech
Gelatine	Sigma-Aldrich
Glygogen	Promega Corporation
Herring sperm DNA	Promega Corporation
Horse serum	Biochrom
Lipofectamine	Gipco
MES Free Acid Monohydrte Ultra pure	Sigma-Aldrich, P/N M5287
MES Sodium Salt	Sigma-Aldrich, P/N M5057
Paraformaldehyde	Sigma-Aldrich
Phenol/chloroform/isoamyl alcohol	Roth
poly-L-Ornithin	Sigma
Laminin	Sigma
Protease inhibitor cocktail	Sigma
Tissue Tek	Miles, Elkhart, Ind. USA
Triton X-100	Sigma-Aldrich
Trizol	Roth
Tween-20	Pierce Chemical
Fetal calf serum	Biochrom

Further chemicals were obtained from Biomol, Merck, Roth and Sigma-Aldrich

Buffers and solutions

Affimetrix

BUFFER AND SOLUTION	COMPOSITION
10mg/ml goat IgG stock	Resuspended 50 mg in 5 ml PBS
100x stock of control cRNA	BioB (150pM), BioC (500pM), BioD (2.5nM), Herring sperm DNA (0.1mg/ml), 1x MES, NaCl (0.926M), Tween 20 0.01%, dissolved in DEPC water
12x SSPE	3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA
12x MES stock	1.22M MES free acid monohydrate, 0.8 MES sodium salt, pH between 6.5 - 6.7
2x Hybridization buffer	(final 1x concentration is 100mM MES, 1M Na^+, 20mM EDTA, 0.01% Tween20) stored at 2-8 ^oC, and shielded from light
2x stain buffer	Final 1x concentration 100mM MES, 1M Na^+, 0.05% Tween 20
5x RNA fragmentation buffer	200mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc
Antibody solution mix	1x MES stain buffer Acetylated BSA 2mg/ml Normal goat IgG 0.1 mg/ml Biotinylated antibody 3 μg/ml
Buffers for heparin hromatography	Buffer A: 50 mM Tris pH 7.5, 20 mM NaCl Buffer B: 50 mM Tris pH 7.5, 2 M NaCl
Gel filtration running buffer	50 mM Tris pH 7.5, 200 mM NaCl
Hybridization mix	Fragmented cRNA 0.05 μg/μl Control oligonucleotide B2 50pM 100x control cRNA coctail (bioB, bioC, bioD, cre) 1.5, 5, 25 and 100pM respectively Herring sperm DNA 0.1 mg/ml Acetilated BSA 0.5 mg/ml Hybridization buffer 1x Minimal final volume 300μl
Non-stringent wash buffer	6x SSPE, 0.01% Tween 20
SAPE solution mix	1x MES stain buffer Acetilated BSA 2mg/ml Streptavidin-Phycoeritrin (SAPE) 10μg/ml
Stringent wash buffer	100 mM MES, 0.1M Na^+, 0.01% Tween 20

Whole mount *in situ* hybridization

BUFFER AND SOLUTION	COMPOSITION
20 x SSC	3M NaCl 0.3M Na-citrate pH 7.0
PBT	PBS + 0.1%(v/v) Tween-20
Hybridization buffer (HB)	1.3% v/v 20X SSC 50% formamide 2% Tween 20 0.5% CHAPS 5 mM EDTA 50 µg/ml yeast RNA
TST	0.5 M NaCl 10 mM Tris, 0.1% Tween 20 pH 7.5
MABT	0.1 M maleate, 0.15 M NaCl 0.1% Tween 20 pH 7.5
B3-buffer	0.1 M Tris 0.1 M NaCl 50 mM $MgCl_2$ 0.1% Tween 20 5 mM Levamisole pH 9.5

Others

BUFFER AND SOLUTION	COMPOSITION
10x MOPS	200mM MOPS 500mM Na-acetate 10mM EDTA pH 7.0
10x TBS	0.5M Tris/HCl pH 7.9 1.5M NaCl
4% PFA	4% paraformaldehyde in PBS pH 7.4
5x Lämmli buffer	60mM Tris/HCl pH 6.9 10% SDS 10% β-mercaptoethanol 50% glycerol 1.5% bromphenolblue
Acetate buffer	Na-acetate 10 mM pH 5
Homogenization buffer	0.1M PBS
Patch clamp buffer - intracellular solution	110 mM KCl

	10 mM Na^+ 1 mM $MgCl_2$ 1 mM EGTA 10 mM HEPES pH7.3, adjusted with KOH
Patch clamp buffer - extracellular solution	140 mM NaCl 1 mM $MgCl_2$ 2 mM $CaCl_2$ 4 mM KCl 4 mM glucose 10 mM HEPES pH 7.4, adjusted with NaOH
PBS	PBS Dulbecco w/o Ca^{2+}, Mg^{2+}
Phosphate buffer	0.1M KH_2PO_4 0.1M Na_2HPO_4 x $2H_2O$
SDS PAGE running buffer	25mM Tris/HCl pH 8.3 190mM Glycine 0.1% SDS
SIF (Synthetic Interstitial Fluid)	2mM $CaCl_2$ 5.5mM glucose 10mM Hepes 3.5mM KCl 0.7mM $MgSO_4$ 123mM NaCl 1.5mM NaH_2PO_4 9.5mM Na-gluconate 7.4mM saccharose set to pH 8.4 with 10N NaOH; carbogene used for oxygenation during the experiment will bring it to pH 7.4
Tail lysis buffer	0.05M Tris (pH8) 0.1M EDTA (pH8) 0.1M NaCl 1% SDS
TCA	20% Trichloracetic Acid
TE buffer	10mM Tris pH 8.0 1mM EDTA

Culture media

DRG medium: 20% HS (Biochrom)
2mM glutamine (Gibco)
100u penicillin/100µg/ml streptomycin (Gibco)
in D-MEM/F12 (Gibco)

OPTI-MEM: Gibco

Hybridoma medium: 1mM Na-Pyruvate
1% non essential amino-acids
10% FCS
in RPMI (Gibco)

Hybridoma protein free medium:Gibco

Enzymes and molecular weight markers

ENZYME	COMPANY
Collagenase TypeIV	Gibco
DNase I (RQ1 RNase free DNase)	Promega Cat. No. M610A
DNAse I	Qiagen Cat. No. 74104
E. coli DNA polymerase I	Invitrogen Life Technologies
Pfu-DNA polymerase	Stratagene
Phosphatidylinositol phospholipase C	Oxford GlycoScience
Proteinase K	Roth
Restrictionendonucleases	Amersham, Roche
RNA-polymerases T7	Invitrogen Cat.No. 18033-019 Promega Cat. No. P-2075
RNA-polymerases Sp6	Invitrogen Cat.No. 18018-010 Promega Cat. No. P-1085
RNaseA (10mg/ml)	Qiagen
RNaseOUT™	Invitrogen Life Technologies
SAP (shrimp alkaline phosphatase)	Amersham
Superscript II™ reverse transcriptase	Invitrogen Life Technologies
T4 DNA polymerase,	Invitrogen Life Technologies
T4-DNA ligase	Promega
T4-DNA ligase	Invitrogen Life Technologies
Taq-DNA polymerase	Gibco
Trypsin	Gibco

Kits

KIT	COMPANY
DIG RNA Labeling Mix (SP6/T7)	Roche Cat. No. 11277073910
GeneClean III Kit	Dianova
QiaEx Gel Extraction Kit	Qiagen
Qiagen Plasmid Midi Kit	Qiagen
Qiagen Plasmid Mini Kit	Qiagen
RNeasy mini kit	Qiagen, Cat. No. 79254
RNA transcript labelling kit	Enzo
Phaselock	Eppendorf
TA-Cloning Kit (pGEM-T Easy)	Promega

Plasmids

PLASMID	COMPANY
pGEM-T Easy	Promega
pTracerCMV6	Invitrogen

Primers

Affymetrix ID	**Gene name**	**Probe**	**SEQUENCE** (5'→3')
Transmembrane- channels			
92919_at	Htr3a	**Ser**	5' gtgcatggctctgctagtga 3' tgaccagggtgatgctgtaa
114949_at	Kcnmb1	**K**	5' ggaagacactcgggatcaaa 3' gtcagggatgggaagctgta
102704_at	Aqp4	**a**	5' acggttcatggaaacctcac 3' ccgaagagtctttccccttc
94060_at	Kctd10	**133**	5' gtgtacccttgcctgagagc 3' gggcctcgtacagcaagata
Transmembrane- receptors			
92469_at	Sfrp4	**25**	5' caccacagcactcaggagaa 3' tcattgcaaccactcctctg
92198_s_at	Daf2	**28**	5' cttggtcatcaccaccactg 3' ccggttggtatgttgatgtg

96186_at	Lrp10	**37**	5' ttggacccaaactggaactc 3' ggcaaaagcacttgtgtcct
103957_at	Tfrc	**38**	5' ttctagacttgtgttggttgtgc 3' tgccctcaggacatatagca
161447_f_at		**67**	5' ggatttagaaacaggcattcaca 3' ggcaaacatggaacacacaa
112363_at	Sorl1	**76**	5' ccagttgcatgtgtttctgc 3' ggtgatggcggtgtctttat
93088_at	B2m	**85**	5' ccttcagcaaggactggtct 3' tgggcacagtgacagacttc
162776_f_at	Spg7	**9**	5' aaagagcgggaagaccagat 3' tggtgaaacgtgccatctta
163934_at	Sema6d	**8**	5' cttcccagaagacgatgagc 3' cagatccatctcccatgctt
114989_at	Prlr	**7**	5' tttggactggtgttgatgga 3' gagcacaaagcacaaaagca
137131_at	Grin3a	**60**	5' tttttcatttgcctgccagt 3' tccacagtcatagaaagcgaag
Transmembrane- transporter			
100315_at	Atp8a1	**63**	5' ccatgctcttcagctctgg 3' cacttttctccccatggac
128583_at	Abcg2	**33**	5' agccttccaagggagagaag 3' acttggaagaacctcatgg
104394_at	Abcb10	**64**	5' tcaccatctgagcaccagag 3' cccagcagagtttcatctcc
93341_r_at	Copb2	**69**	5' ggcacgagctttaaggaaaa 3' tcaattccttatcccccaga
116635_at	Abca3	**34**	5' ttgagcagcttcaaccac 3' cagctgttaagcccctcat
170059_r_at	Tm9sf2	**35**	5' atcagatgggcgtctaggtg 3' agcaaacacaatcccaggac
168346_r_at	Syt11	**10**	5' gatccagggcctgaaagatt 3' atggggcttagaatgctcct
Transmembrane			
105830_at	Tmem30a	**1**	5' ggataacacggccttcaaac 3' tggaataggcttgggatcag
162747_at	Tspan14	**11**	5' aaaggcttgatggcccttat 3' actgggtaaggctgctgaga
165385_at	Gpsn2	**57**	5' acttctgcggtgagttcctg 3' attgttgctgctggagaacc
133183_f_at	Mal2	**2**	5' ttctcctcgcaaaccctgt 3' ggaaggactgaaggagctga
109460_at	Tmem24	**142**	5' ggaagcctcaaggatcacaa 3' cgaaaacaaaccctcaggaa
135667_at	1110021J02Rik	**59**	5' acatgtgcgatgttcaggtg 3' ctgctgttgtccacagtgct
111067_at	A930031D07Rik	**9tr**	5' atcaatgtgggagtggtggt 3' agggaaggaagacacaagca
Cell adhesion molecule			
166811_at	Negr1	**26**	5' catttttgctggaggtgaca 3' tatcagtccactgcgtccag
100095_at	Scarb1	**39**	5' ggaagtctttctgtctttctcca

			3' tggttaattcagtgacatagcagag
115537_at	Btbd9	**150**	5' gtgtcggtcttggcaaaagt 3' atgaaggatgcaggctgtct
102327_at	Aoc3	**47**	5' attgcccctctgttcttcct 3' caggttctgtccctggtgtt
97773_at	Cd34	**52**	5' catgtagctgtacctgctctgc 3' tgggcaaaaggcagtttatt
Extracellular secreted			
133116_at	Edil3	**24**	5' ctcacagtcatcagatggaagg 3' tgacaaagaatattggcctgttt
98374_at	Il5	**89**	5' acgcaggaggatcacatacc 3' gtaaactgggggaggcttct
96049_at	Bgn	**27**	5' ctacgccctggtcttggtaa 3' gaagttccctcagggtaggc
160458_at	Mcam	**83**	5' ctccctgctcagacttcacc 3' cagggatgtgtctggaggac
101078_at	Bsg	**32**	5' actggggaagaagaggcaat 3' aggttgggttgggtaggttc
114835_s_at	Angptl6	**147**	5' agcaccagagagagcagagc 3' tgtgccaagagagggagtct
95706_at	Lgals3	**136**	5' ctggagcttatcctggctca 3' gtcaccactgatccccagtt
95651_at	Myg1	**175**	5' ccaaagtggccattaccttt 3' ggatctcctggagctggagt
Neurotransmiter related			
106075_at	Cplx1	**73**	5' ccataacccttcctgtccac 3' attggctgtgattccctcag
100690_at	Th	**166**	5' aggagagggatggaaatgct 3' cttcagcgtggcgtatacct
105700_at	Syt1	**54**	5' aaggggagcctgctacctaa 3' cccctcatttgagtctcctg
102116_f_at	Syt3	**46**	5' gtgcacaggaagacttgaa 3' taccactgcgatgctgagac
138946_at	Syt7	**62**	5' catgggggaggttgtgag 3' ctgttttcctttgtcccttcc
Cytoskeletal binding			
160162_at	Tagln2	**15**	5' agcagacattccctgagagc 3' acctttgctccacccccttac
115895_at	Kif1b	**13**	5' gagaaggaggaagccgatct 3' cctttcccagtgaccatcat
116311_at	Ivns1abp	**12**	5' gggaacccactagatggaca 3' gagcaaggaaggaccagtga
117231_at	Kif21a	**14**	5' tagcattgccgactgtcaag 3' aggaggcatctcctgcacta
163135_at	Cttnbp2	**16**	5' acaagtcataaatgcaaactcagaa 3' aagattggtgtagatgaaatgtgtgc
92231_at	Epb4.1l4a	**17**	5' agaccacgacccatacaagc 3' atggacccaaacttgctgag
101056_at	Rdx	**20**	5' gagaaagacgttgcctttgc 3' tgcacagccactgtacaaca
160065_s_at	Csrp1	**65**	5' gaggcaagaaatgtggggta 3' cccaaagtttttggcatagc

111423_at	Mtss1	**74**	5' ctcccctcacgcttttgtag 3' ccccttggatgtatttgga
112032_at	Cgnl1 LOC677485	**75**	5' gatgagggcatgtctcctgt 3' cccatggaggtagctgtgat
171486_at	Coro1b	**36**	5' ggaaggtgttcaccacaggt 3' tactgggcacataggcttcc
136755_at	Hook3	**78**	5' tgggagaaatatcgctcatgata 3' ccctggttagaaggcacaga
G protein coupled receptor			
163690_at	Ednra	**18**	5' atcgggatccccttgattac 3' gaattcatggttgccaggtt
133456_f_at	P2ry14	**19**	5' aagacaacttgtttattggcaca 3' ccatgcaaaatggaagtctg
101001_at	Gpr177	**7tr**	5' tcacattgcagggtactgga 3' tgggatggtgcatacaagaa
95474_at	F2r	**51**	5' tccttggctggagatcctaa 3' gttactgcctcccaagatgg
G protein signalling			
94155_at	Rgs4	**86**	5' tctttgcagagcagaagcaa 3' cctctctggtgcaagagtcc
99597_at	Gnai2	**30**	5' catcttctgtgtcgccttga 3' tcagaagaggccacagtcct
Signalling protein			
92949_at	Pacsin1	**68**	5' ccaatgagcatttgcagaga 3' cccacacccagaatccttta
170365_at	Slc9a3r2	**81**	5' tctttaaggggccagaggat 3' ggcaaggcctgtttaatttg
167275_f_at	Nradd	**3**	5' cgtcttcgtggactctcctc 3' ttggagaggatggagaagga
108294_at	Rab14	**93**	5' gaaaacggtgggtttgagac 3' ttctgctcactgctctcctg
113193_at	Rapgef1	**31**	5' gggagagctgtgaagattcg 3' agtcagctctctcctgcaca
104300_at	Iqgap1	**21**	5' tggtctagcgctcacagaga 3' gacggccagtccaaattct
166736_at	Plaa	**158**	5' actggcgagtgtctggaagt 3' atgagccaacaacatcacca
166647_at	Snx27	**156**	5' ttcgcaagtggctctttaca 3' cacttgagctcgcagaacac
136797_s_at	Krit1	**155**	5' acaggcagagaagcagcact 3' tttcctgtttgggaatttgg
167630_f_at	Kndc1	**161**	5' ggagacaggaggtttcacca 3' gggatggggctaggttagag
109683_at	Oxa1l	**186**	5' cgagtcgtacatgaccctga 3' agggacctgggagaagacag
101866_at	Arfrp1	**167**	5' atcaccactaccgtgggtct 3' cacgacacacttcaccatcc
166998_f_at	Pde2a	**198**	5' ggtaggattggtacccttgga 3' tggatatggccttgaagagg
111006_at	Plcb1	**187**	5' ggcctgtttgaggatagcag 3' ggtttgcgcctctacttctg
114665_at	Epas1	**115**	5' ctacaccctgccacagatca

			3' ctgaggcaacaacacaggaa
102382_at	Arntl	**100**	5' ttagccaatgtcctggaagg 3' ccaagaggctcatgatgaca
Kinase receptor			
99956_at	Kit	**41**	5' tcaggtatgttgccttcacg 3' tgggctcaggaatattcaaa
Kinase			
96284_at	Csnk1g2	**88**	5' tactacttcggcccttgtgg 3' cgcttggtgtctccaatctt
99510_at	Prkcb1	**42**	5' tgtggaactgactcccactg 3' tttgaccaggaacatcagca
104847_at	Braf	**92**	5' accaaacaagcaagtgatgg 3' aaagttttcccaattctctgc
166555_f_at	Csnk1e	**98**	5' gatgtgaagcccgacaactt 3' tgagcatgttccagtcgaag
160121_at	Galk2	**82**	5' acccaaatcctgactccaaa 3' cccgccagctccttaatac
98618_at	Dtymk	**90**	5' gtgctggagggtgtggac 3' tttgcagagaagagcaggtg
110368_at	Itpkc	**43**	5' cagcactgtgaccatccatc 3' ttttgcctcaattccctgac
134878_at	Mast2	**44**	5' ggggtatctcagccatctca 3' caaactcatgaggccgattt
162954_at	Irak1bp1	**77**	5' gagtgagcagcaccaagga 3' catggatggttgcacttttg
Phosphatases			
93942_at	Inpp1	**45**	5' ccagcggcttcagtgtgta 3' cccgtgtctggacttctctc
113964_at	Ppp1r11	**95**	5' cgggataagtgagaccgtca 3' gtgctgcataggtcctggag
130608_at	Ptprg	**96**	5' ccccctcttttattgtgtcc 3' aacacagtgccaggcataca
Transferases			
95591_at	Extl3	**40**	5' tgggtatcattgatctgtgtttg 3' ctggaaaacaaagctgtcctg
106117_at	Zdhhc17	**6**	5' tgcagtacctgcttgattcg 3' ccacagcatcgaaattcaaa
94393_r_at	Elovl2	**4**	5' taatggcatgacggacaaga 3' aaggatcccgcttaaatgct
108878_at	Pdhx	**185**	5' atcacctctggggatgagc 3' gacaaggacagccttggatt
96746_at	Dlat	**177**	5' agctggacactcctagtgcag 3' catccggctcatctcagaa
96763_at	Sardh	**178**	5' cctacagcaggttgctaggc 3' gttgctcaggtcagcatcaa
98291_at	B3galt6	**181**	5' gtggacttcgagttcgtgct 3' acataggaaaggcgcaactg
92403_at	St6galnac5	**171**	5' ccccaggtctctgggagtat 3' tgttgcacatttccacatca
116858_at	Gnptab	**118**	5' ttcaatgttgcgaaaccaaa 3' gcagcgagtacctcagctct

Synthase			
92545_f_at	Ptgds	**23**	5' agtgcagcccaactttcaac 3' ccagccctctgactgacttc
Ca binding			
96203_at	Calml4	**70**	5' tctccaccttcctgaccatc 3' ctggcacacaaccctgacta
166983_at	Cabp2	**160**	5' agcatgcatcttccttcgac 3' cgagacatcatccgaacaaa
113065_at	Sri	**145**	5' tgttgggtggcttactactgc 3' ttggaaattgagttggaagga
Protein transport			
93711_at	Sec23a	**29**	5' gcacgacattgagaaggaca 3' aaattggactgcacctcgtc
95034_f_at	Ipo4	**87**	5' ctggatgacccctcacagat 3' caagctgacagcattcctca
99356_r_at	Tnpo1 LOC634263	**91**	5' caaggcatgattccgtacct 3' tgtggcaacagacgagagac
106309_at	Wbscr18	**141**	5' gtcaccgctatggagatggt 3' cgccgttctctctctagctg
94131_at	Xpo1	**134**	5' gctgttggtcatccctttgt 3' gctaggaatgctgggaaaca
160581_at	Atg16l1	**66**	5' tcctcccagaaaagagctga 3' aggaagggagttcagggttc
167834_f_at	9130023D20Rik	**80**	5' agttattcgggagcaccatc 3' tcttttatgcagaaccgttgc
161015_at	4933402J24Rik	**50**	5' cactgccaacacttgcaaaa 3' ccttgccagtgtccaaaaat
164695_f_at	Dnajb10	**123**	5' ccagacaagaacccggataa 3' caagcctagtgccaggtcat
Transcription factor (classical)			
97937_at			5' acgtacaccatgccaagtca 3' agttctggtggcgcttcat
167579_r_at	Elf1	**128**	5' caggaggaagcagcaaattc 3' tcaaatcatcttcggccttt
93010_at	Pqbp1	**107**	5' ccgagctggtacaaggtgtt 3' gggagtcctgttgaccatgt
103765_at	Hkr3	**101**	5' aagccaggaattgcatgaac 3' ggctgcagaactcacagaca
104215_at	Atf6	**102**	5' acactggtgggctttttgtc 3' tcctgcatttcttccacaca
161030_at	Scx	**105**	5' ggtcgctacctgtaccctga 3' gtttgggctgggtgttctc
94135_at	Tcfcp2	**109**	5' gcttcaacagctcccatagc 3' atctggctgatctggtgagg
161287_f_at	Mybbp1a	**106**	5' ccaacaccccaaccacttac 3' caaaatggcggatcaagtct
93054_at	Ankrd46	**108**	5' ttggaaagtggctttgatcc 3' aacagcaagaccctccagaa
114158_at	Ankhd1	**114**	5' cacactttgtgctggggtta 3' agcccaggctagcaacaat
106255_at	Cand1	**110**	5' aagtgcctggacgctgtagt

			3' tcccgggaaggaaagtaaag
110650_at	Nrip1	**111**	5' acagttgcaagtctggacaaa 3' cacactgcggtatgtacaattca
116970_at	Zfp297b	**119**	5' tgttgcttgaagcctgtgtc 3' tgtcatacaaaaggacatggtctc
166563_f_at	Gabpb1	**127**	5' tcaagaggtggtggagcttt 3' atcccactggttggtatgga
170560_r_at	Hey1	**129**	5' gttgcccgttatctgagcat 3' atctctgtcccccaaggtct
135401_at	Sox8	**126**	5' ccagaacatcgacttcagca 3' ggtagaggctgggagggtag
115635_at	Med28	**151**	5' cctgggagggatgttcact 3' tcagaggtgcaggaatgttg
97438_r_at	Ankib1	**139**	5' gtcctaggccaagtgaacga 3' cacagggtaagggaacagga
105120_f_at	Jarid1d	**140**	5' tcacatcaactcagccaagc 3' gtcaaaggcaaagcctgaag
164068_at	Narg1	**194**	5' ctgcagattcaaatgcgaga 3' tcccctggggtatttagtcc
140942_at	Asxl1	**79**	5' cctccgaaacttgaaccaaa 3' ggcattcttttcagacacacc
105640_at	D630045J12Rik	**53**	5' acccctgttctgggcttatt 3' tggcatgtttcacagcagtt
112001_at	AI480556	**113**	5' caccaaggaaggagtccaag 3' caggctgaagggctaggtc
114681_at	Cugbp2	**116**	5' aagatgaacggagctttgga 3' tgattgcattctgtgccatt
101528_at	Tcea1	**99**	5' ggcaccaagcacttctgatt 3' cttccaccgatttccacatt
Translation			
164324_f_at	Eif1ay	**121**	5' aggaaaaggaggcaaaaacc 3' catcatcatccccaatgtca
160365_at	Eif2s2	**104**	5' ggagccagaaccaactgaag 3' ttcctactcggacgacctgt
Other Enzymes			
135093_at	Ganc	**97**	5' ttgactcccaataatcagagagc 3' tgtttcagacacaacgaagagag
134082_at	Ube2n	**197**	5' catggaatttctgattcttgct 3' taggagcagctctggctttc
98410_at	Iigp2	**182**	5' gtctcacgatggtgggattg 3' attcacaccctgcttctcca
96184_at	Rnpepl1	**176**	5' cagagcgtgtacgtggaaga 3' aggctgtctctaggcaggtg
94852_at	Glul	**174**	5' ttggtgggattagcatgtca 3' gcctggcagttacagtctgttt
98042_at	Scpep1	**180**	5' cggagaaagctatggaatcg 3' catcatcttcagggccatct
94350_f_at	Nqo1	**173**	5' ttcaaccccatcatttccag 3' tccagacgtttcttccatcc
92592_at	Gpd1	**172**	5' ccaagatcgtgggtagcaat 3' ggtgtctgcatcaggtcctt
97487_at	Serpine2	**179**	5' cctctgcctctgagtccatc

			3' taatgccaagggctttcagt
161725_r_at	Ube2v2	**170**	5' ggggtctcgaatttccctat 3' tttcatctcaaaatcacatatcca
113025_at	Lactb LOC677144	**189**	5' gacaagggaggcaaaaacaa 3' acagcattgcgtttccaaat
117284_at	Glul	**192**	5' atgaacggaggaccaagaaa 3' ggaagggtaacccactgctt
106104_at	Gfod2	**184**	5' gaattgggaagaacgtggtg 3' accatgacctcatgcacaaa
162834_at	Ddah1	**193**	5' ggaaggaggttgacatgatga 3' catctccgagttgctcacag
116042_at	L2hgdh	**190**	5' tcctgaccctcaaattgtcc 3' cgcatttctcacatgaagga
164889_f_at	Angel2	**195**	5' cctctcgcaggatttactgg 3' catctttgcgatgggtcac
168164_r_at	Acad8	**199**	5' gagctgttccctgtggatgt 3' ccccaccttcttctccttct
166327_i_at	Rnf13	**61**	5' accaatgagtgttgggttgg 3' tcaaaattaaaatttcttttcctagtg
164115_at	Cybrd1	**56**	5' ggactggtgacaagtggtttc 3' gaaaaatagatgcacatcttcccta
Miscellaneous			
105736_at	-	**72**	5' cagagtgcagccattcacat 3' gcccatgggttctcaataca
104415_at	-	**103**	5' gccataataagagaaagccttgg 3' tgtccacagacatggaatgg
163229_at	2610024B07Rik	**120**	5' tgtataggatgggcttgtgg 3' cctgcaggaactgacacaaa
134649_at	-	**124**	5' tgttctttggtcttactcctcct 3' agccaaaaatcagaggcaga
134756_at	3110027N22Rik	**125**	5' ttcctgggagtttgtgctct 3' tttgaggttcagatcttttatttca
160491_at	-	**169**	5' gggatgatggtctcaaagga 3' tccactttgtcccgaaaatc
116557_at	-	**191**	5' gttgctttgtgtggatttgg 3' tccggtgacaagaacacaga
111661_at	-	**188**	5' ggtgcatgcctctaattcct 3' ctatcctccatgtgcccttc
132325_f_at	-	**196**	5' ctcaccaatcgacgctacct 3' gcttgggtggaagttgaaaa
102094_f_at	-	**168**	5'gcatgctcctggaatacaca 3' caggaagtccctcaggtttg
96728_at	Wdr45	**71**	5' cgactcctccttcctctgtg 3' cacaaaaagtgctcgtggaa
161610_at	Ndrg2	**84**	5' gttcggggatatgcaagaga 3' cctcgctcaaagttcaggtc
164358_f_at	Zswim1	**122**	5' agggtatggcgtctgtgttc 3' agagtcacagccaggtgctt
130534_i_at	Lrrn6a	**58**	5' tgccctcctcacacactctc 3' ttgaaacccaagttacagaggaa
160591_at	St7	**49**	5' tgggtcccagtatgaagctc 3' tgtgcgagatgaaagaatgc

103691_at	Trim13	**48**	5' ccacaagccctttaatggaa 3' tcctgtgttctccaacaatcc
109128_at	Thoc5	**94**	5' aagcgcaaggagatgctaaa 3' cacaatgccatgttccagag
101551_s_at	Tes	**130**	5' aatgaggaggaccggaaagt 3' gcagacttgtccttggaagc
161884_r_at	Fxr1h	**131**	5' gctgcttgtgatgccactta 3' aacgttccggtgtcttcatc
92435_at	Rlbp1	**132**	5' acactttgcagaaggccaag 3' catggtgaagcccttgaagt
95517_i_at	BC004004	**135**	5' gtccttgcccattaccaaga 3' cttgctacccaccacaggtt
96900_at	Ndg2	**137**	5' gggtcttatggctcagatgg 3' ggagctcagaccgtgattgt
96921_at	Ttc1	**138**	5' gtcgcagatcctcaagaagg 3' gctttgctgcagtcagtgat
109690_at	Srrm2	**143**	5' ctacgttcagcgaaacctgtc 3' aactgctggtctctcgaacaa
110217_at	Palmd	**144**	5' acttgccaggaggagacaga 3' atcacctttttccccagctt
113877_at	-	**146**	5' gcacaggaggatgctgactt 3' gttgctgaggaggctgaaac
114947_at	Drr1	**148**	5' acagagctccctgctttctg 3' tggaatctgattgggtatgg
115336_at	Atg16l2	**149**	5' ggttgttcatgggtctccac 3' tggatacacacacataccacaca
116576_at	Zcchc6	**152**	5' ccaaaaatggatttgggtca 3' acaggtggagacctttgctg
162926_at	Topors	**153**	5' accctgaggttcgaaggttt 3' cacaaatgcctgactctcca
163272_at	Trim12	**154**	5' gtgttcatttgccctgtgtg 3' cagactctgccaggttgttg
166691_r_at	6330500D04Rik	**157**	5' gaccttgcacccagcaac 3' aacttcggttccatgacgac
166970_at	Suv39h1	**159**	5' aaaggttgcagtgtgtgctg 3' atgccctcaccaactcgata
168483_r_at	Angptl7	**162**	5' aaccacagattatccgtcatca 3' tttgaaaaataggaattgtaaatgagg
169162_at	Psmf1	**163**	5' cgggtctcgaggttctcttc 3' agtgggtccacctctctgg
170313_at	Usp13	**164**	5' ttccacaccaaccgtgacta 3' cccccaatttagacgcaaag
105778_at	E130309F12Rik	**183**	5' gctgccactccaactgctat 3' gtcaggaaggcggtacagag
100588_at	Psme2	**165**	5' ccaaggatgacgagatggaa 3' atcgatggcttttcttcacc
164036_at	Narg3	**55**	5' ttttggaatcaaatgtgctga 3' caaggaggataaatgagcatga

Primer and probe sets for quantitative real time PCR:
(all primer and probe sets were obtained from Applied Biosystems: Assay-On-Demands™ Gene Expression Products)

Gene	Exon	TaqMan expression assay
c-kit	ex 7-8	Mm00445212_m1
rgs4	ex 1-2	Mm00501389_m1
sorl1	ex 1-2	Mm01169532_m1
Ntrk2	not given	Mm00435422_m1
calca	ex 2-3	Mm00801463_g1
Htr3a	ex 1-2	Mm00442874_m1
Gpr177	not given	Mm00509695_m1
Tes	ex 3-4/5-6	Mm00501892_m1
ST6	ex 2-3	Mm00488855_m1
rdx	ex 12-13	Mm00501337_m1
control	not given	Mm00482162_m1

Antibodies

ANTIBODY	COMPANY
Anti-β (2.5 S, 7S) NGF (klone 27/21)	Roche
Anti-VR1	Oncogene
Anti-DIG antibody coupled with alkaline phosphatase	Boehringer (Cat No. 1093274)
Secondary antibodies	Dianova, Hamburg

Consumables

PRODUCT	COMPANY
15ml and 50ml tubes	Falcon, Greiner

Cell culture dishes	Falcon
Centricon	Millipore
Coverslips	Roth
Dissection scissors	FST
Dissection Forceps	FST
Dounce homogenizer	Roth
Eppendorf tubes	Eppendorf
Glass rod	In house made
Hybond-N	Amersham
Hybridization plates	Multiscreen TM- MAHVS4510
Insect needles	FST
Micro spin columns	Amersham
MicroAmp Optical 96 well reaction plate	Applied Biosystems
Needles	Sterican
Quartz cuvettes	Roth
Pipettes and multichannel pipettes	Eppendorf or Biohit
Slides and coverslips	Roth or Menzel-Gläser
Sterile filters	Nalgene, Millipore
Syringes	Braun
Whatman filters	Schleicher & Schuell

Animals

C57BL/6N mice were obtained from Charles River Breeding Laboratory, Inc., Wilmington, Massachusetts and kept in the animal house of the MDC until they were used for experiments.

RGS4$^{+/-}$ mice were generous gift from Dr. Jean-Francois Brunet. Animals were bred in the animal house of the MDC.

c-Kit$^{-/-}$ (dominant spotting W allele) mice were used in collaboration with Dr. Alistair Garratt, MDC. c-Kit$^{-/-}$/Epo^{tg} mice were generated by inter-crossing of c-Kit$^{+/-}$ and Epo transgene mice. Animals were bred in the animal house of the MDC.

Methods

Molecular biology

Standard methods were performed according to Sambrock et al. (1989) and Asubel et al. (1997).

DNA cloning

Gel extraction

The QiaEx or the GeneClean Kit was used according to the manufacturer's instructions to extract DNA fragments from agarose gels.

Ligation

The final ligation volume was 10µl. 50ng of vector were used with the molar ratio of vector to insert being set at 1: 3.

50ng vector
3x insert
1µl 10x buffer
1µl T4-DNA-ligase
ad 10µl with H_2O

3µl of the ligation mixture was used for transformation of competent bacteria (*E.coli* Top10).

Electroporation

Electrocompetent bacteria were thawed on ice for 5–10min. 50 µl of bacterial suspension were mixed with 3µl of the ligation product. Immediately after the electroporation (U = 2.5kV; C = 25µF; R < 200Ω; t_{impuls} = 5 ms), 1ml of SOC-medium was added before transferring the suspension to an Eppendorf tube. Cells were incubated at 37°C for 1h. Subsequently 100µl of the cell suspension were plated on an agar plate containing the appropriate selection antibiotic for the plasmid and grown at 37°C overnight.

Isolation of plasmid DNA from small amounts of bacteria (mini-prep)

Mini-preps were performed in accordance to the manufacturer's instructions using the Qiagen Plasmid Mini Kit. The final DNA pellet was dissolved in 50 µl H_2O or TE buffer with the concentration set to 1µg/µl and stored at 4°C.

Isolation of plasmid DNA from medium amounts of bacteria (midi-prep)

Midi-preps were performed in accordance to the manufacturer's instructions using the Qiagen Plasmid Midi Kit. The final DNA pellet was dissolved in H_2O or TE buffer with the concentration set to 1µg/µl and stored at –20°C.

Determining nucleic acid concentrations

Using quartz cuvettes to measure the OD_{260}, the nucleic acid concentration was calculated as follows:

DNA	OD_{260} x 50µM/ml
RNA	OD_{260} x 40µM/ml

Sequencing

DNA sequencing was carried out by InViTek, Berlin-Buch or Seqlab, Göttingen.

PCR reactions using Taq DNA polymerase

DNA was amplified by PCR using a reaction volume of 50µl:

1µl template
5µl 10 x PCR buffer
2.5µl $MgCl_2$
1µl dNTPs (10mM)
1µl primer 3´
1µl primer 5´
38µl H_20
0.5µl Taq DNA polymerase.

PCR cycling:

Initial denaturation:	94°C	2min
Denaturation:	94°C	30–60sec
Annealing	55 – 60°C	30–60sec
Extension:	72°C	30–60sec
Cycles:	25–35	
Final extension	72°C	2min
Storage:	4°C	

PCR products were loaded on an agarose gel, bands of the expected size were gel-extracted and where required cloned into the TA-cloning vector pGEM-T Easy, in accordance to the manufacturer's instructions. PCR fragments were analysed by restriction digest and/or sequencing.

Affymetrix gene chip experiment

Gene chip® micro arrays are manufactured using a combination of photolithography and combinatorial chemistry. Representative oligonucleotides of 25 bases called probes are synthesized on to the chips and the specific area where one single type of probe present is called a probe cell. Probes are designed to detect complementary RNA or DNA in the sample. Millions of copies of probes are present in one probe cell. The Affymetrix Murine Genome U74v2 set contains about 36000 putative genes, 20% known and 80% EST sequences. Each gene on the array is represented by up to 20 different oligonucleotides spanning the entire length of the coding region of that gene. Moreover each of these oligonucleotudes is paired with a second mismatch oligonucleotide in which the central base in the sequence has been changed. The combination of probe redundancy and inclusion of mismatched control sequence greatly reduces the rate of false positives obtained from this type of approach.

For expression profiling based comparison, fluorescently labeled probes are generated from test and reference samples (Figure 3). Fluorescent probes are generated by reverse transcribing total RNA using primer containing a T7 polymerase site. Amplification and labeling of the cDNA probe is achieved by carrying out an in vitro transcription reaction in the presence of a biotinylated dNTP, resulting in the linear amplification of the cDNA population (approximately 30-300 fold). The biotin labeled cRNA probe generated from the test reference samples is then hybridized to separate oligonucleotide arrays, followed by binding to a streptavidin-phycoerythrin conjugated fluorescent marker. Detection of bound probe is achieved following laser excitation of the fluorescent marker and scanning of the resultant emission spectra using a scanning confocal laser microscope. Presence of targets is detected by the light emitted at 570nm and the amount of light is proportional to the target hybridized to the probe. Location of the probe is detected for the identification of the corresponding target. The differential fluorescent signal is then represented as alternations in the transcriptional profile between the two samples compared.

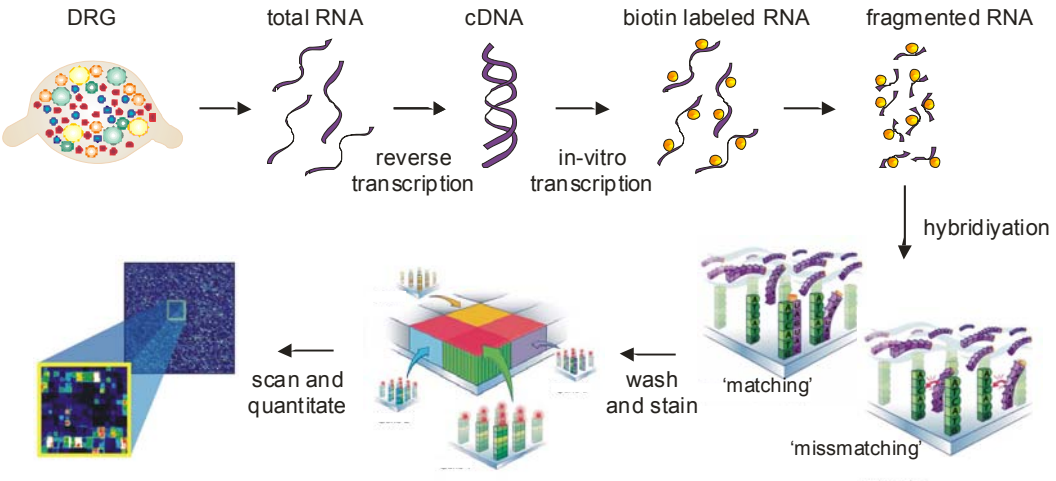

Figure 3. Overview of the Affymetrix experimental procedure

In the experiment, two different pools of cRNA targets were used. cRNA targets from total RNA isolated from DRGs of control and the anti-NGF treated mice were used to find out the comparative expression level of transcripts. The protocol was followed according to the instruction manual and all the internal quality control criteria were met. In brief, DRGs from all levels of age matched control and anti-NGF treated mice were dissected out and RNA prepared by the Trizol® method according to the manufacturer's instructions. First strand synthesis and further steps were conducted until fragmentation of the cRNA probes. After this step, the probes were given to the common facility for hybridization and scanning. The raw data and comparative data were further analyzed to find out genes with altered expression level. Genes regulated in two out of three independent experiments comparing the anti-NGF treated and control transcripts in DRGs were taken into consideration. Decision about presence or absence of the selected gene and calculation of the abundance of the RNA was done by a algorithm developed by Affimetrix, which mainly used the PM:MM ratio, background subtraction and discarding of outliers. The software used to analyze the data was Data Mining Tool Version 3.0 from Affymetrix. The average difference, the absolute call (present, absent, marginal) and a fold change (in comparison analysis) were taken into consideration.

Whole mount in situ hybridization

Whole mount *in situ* protocol on adult DRGs was based on a protocol developed for whole mount embryonic spinal cord (Carroll, et al., 2001). Mice were sacrificed by CO_2 inhalation and the spinal column was removed. DRGs from all the levels were dissected out into fresh 4% PFA prepared in Ca^{2+} and Mg^{2+} free PBT and fixed either by incubating at room temperature (RT) for 2 h or at 40^{o}C overnight with gentle rocking. After washing twice with PBT (10 min, gentle rocking at RT), DRGs were progressively dehydrated for storage by washing successively in 25%, 50%, 75% PBT-ethanol and 100%(2x) ethanol. These could be stored in 100% ethanol for several weeks. Fixed DRGs were distributed into 96 well plates (3 or 4 per well) with a porous bottom (Multiscreen™ - MAHVS4510), which enables holding of solution and solution removal by suction. All the following steps were done in these plates. For the hybridization, DRGs were progressively rehydrated by washing with 75%, 50%, 25% PBT-ethanol and twice with PBT successively. Proteinase K (10 µg/ml final concentration) treatment is conducted in PBT for 45 min at RT. After washing in PBT, post-fixation was carried out by 4% PFA, 0.1% gluteraldehyde (available as 25% solution in water, Serva-23115) in PBT for 20 min at RT. Washing was carried out twice to remove the fixatives by rinsing with PBT-HB (1:1) and washing with HB, before changing to hybridization buffer (HB). Pre-hybridization was performed in HB for at least one hour at 70°C. For hybridization, DNase treated, purified and diluted labeled probe was added to the HB and incubated overnight at 70°C. Unhybridized probes were removed by rinsing and washing twice with pre-warmed HB at 70°C. Prior to the RNAse treatment, washing was carried out with pre-warmed TST-HB at 70°C and with TST at RT. RNase treatment was performed (10 µg/ml in TST) at 37°C for 30 min. To inactivate RNase, DRGs were washed once with TST, once with HB at RT and incubated twice in HB at 65°C for 30 min. Washing with pre-warmed MABT-HB (1:1) at RT, rinsing twice with MABT, washing once with MABT and incubating in MABT at RT for 1 hr was done prior to blocking step. Blocking was done by 20% goat serum in MABT for 1.5 h. After blocking, DRGs were incubated overnight at 4°C with anti-DIG antibody coupled with alkaline phosphatase (1:2000 dilution) in the presence of 2% goat serum in MABT. Excess antibodies were washed off by rinsing 3x with MABT and incubating 3x in MABT at RT for 1 h each with gentle agitation. For color development, DRGs were washed twice with B3 and incubated with the substrate NBT/BCIP (1.5% v/v) in B3 without shaking till optimum color development occured (kept at RT or at 4°C during the night) with intermittent changes of the solution after every 12 h. After the color development, staining was stopped by washing three

times with PBT at RT and post fixed in 4% PFA in PBT for 30 min at RT. DRGs were soaked in 4% PFA,75% glycerol mix for two to three hours and mounted with the same solution, and observed under light microscope (Leica) using Metamorph® software.

Synthesis of riboprobes

One µl of the bacterial culture was taken as the template in 20 µl volume of reaction mix prepared according to the instruction manual (Taq Polymerase, Invitrogen). M13 Forward (GTT TTC CCA GTC ACG AC) and M13 Reverse (CAG GAA ACAGCT ATG AC) primers were taken to amplify the inserts along with the flanking T7 and SP6 polymerase binding sites. PCR cycling:

Initial denaturation:	94°C		3min
Denaturation:	94°C		45sec
Annealing	56°C		30sec
Extension:	72°C		90sec
Cycles:		30	
Final extension	72°C		10min
Storage:	4°C		

96 well PCR plates (ABgene-Cat.No.AB-0800) were used with an adhesive film (AB gene-Cat.No.AB-0588) to prevent the evaporation. 1µl of PCR product is used for making each sense and anti-sense ribo probe using T7 and SP6 RNA polymerases. The reaction mix was made with DIG RNA labeling Mix according to the manuals. After incubating at 37^{o}C for 2h, DNase I treatment (3U/well) was done at the same temperature for 15 min to eliminate the DNA template. After inactivating the DNase I, the volume of the reaction mix is made up to 50 µl and the probe is purified using PCR product Purification Plates (Qiagen) according to the maufacturer's instructions. The probes were eluted with 50 µl of RNase, DNase free molecular biology grade water. 1:600 dilution of T7 made probes and 1:450 dilution of SP6 made probes were used for hybridization.

Quantitative Real Time PCR

Isolation and cleaning of total RNA from mouse DRG was performed in accordance to the manufacturer's guide using Qiagen RNeasy mini kit (Qiagen) with on column DNAse I digestion (Qiagen). 2000ng of total RNA was used as a template for reverse transcription in 50µlvolume. The same protocol was followed as described in Superscript

II RNAse H-(Invitrogen) manual with random hexamer primers. For the first strand cDNA synthesis the following protocol was used:

1µl random hexamer primers

2µg total RNA

1µg dNTPs (10mM each)

ad 12µl distilled water

The mixture was heated to 65°C for 5 min and quickly chilled on ice. The content was collected by brief centrifugation and the following components were added:

4µl 5x first-strand buffer

2µl 0.1M DTT

1µl RNaseOUT™ (40U/µl)

The contents were gently mixed and incubated at 42°C for 2min. One µl Superscript II™ reverse transcriptase was added and the reverse transcription reaction was performed at 42°C for 50min. The reaction was terminated by heat inactivation of the enzyme at 70°C for 15min.

TaqMan Gene Expression Assays obtained from Applied Biosystems were used for quantitative Real Time PCR amplification reactions in accordance to the manufacturer's instructions. Template for the Real-time PCR was 1:200 dilution of the first strand reaction mix for. All the samples to be compared were processed in parallel and 5 or 6 independent experiments were performed. Probes in the primer probe mix from Applied Biosystems had FAM and a nonfluorescent quencher in the 5' and 3' positions respectively. Reaction conditions were as follows:

Initial denaturation:	50°C	2min
	95°C	10min
Denaturation:	95°C	15sec
Annealing and extension	59°C	1min
Cycles:	40	

The reference gene used for the experiments was prenylcysteine oxidase. It was chosen because no change was observed in three independent Affymetrix gene chip experiments to compare the expression level of transcripts in anti-NGF treated and control DRGs. One single reaction mixture contained 1µl 20x TaqMan Expression Assay (containing amplification primer and probe sets), 9µl cDNA and 10µl 2xTaqMan Universal PCR Master Mix. All reactions were carried out in sextuplets. The threshold cycle number for product detection (ΔCT value) was used to calculate the relative expression levels. Real Time PCR reactions were run using the Applied Biosystems PRISM 7000 Sequence Detection System.

Cell cultures

Cultivation of sensory neurones

Mouse DRGs were dissected and collected in a 1.5ml tube in PBS on ice. DRGs were washed once with PBS before incubation with 1µg/ml Collagenase TypeIV in 1ml PBS at 37°C for 30 min. DRGs were centrifuged briefly (170 x g), the supernatant was removed and DRGs were incubated with 100µl 0.5% Typsin in 1ml PBS at 37°C for 30 min. The supernatant was removed and 1ml D-MEM/F12 medium was added. The suspension was passed through 1-2 different siliconised Pasteur pipettes to dissociate them into single cells and centrifuged at 170 x g for 4min. The supernatant was removed and DRGs were resuspended in 1ml DRG medium. Cells were plated on poly-L-Ornithin and laminin coated coverslips (about 60-120µl of cell suspension per coverslip) to let the cells attach to the coverslip. After 4 hours an additional 150µl of the DRG medium was added to the coverslips. Cells were cultured for 24h at 37°C in a Steri-Cult 200 incubator.

Cultivation of embryonic chicken DRGs explants cultures

Embryonic chicken DRGs (E7-8) were dissected and collected in a 1.5ml tube in PBS on ice. DRGs were washed once with PBS. DRGs were plated in 6x4 well plates (3 per well) in DRG medium. NGF (100ng/ml) and different concentrations of anti-NGF were added to observe the neurite outgrowth in the next three to four days.

Cultivation of hybridoma cells

Frozen hybridoma cells (anti-NGF clone 27/21) were thaw, cultured in the hybridoma medium and passaged in progressively bigger culture dishes. When high density culture was obtained in 500µl dish, the medium was replaced with protein free hybridoma medium. After two to three days the cells were discarded and supernatant was collected.

Protein chemistry

Immunocytochemistry

Immunostaining of tissue sections

Animals were anesthetized with sodium urethane 0.1 mg/ml (0.1-0.5 ml or more) and perfused with 4% PFA in 0.1M PBS, pH 7.4 and 4°C. Immediately after perfusion the DRGs were dissected and post-fixed in the perfusion fixative at 4°C for 4 hours.

Tissue was immersed in 25% sucrose in PBS for 1-3 days until the DRGs sank to the bottom of the sucrose solution. Fresh sucrose solution was replaced daily.

DRG sections were cut on a freezing microtome into 40 µm sections.

DRG sections were pre-incubated in 1% serum albumin (BSA) and 0.3% Triton X-100 in TBS for 1h and incubated overnight at RT with PGP9.5 antibody 1: 2,000 diluted in TBS with 0.3% Triton X-100 and 5% normal goat serum. DRG sections were washed by rinsing slides in excess TBS for 30 min and then incubated for 1 hour at room temperature with Cy-3-conjugated secondary antibodies diluted 1:800 in TBS containing 0.3% Triton X-100 and 5% normal goat serum.

DRG sections were washed twice in excess TBS for 30 min and then once with water before mounting them in Aqua-Polymount. Cy3 light emission was captured with the XF22 filter (excitation 535nm, emission 605DF50, Omega Optical).

Production of functionally blocking anti-NGF

The antibody used to test the dose-dependent effectiveness of anti-NGF treatment was the commercially available blocking anti-β (2.5 S, 7S) Nerve Growth Factor (clone 27/21). Hybridoma cells (clone 27/21) were cultured to produce sufficient amounts of antibody needed for the anti-NGF treatment. The cells were cultured in protein free medium and the supernatant was collected. Centrifugal filter devices (Centriplus, YM-50, amicon®) were used to concentrate the supernatant to approximately 10x lower volume. The concentration of proteins in the supernantant was measured assuming that the only protein content of the supernatant was anti-NGF. ELISA protein concentration measurements were performed using a commercial antibody as a reference. The protein concentration ranged from 256ng/µl to 1µg/µl in different preparations. To test the biological activity of the antibody produced a bioassay was performed. The NGF dependant neurite outgrowth of isolated embryonic chicken DRGs was observed and

quantified. The capacity of hybridoma supernatant to block NGF and the neurite outgrowth was compared to the biological activity of commercially available anti-NGF. Commercially antibody had the same effectiveness at concentrations up to 10 times lower compared to hybridoma supernatant antibody. For *in vivo* injection hybridoma anti-NGF was used at concentration extrapolated from the bioassays corresponding to 0.5μm/g of the commercially purified antibody. The effectiveness of each treatment was additionally confirmed by its *in vivo* effect - the phenotypic switch seen in Aδ fibers. The control group of the mice was injected, during the same time course as the anti-NGF treated, with the flow thru obtained after concentrating the cell culture supernatant.

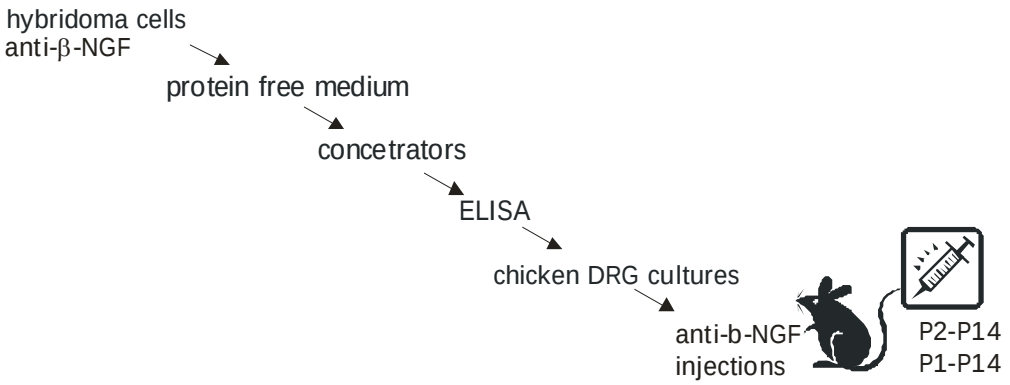

Figure 4. Overview of the anti-NGF production

Electrophysiology

The in vitro skin nerve preparation

Adult mice were sacrificed by CO_2 inhalation and the hair from the hindlimb was removed. The saphenous nerve up to the lumbosacral plexus and a piece of the innervated skin reaching from the toes up to halfway between the ankle and the knee were excised. To facilitate oxygenation of the tissue, the skin was placed corium-side up in an organ bath, where it was fixed with insect needles, and superfused with 32°C warm oxygen-saturated synthetic interstitial fluid (SIF) at a flow rate of 10ml/min. The saphenous nerve was pulled through a gap to the recording chamber and laid on top of

a small mirror that served as the dissection plate. The aqueous solution in the recording chamber was overlaid by mineral oil in such a way that the interface of the two phases was located just below the surface of the mirror. Dumont's 55 forceps were used to desheath the nerve, carefully removing its surrounding epineurium, and to tease small filaments from the nerve so that the activity from single units could be recorded by placing the individual strands of the nerve onto the silver recording electrode installed in the chamber's wall.

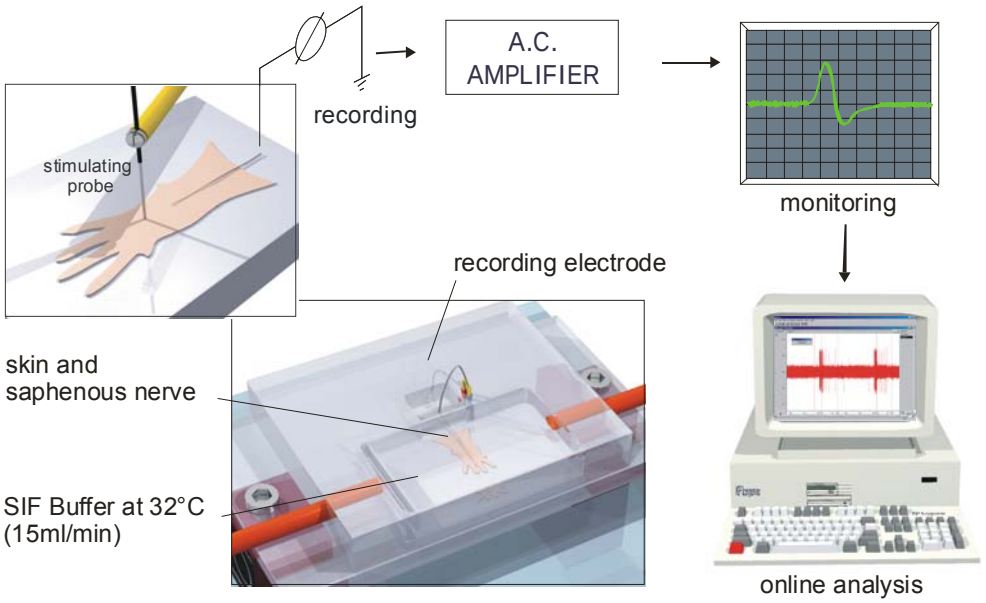

Figure 5. Overview of the skin-nerve preparation
(Modified from Dr. Paul Heppenstall)

Electrophysiological recordings were performed using the NeuroLog™ system from Digitimer Ltd., which included a low-noise differential amplifier for recording from identified units. The recordings were visualized on a connected Tektronix TDS 220 two-channel digital real time oscilloscope, while simultaneously acquired on a PC by a PowerLab/4s converter from ADInstruments. The receptive fields (RF) of individual units were identified by manually probing the corium-side of the skin with a mechanical search stimulus, a blunt-end glass rod (Koltzenburg et al., 1997). Individual units were characterised by the constant shape of the action potentials they fired in response to stimulation, as the shape of an action potential depends on the composition of voltage-gated ion channels expressed by each neuron.

Using the Chart v5.2 software from ADInstruments, the recorded action potentials of individual units were discriminated by their width (in µsec), height (in points) and conduction velocity. In this way, about 10-20 units could be characterised per experiment, so that their mechanical activation threshold, their conduction velocity, their stimulus-response function and spike frequency adaptation could be recorded.

Determining the mechanical activation threshold

The receptive field of a unit was identified using the glass rod. To determine the mechanical activation threshold, i.e. the minimal amount of force needed to elicit an action potential from that unit, calibrated von Frey hairs were used on the most sensitive spot of the RF. The von Frey hair nylon monofilaments were of various thicknesses, which accordingly produced bending forces from 0.4mN up to 32mN.

Determining the conduction velocity

A teflon-coated silver electrode with a non-insulated tip (∅ <0.5mm) was set on the most sensitive spot of the receptive field, and electrical pulses of constant current in square-wave pulses were used to excite the unit. The stimulus intensity was set at approximately two-times the threshold with a pulse duration of 50-500µsec depending on the afferent under investigation. The latency between the stimulus artefact and the resulting action potential was measured. To calculate the conduction velocity the distance between the stimulating and the recording electrode was divided by this latency. Units could thus be grouped into three classes: A-β fibers, which are thickly myelinated units, have a conduction velocity faster than 10m/sec, A-δ fibers are thinly myelinated units with a conduction velocity of 1-10m/sec, and non-myelinated C-fibers conduct slower than 1.2m/sec.

Measurement of mechanosensitivity

To determine the mechanical sensitivity of the sensory afferents of the saphenous nerve, the desired receptor was first identified by electrical stimulation. In order to determine the starting point, i.e. the mechanical threshold at which the unit could be reproducible excited, the Nanomotor™ (Nanotechnik, Kleindieck) was placed over the most sensitive spot of the RF and moved slowly towards the skin.

In this way, the recording was started at threshold for each unit. For producing a stimulus response function an ascending series of increasing displacement stimuli was then applied to this spot. The elicited action potentials were acquired by PC and analysed using the Chart v5.2 software.

Measurment of heat sensitivity

To measure the heat sensitivity of C-fibers a contact thermal stimulator was used (Yale University, Instrumentation Repair and Resign Shop). The temperature of the contact surface (1cm^2) was measured by tungsten-constantan thermocouple placed on the top of the Peltier device. The thermal stimulator is designed to hold a preset temperature and on command change to a new temperature. Heat ramp stimuli (32-60^{o}C) were applied at the rate 2^{o}C/second, keeping the temperature at 60^{o}C for five seconds and cooling it down to the bath temperature at the same rate.

Patch clamp experiments

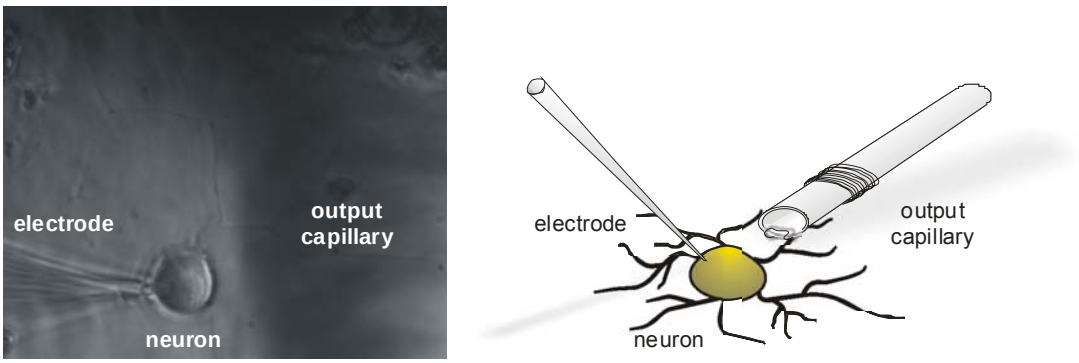

Figure 6. Overview of the patch perfusion system used to record I_{heat}

Whole cell recordings were made from DRG neurons 24-48 h after plating using fire polished glass electrodes with a resistance of 4-9 MΩ. The recording chamber was perfused with extracellular solution contained (in mM) 140 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 4 KCl, 4 glucose, 10 HEPES, pH 7.4 and electrodes were filled with solution containing (in mM) 110 KCl, 10 Na^+, 1 $MgCl_2$, 1 EGTA, 10 HEPES, pH7.3. Membrane current and voltage were amplified and acquired using an EPC-9 amplifier sampled at 10-40 kHz. For whole cell recording, the membrane voltage was held at –60mV. Heat ramp stimuli (24-56°C) and drugs were applied using an automated perfusion system (WAS02, (Dittert et al., 2006). The outlet of the output capillary (400 µm inner diameter) with

heating Cu-coil was placed not more then 100 µm away from the patched cells. The applied drug (SCF, 10 nM) was dissolved in extracellular solution. Acquired traces were analysed using Pulse and PulseFit software (HEKA).

Electron microscopy

Mice were perfused with freshly prepared 4 % PFA in 0.1 M phosphate buffer. Saphenous nerves were dissected and postfixed in 4% PFA 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 days.

Following treatment with 1% OsO_4 for 2h, nerves were dehydrated in a graded ethanol series and propylene oxide and embedded in Poly/Bed^R 812 (Polysciences, Inc., Eppelheim, Germany). Semi-thin sections were stained with toluidine blue. Ultra-thin sections (70 nm) were contrasted with uranyl acetate and lead citrate and examined with a Zeiss 910 electron microscope.

Digital images were taken with a 1kx1k high-speed slow scan CCD camera (Proscan) at an original magnification of 1,600 x. Two ultrathin sections per nerve and genotype were analysed. On each ultrathin section, four images were taken representing an area of 18.25 x 18.27 µm. Myelinated and non-myelinated axons were counted using the analySIS 3.2 software (Soft Imaging System, Münster, Germany).

Behavioural experiments

Paw withdrawal test –latency to noxious heat stimuli

Thermal latency was measured based on method described in Hargreaves K., et al., (1988) using Plantar Test 7371 (Ugo Basile, Italy). In the radiant paw-withdrawal test the mice were placed in a Plexiglas chamber (15 cm diameter, 22.5 cm in height). The stimulus was a high intensity beam directed at the plantar surface of the hindpaw; movement of the paw away from the beam results in the heat source being terminated, with the withdrawal latency indicated on a digital screen. Paw withdrawal latencies were determined alternately from both hindpaws (stimuli given every 2- 3 min), each paw was

tested 5 times per experiment. Criteria for inclusion of a data point were calm behavior of the mouse just prior to testing, and a clear paw withdrawal from the hot beam associated with paw flicking and grooming. Baseline values were taken once a day at the same time for a period of 5 days.

Paw withdrawal test –latency to noxious mechanical stimuli

The sensitivity to a punctuate mechanical stimulus was assessed using the dynamic plantar aesthesiometer (Ugo Basile, Milan, Italy). Each mouse was placed in a Plexiglas chamber (15 x 15 x 22.5 cm, wire mesh floor). 10 minutes later a mechanical stimulus (a small diameter blunt metallic filament) was applied to the plantar surface in an increasing vertical force. The force was increased with a ramp of 0.5 g/second to the maximum of 10 g (20 secs). The force applied and the withdrawal latencies were measured. Paw withdrawal latencies were determined alternately from both hindpaws, each paw was tested 5 times per experiment. Criteria for inclusion of a data point were calm behavior of the mouse just prior to testing, and a clear paw withdrawal from the hot beam associated with paw flicking and grooming. Baseline values were taken once a day at the same time for a period of 3 days.