2 Materials and Methods

2.1 Solutions and Buffer

10x Oligo annealing buffer 100 mM Tris HCl (pH7.5) 1M NaCl 10mM EDTA in molecular biology grade H_20	**0.5 M PB** 7.10g Na_2HPO_4 in H_20
10x PBS 1360mM NaCl 20mM KCl 106mM Na2HPO4*2H2O 15mM KH2PO4	**PBST** 1ml Tween 20 in 1l 1x PBS
10x white Laemmli-buffer 25mM Tris base 192mM Glycine 3.5mM SDS (1%) in aqueous solution Do not adjust pH, store at RT	for **1l** of buffer prepare: 30.3g 144g 10g H_2O bidest to 1 liter
white Laemmli+PI Add 1 tablet of Complete Mini Protease Inhibitor (Roche, Mannheim, Germany) to 10ml of 1x white Laemmli. Protease Inhibitor containing buffer can be stored for approximately 12 weeks at -20°C.	
Brain nuclear extraction buffer (non-ionic detergent) 50mM Tris pH 8.0 2mM EDTA pH 8.0 0.1% NP40 in aqueous solution	for **10ml** of buffer prepare**:** 0.5ml of 1M Tris-HCl, pH 8.0 0.04ml of 0.5M EDTA, pH 8.0 0.01ml NP40 (very viscous! Use 10% stock solution in water and dilute 1:100) H_2O bidest to 10ml
5x Blotting buffer 29.1g Tris 14.65g Glycine 18.75ml 10% SDS ad 1l with bidest H_2O	**10x Erythrocyte Lysis buffer** 41.45g NH4Cl 5g KHCO3 0.17g Na-EDTA ad 500 ml with H_2O bidest, then adjust pH to 7.4 with 1M HCl or 2M NaOH.
Tris-Na-EDTA-buffer (TE or TNE buffer) 10mM Tris pH 7.5 1mM Na-EDTA pH 8.0	**Narcotic** 3 mg/ml Xylazine 15 mg/ml Ketamine for zebra finches: 3 µl/gram body weight

TDMH (Taq polymerase reaction buffer)	for **2ml** prepare**:**:
10x PCR-buffer without $MgCl_2$ (Roche)	521.1µl
25mM $MgCl_2$	416µl
H_20	37µl
	1025.7µl
HEK293-T / Hela cell culture medium	
500ml DMEM (Invitrogen, Carlsbad, USA)	
55ml Fetal Calf Serum (this corresponds to ~ 10 %)	
6ml L-Glutamin (200mM; Invitrogen)	
7ml ready-to-use Penicillin/Streptomycin-Mix (Penicillin 10.000 U/ml, Streptomycin 10.000µg/ml (Invitrogen)	

2.2 Enzymes

All restriction enzymes were purchased from New England Biolabs (Ipswich, USA). Recombinant Taq polymerase was provided by the "Sequencing Service Unit" of the MPI for Molecular Genetics, headed by Dr. R. Reinhard.

2.3 Nomenclature

For avian brain regions, the recently revised nomenclature proposed by the Avian Brain Nomenclature Forum (Reiner et al., 2004) and (http://avianbrain.org/) was used. In order to increase readability of this manuscript I use a unified nomenclature for the FoxP2 gene and protein from all species. This nomenclature is based on (Kaestner et al., 2000) with the exception of human FOXP2 and mouse FoxP2 being all written as FoxP2.

2.4 Molecular Biology

2.4.1 Sex determination of young zebra finches

The sexing protocol is based on the detection of a length polymorphism in the chromobox helicase DNA binding gene (CDH) located on both bird sex chromosomes called by "W" and "Z" (Griffiths et al., 1998). A fragment of CDH encompassing the length polymorphism can be amplified from genomic DNA in a PCR reaction with primers *sexing-for* and *sexing-rev.* (For exact primer sequence see Table 2.12.1). Amplification generates two different bands (PCR-products 389bp and 353bp) in the heterogametic females (karyotype ZW) and a single band (PCR-product 353bp) in the homogametic males (karyotype ZZ).

A drop of blood from the bird's vene was transferred into a 2ml tube containing 1ml of 1x erythocyte lysis buffer. After incubation for 15min at RT, the blood was centrifuged for 5min at 1000xg. Supernatant was removed and 600µl TNE buffer, 120µl SDS 10% and 15µl Proteinase K (stock 10mg/ml from New England Biolabs, Ipswich, USA) were added to the tubes. Next the samples were incubated in a hotplate at 65° C overnight. The next day, 60µl $NaClO_4$ were added and the samples were mixed immediately to avoid precipitation of salt. Then, genomic DNA was precipitated by addition of 700µl isopropanol and subsequent overhead inversion of the tubes. The genomic DNA was pelleted by centrifugation at maximum speed of the table top centrifuge (16,000xg) for 5min. Supernatant was removed carefully and the DNA-pellet washed once with 70% EtOH (molecular biology grade). Ethanol was removed by careful decanting. Pellets were dried for 5min at 55°C (tubes with open lids) in heating block. DNA was resuspended in 200-400µl H_20 (molecular biology grade)

PCR reaction (1x):

10 µl TDMH
0.5 µl sexing forward primer
0.5 µl sexing revers primer
9.5 µl H_2O
0.5 µl Taq Polymerase
5 µl genomic DNA

total volume 26 µl.

PCR-program:

1)	94°C	5min:	DNA denaturation
2)	35 cycles of		
	94°C	45sec:	DNA denaturation
	50°C	1min:	annealing of primers
	72°C	1min:	elongation by Taq polymerase
3)	72°C	5min:	Final elongation.
4)	04°C	forever	

2.4.2 RNA extraction from zebra finch tissue

Tissue was removed from anesthetized animals and transferred into liquid N_2. The frozen tissue was disrupted with a mortar and a pestle, both precooled in liquid N_2. Tissue powder was weighed and stored at -80°C. RNA extraction was the performed either with Trizol reagent (Invitrogen, Carlsbad, USA) or the NucleoSpin RNAII Kit from Macherey and Nagel (Düren, Germany). Both extraction procedures were carried out according to the manufacturers protocol.

For the quantification of the FoxP2 expression in Area X, animals were decapitated, their brains dissected out immediately and shock-frozen on liquid nitrogen. Then the brains were brought to a temperature of -10°C by incubation in a $NaCl_2$-saturated ice/water mixture. Next, a cooled razorblade was used to cut slices of approximately 1mm thickness. The slices harboring Area X were thawed on a glass slide and as soon as Area X became visible, it was punched out with a 1mm diameter glass capillary. The weight of the dissected tissue was usually in the range between 1 and 2µg. For the RNA extraction from these small amounts of tissue material I used TRIZOL. RNA yield was determined by UV spectroscopy at 260/280nm with a NanoDrop Fluorospectrometer (NanoDrop Technologies, Wilmington, USA) device.

2.4.3 Northern blotting

20µg of total RNA from adult male zebra finch brain and lung were separated on a 1%denaturing agarose gel and blotted as described in (Sambrook and Russell, 2001) onto a nylon membrane (Amersham Biosciences, Piscataway, USA) overnight. The membrane was then incubated with a 32^P-labeled FoxP2 fragment spanning bp 114-959 relative to the first start codon of isoform III at 65°Cfor 3hr. The blot was washed and exposed to an MS-intensifying screen (Eastman Kodak), which was then scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, USA) and analyzed with ImageQuant software 5.2 (Molecular Dynamics).

2.4.4 Cloning of FoxP2 and FoxP1 from zebra finch

Primers specific for the mouse FoxP2 sequence were used to amplify zebra finch FoxP2 from adult male zebra finch brain total RNA. For rerverse transcription of RNA into cDNA

we used Superscript II (Invitrogen, Carlsbad, USA) and followed the manufacturers manual. We amplified a fragment covering bp 114-959 of isoform III, relative to first start codon, with primers *FoxP2-SH1-f* and *FoxP2-SH1-r* and the entire FoxP2 ORF with primers *FoxP2-SH2-f* and *FoxP2-SH2-r*. A 180bp FoxP1 fragment was obtained using degenerated primers. For PCR program see above in 2.4.1. All PCR products were examined on agarose gels, cleaned from nucleotides with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into the pGEMTeasy vector (Promega, Madison, USA). Inserts from 15 independent FoxP2 clones and 6 FoxP1 clones were then sequenced on both strands with primers *M13-f* and *M13-r*. We obtained additional cDNA sequence for each gene using the SMART-RACE kit (Clontech, Palo Alto, USA). All zebra finch FoxP2 and FoxP1 sequences were deposited into GenBank (accession numbers AY549148, AY549149, AY549150, AY549151, and AY54952) and the songbird cDNA database (http://www.dbsr.duke.edu/songbird). One full ORF FoxP2 clone and one containing the fragment covering bp 114-959, relative to the first start codon, as well as the 180bp FoxP1 clone, were selected for the generation of riboprobes used in *in situ* hybridization experiments. Table 2.12.4 summarizes all plasmids used in this study.

2.4.5 Cloning of FoxP2 and FoxP1 from other avian species and crocodile

Using primers *FoxP2-SH2-f* and *FoxP2-SH2-r* the FoxP2 genes from *Glaucis hirsuta* (rufous-breasted hermit hummingbird), *Gallus gallus* (chicken), *Melospiza melodia* (song sparrow), *Sayornis phoebe* (phoebe), *Melopsittacus undulatus* (parrot, budgerigar), *Archilochus colubris* (North Carolina hummingbird, ruby-throated hummingbird), *Serinus canaria* (canary), *Columbia livia* pigeon (rock dove), *Aphantochroa cirrhochloris* (sombre hummingbird) and *Alligator mississippiensis* (american alligator) were amplified by PCR from cDNA, provided by K. Wada from Duke University, Durham, North Carolina, USA. PCR products were then cloned into pGEMTeasy (Promega). 3 clones from every of the 11 species were fully sequenced with primers *M13-f* or *M13-r* on both strands. All sequences from one species were assembled together, yielding an average coverage of ~14 times per FoxP2 gene from each species.

2.4.6 Cloning of V5-tagged FoxP2 expression constructs

A mammalian expression vector encoding V5-tagged FoxP2 was generated by first amplifying the entire ORF of zebra finch FoxP2 with primers *FoxP2-BamH1-Kozak* and *FoxP2-EcoRI-V5*. *FoxP2-BamH1-Kozak* adds the recognition sites for the restriction enzyme *BamHI* and a Kozak sequence before the 5´ end of the FoxP2 ORF. *FoxP2-EcoRI-V5* removes the STOP codon of the FoxP2 ORF and adds the recognition sites for the restriction enzyme *EcoRI* to the 3´ end. Further it was designed such that after cutting the PCR product with *BamHI* and *EcoRI* it can be ligated in-frame into the multiple-cloning-site (MCS) of pCDNA4/V5-His B (Invitrogen, Carlsbad, USA). The resulting expression construct was named pcDNA-FoxP2V5.

2.4.7 Cloning of constructs encoding short-hairpin RNA

A list of putative shRNA targets within the zebra finch FoxP2 gene were generated using the web-based software from Qiagen (http://www.qiagen.com) by sequence search with the minimum common sequence of all isoforms (ORF of isoform **IV)**. With this approach knockdown of all known zebra finch FoxP2 isoforms can be achieved. Since shRNA´s were to be expressed from a plasmid via U6 promoter driven RNA polymerase III, it was absolutely crucial NOT to include more than 4 consecutive thymidines (uracils), which are recognized as a stop signal by the polymerase. All proposed targets that contained more than 2 thymidines in a row were excluded. In order to reduce the risk of cross-reactivity with other genes, all target sequences were checked for homology to chick expressed sequence tags (EST) with the internet-based BLAST tool "search for short nearly exact matches" (http://www.ncbi.nlm.nih.gov/BLAST). They were further directly compared to the FoxP1 sequence to avoid cross reactivity with the closest homologue of FoxP2. Only target sequences with at least 6 non-homologous bases were chosen. Target sequences within the known protein domains of FoxP2 were also avoided. In a last step, all chosen targets were checked for ambiguity in the sequence raw data of all available zebra finch FoxP2 clones, to rule out interference with single-nucleotide-polymorphisms. All target sequences have a GC-content of approximately 50%. Sequences are shown in Table 2.12.2. For each target sequence meeting the above mentioned criteria, a DNA sequence encoding the corresponding short hairpin RNA was generated. The general composition of the sequence was: sense → hairpin loop → antisense. The sequence of the hairpin loop was GTGAAGCCACAGATG. A *BbsI* and a *BstBI* restriction site were added to the 5´

and the 3´ end respectively, which allow to clone the DNA fragments into the short-hairpin expression vector pBudΔU6. A non-silencing control shRNA was designed accordingly, based on the non-silencing control sequence from Qiagen (Hilden, Germany). A BLAST homology search with the sequence of this control shRNA reveals a 16 base overlap with section 21 of 136 of the complete *Thermotoga maritimia* genome, but no other match to any other sequence deposited in GenBank. Another shRNA was designed to target the green fluorescent protein (GFP). The sequence for this shRNA was provided by Pawel Licznerski from MPI for Medical Research, Heidelberg, Germany). Table 2.12.3 lists the sequence of all ssDNA fragments. For cloning of the DNA fragments encoding the different short hairpin RNAs into pBudΔU6, we first generated double stranded DNA fragments from single stranded synthetic oligonucleotides. Each pair of complementary strands was diluted in annealing buffer. The tubes were placed in boiling water to denature the DNA. Next the tubes and the water were slowly cooled down to RT. After that, the now double-stranded DNA was digested with enzymes *BbsI* and *BstBI* and ligated into pBudΔU6, cut with the corresponding enzymes before.

All hairpin constructs were tested for their knockdown efficiency *in vitro* (see below Figure 3.18). Functional U6-shFoxP2 expression cassettes (U6 promoter + shRNA) and the U6-shGFP and U6-shControl control constructs were subcloned into the viral transfer vector pFUGW_linker with the enymes *NheI* and *BstBI*. This vector was subsequently used to generate lentiviral particles. We confirmed the sequence of all pFUGW-shFoxP2 constructs by sequencing with primers *Seq pFUGW-f* and *Seq pFUGW-r*.

2.4.8 Preparation of Plasmid DNA

All vectors were transformed into chemically competent TOP10 (Invitrogen, Carlsbad, USA) *E.coli* cells as described in (Sambrook and Russell, 2001). Small and midi-scale plasmid extraction was performed with Qiagen Mini- and and Midi-plasmidpreperation kits (Hilden, Germany). Large scale extraction was done with Cesium chloride by L. Vogt, MPI for Molecular Genetics according to (Sambrook and Russell, 2001).

2.4.9 Sequencing

Sequencing was performed according to the "Sanger"-method with fluorescently-labeled didesoxynucleotides. The amplification/labeling reaction was carried out as described below, followed by DNA precipitation. Electrophoresis and base calling was done by the "Sequencing Service Unit" of the MPI for Molecular Genetics, headed by Dr. R. Reinhard.

PCR reaction (1x):

1 µl Primer (10pmol)

2 µl Terminatormix (Applied Biosystems, Foster City, USA)

10 µl H_2O

10ng/100bp DNA for sequencing of PCR products

150-200ng DNA for sequencing of plasmid DNA

total volume 20µl.

PCR program:

1)	96° C	3 min
2)	24 cycles of	
	96°C	10 sec
	primer annealing	5 sec
	60°C	4 min
3)	04°C	forever

DNA-precipitation:

First 25 µl EtOH abs. (RT) were mixed with the sequencing reaction by pipetting up and down. The mixture was incubated for 1 h (RT) and then centrifuged for 1h at 4000 rpm (Eppendorf 5810R). The supernatant was carefully removed and the pellet washed twice with 150 µl 70% EtOH. After each washing step the samples were centrifuged at 4000 rpm for at least 15min. After the second washing step, the pellets were dried by carefully centrifugating the tubes inversed (acceleration and brake 3) at 500rpm. Samples were stored at -20°C .

2.4.10 Sequence analysis

Sequence assembly and analysis was conducted with GCG 10.1 (Accelrys, Cambridge, UK) and the Staden package (Staden et al., 1998).

2.4.11 *In situ* hybridization

In situ hybridizations were performed according to two protocols using ^{33}P-labeled (Vortkamp et al., 1996) or ^{35}S-labeled (Mello et al., 1997) riboprobes. Both protocols yielded identical labeling patterns in the brain. Riboprobes were *in vitro* transcribed from T7 and SP6 promoter sides of the pGEM-T-easy cloning vector containing the FoxP2 and FoxP1 cDNA clones. Slides were exposed to Bio-max x-ray film (Eastman Kodak, Rochester, NY) for 2-3 d (^{35}S-labeled material) or 1-3 d (^{33}P). For species comparison and developmental studies, a set of 163 slides with sections from 11 different species and from zebra finches of 12 different developmental ages [embryonic stages 10, 23, 26, and 28 and featherbud stage embryos corresponding approximately to chick stages 34 and 37, and post-hatch days (PHDs) 15, 25, 35, 45, and 75 and adults >90 d] were hybridized at the same time with a FoxP2 mastermix, with the same counts per minute radioactive count per slide. For the seasonal comparisons, all sections were also hybridized with a master mix. FoxP1 was hybridized on another day to avoid the possibility of cross-contamination. For *in situ* quantifications, the exposed film was placed under a high-power dissecting scope (Wild M420; Leica, Deerfield, USA) and scanned into a computer using a Spot III camera and Spot software version 3.2.4 (Diagnostic Instruments, Sterling Heights, MI). Images were transferred to Photoshop (Adobe, San Jose, USA) and converted to grayscale. Vocal nuclei and adjacent non-vocal areas, i.e., the surrounding brain subdivisions (caudal nidopallium ventrally adjacent to HVC; nonauditory arcopallium abutting the robust nucleus of the arcopallium (RA); nidopallium rostral to lateral magnocellular nucleus of the anterior nidopallium (lMAN) and the surrounding shell region; caudal striatum (CSt) immediately caudal to Area X) were outlined with a selection tool, and the average pixel density was calculated using the Photoshop histogram function. To calculate ratios of differential expression in vocal nuclei relative to their surrounding brain subdivision, we divided the pixel density values of vocal regions by the pixel density values of the respective adjacent region, using comparably sized areas for quantification. When expression within a given vocal nucleus is the same as the expression of the region surrounding it, the ratio is 1; when the expression within the vocal nucleus is higher than expression in the region surrounding it, the ratio is >1; when lower, the ratio is <1.

2.4.12 Real-time PCR

For the quantification of FoxP2 expression levels in Area X we used the real time PCR system ABI 7900HT (Applied Biosystems, Foster City, USA). DNA quantification was performed with the Sybr Green MIX containing the Rox passive control. We determined FoxP2 expression levels by relative quantification based on the normalization of expression levels to internal control genes. A list of 10 mouse control genes was kindly provided by M. Sultan, MPI Molecular Genetics, Berlin, Germany. The search for the corresponding zebra finch homologues in the "Songbird Brain Transcriptome Database" (http://songbirdtranscriptome.net/) and the database from the "Songbird Neurogenomics Initiative" (http://titan.biotec.uiuc.edu/songbird/) yielded 6 sequences that unambiguously identified the genes Vimentin, Pgk1, Pfkp, Hmbs, Hprt and Gapdh. Primers for these 6 genes, GFP and actin were designed to yield PCR products of approximately 100bp length (for primer sequences see Table 2.12.1). These short amplicons are likely to achieve optimal amplification efficiency. T_m was set to 64°C for high primer binding specificity. For determination of relative expression levels we used the comparative C_t method. The C_t value of each PCR reaction is defined as the threshold cycle in the linear exponential phase of the amplification, at which the PCR product is first detected to increase significantly. Differences in expression levels can be calculated by comparing the different threshold C_t values for each gene of the same cDNA. E.g. the expression level for FoxP2 can be expressed as $\lambda C_{t\ FoxP2}$ by simply subtracting $C_{t\ FoxP2} - C_{t\ control\ gene}$. In order to compare expression levels between two different cDNA samples from the same animal, normalized C_t values (λC_t) were calibrated to one cDNA. In this study, we always calibrated the knockdown treatment to the control treatment or left hemisphere-derived cDNA to right-hemisphere derived cDNA. Given that under ideal conditions, one amplicon is amplified once per cycle, the amount of a target gene relative to the internal control gene and calibrated to one cDNA is then $2^{-\lambda\lambda Ct\ FoxP2}]$ with $\lambda\lambda C_{t\ FoxP2} = \lambda C_{t\ FoxP2\ cDNA\ knockdown} - \lambda C_{t\ FoxP2\ cDNA\ control}$. For the C_t method to be valid, it is important that all amplicons are amplified with similar efficiency. All primers used in this study fulfilled this criterion in a validation experiment, where all primers were simultaneously tested in a cDNA dilution series.

2.5 Protein Biochemistry

2.5.1 Protein extraction from cultured cells

Cells were harvested with trypsin (BD Biosciences, Heidelberg, Germany). Detached cells were transferred into a 1.5ml tube and centrifuged for 5min at 500xg. The pellet was washed once with PBS. Next, the cells were pelleted again by centrifugation for 5min at 500xg. Then 100-400µl of white Laemmli+PI was added to the pellet. Importantly, the pellet was dissolved immediately by vigorously pipetting up and down. Samples were stored at -20°C.

2.5.2 Protein extraction from zebra finch brain tissue

Tissue powder was generated as described in 2.4.2**.** A small sample (0.1-0.3 g) of the powder was transferred into an 1.5ml Eppendorf tube containing 600µl of brain nuclear extraction buffer. The tube was vortexed vigorously. The tissue was disrupted by pipetting up and down approximately 15 times, followed by incubation on ice for 20min. While on ice, the tube was vortexed from time to time (~3x). Next, the sample was centrifuged 5min at 1500xg at 4°C. The supernatant, which contains mainly the cytoplasmic fraction of the sample was pipetted off for subsequent Western blotting. The remaining pellet was redissolved in brain nuclear extraction buffer and incubated on ice for another 20min. After a second centrifugation step (5min at 1500xg at 4°C) the supernatant was discarded and the pellet, containing mainly cell nuclei, redissolved in 200µl white Laemmli+PI. The resulting solution was passed through a syringe to break the nuclei and reduce the viscosity of the solution. All samples were processed as described in Western blot for western blotting.

2.5.3 BCA assay for protein quantification

The concentration of protein extracts was determined with the Bicichinonic Acid kit from Sigma (Munich, Germany) according to the manufacturers manual.

2.5.4 Western blot

Protein samples were prepared in a total volume of 30µl: The protein (usually 10-20µg) was diluted in 2xLaemmli containing 0.1M DTT. The sample should contain at least 7µl of

2xLaemmli. Samples were denatured at 95°C for 5min, cooled briefly on ice and loaded on a denaturing acrylamide gel (Sambrook and Russell, 2001). Electrophoresis was performed according to (Sambrook and Russell, 2001). After the gel run, the gel was blotted onto a Polyvinylidene fluoride (PVDF)-membrane with the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell from BioRad (Munich, Germany) according to the manufacturers manual for 25min at 15V. After that, blots were blocked in PBST/5% dry milk for 30min at RT. Before incubation with the antibody, the membranes were briefly washed in PBST. Antibodies against the protein of interest were then diluted in 1.5ml PBST/1% BSA. Table 2.12.5 lists all antibodies and dilutions used in this study. Blots were transferred into the antibody solution and incubated overnight at 4°C. After that, membranes were washed 3 times for 5min in PBST and subsequently incubated for 30min with the corresponding Hrp-conjugated secondary antibody diluted in PBST. Next, they were washed 3 times for 5min in PBST. The blots were then wetted with 1ml of the final detection solution from the Western lightning kit (Perkin Elmer, Rodgau, Germany). Chemiluminescense was detected by exposure to an X-ray film (Kodak, Stuttgart, Germany). Films were developed in a Curix 60 developing machine (Agfa, Cologne, Germany).

2.6 Knockdown Efficiency of Hairpin Constructs *in vitro*

Since the optimal sequence of a short hairpin RNA (shRNA) targeting the *FoxP2* message RNA with maximum efficiency cannot be predicted, 8 different shRNA constructs were tested experimentally *in vitro* to identify those resulting in maximal knockdown. Knockdown efficiency of shRNA constructs *in vitro* was determined by cotransfecting each hairpin construct (pBudΔU6-shFoxP2 a-i) together with V5-tagged FoxP2 into Hela cells. $1.5x10^5$ Hela cells were seeded into each well of a 6-well plate (Corning, Corning, USA). One day later, 3µg of each hairpin construct and 1µg of FoxP2-V5 were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) as described in the manufacturers protocol. 48h post transfection total protein was extracted and analysed by western blot.

2.7 Generation of Lentivirus

Recombinant lentivius was generated as described in (Lois et al., 2002) with the following specifications and modifications. HEK293-T cells (kindly provided by D. Vanhecke, MPI

Molecular Genetics, Berlin, Germany) were used for transfection of viral constructs and titration of virus. Four cell culture plates (10cm diameter CELL+ from Sarstedt, Nümbrecht, Germany) each containing $8x10^6$ cells with 12ml HEK293-T medium, were transfected with 40 µg viral transfer vector, 20 µg envelop vectore pVsVg and 30 µg packaging vector λ8.9 using 225,2 µl Lipofectamine 2000 (Invitrogen, Carlsbad, USA). For transfection cells were kept in antibiotic-free cell culture medium. Approximately 4-6 hours post transfection, the culture medium was changed.

Collection of virus

Lentiviral particles were collected and concentrated 36h-48h post transfection. The culture supernatant was cleared by centrifugation at 500xg for 4min (RT) and then filtered through a 45µm pore size ZAP CAP filter (Schleicher & Schuell, Dassel, Germany), that was prewetted with culture medium. Next 2 ultrazentrifugation tubes were cleaned with 70% EtOH and subsequently rinsed with culture medium to remove traces of alcohol. Next, the virus containing medium was transferred to the ultracentrifugation tubes and virus was concentrated by ultracentrifugation at 25.000 rpm in a Beckmann Coulter Optima L-80 (Krefeld, Germany) with rotor SW32 for 90min at 4°C. After the centrifugation run, the supernatant was carefully removed, without disturbing the pellet. Tubes were inversed and placed on Kim wipes for 10min to remove remaining medium. Then 20µl of Hanks' Balanced Salt Solution (Invitrogen, Carlsbad, USA) was added to each tube. Virus redissolved overnight at 4°C. Finally, virus solutions were aliquoted into 2µl aliquots in Eppendorf tubes, shock frozen in liquid nitrogen and stored at -80°C.

Titration of the virus by infection of HEK293-T cells

The virus titer was determined by infection of $4x10^5$ HEK293-T cells, seeded 12hours prior to titration per well of a coated 6-well plate (CELL+, Sarstedt) with various dilutions of virus. For infection, 1µl of undiluted, 1:10, 1:100 or 1:1000 diluted virus solution was added directly to the culture medium containing antibiotics. Infection was quantified after 72h by flow cytometry with a FACScalibur (Beckton Dickinson, Heidelberg, Germany). All virus constructs generated in this study encode the green fluorescent protein (GFP), thus the 530nm channel of the FACS was used to determine the number of infected cells. Usually the percentage of green cells in the 1:10 and 1:100 dilutions were used to calculate the titer. The percentage of GFP positive cells was divided by the total number of cells

present in the dish before infection (here $4x10^5$) and multiplied with the dilution factor. Titers of virus solution were usually in the range of $1\text{-}3x10^6/\mu l$.

2.8 Surgery and Stereotactic Injection of Virus

Birds were anaesthetized with Xylazine/Ketamine. After that, animals were head-fixed in a Bechnmark stereotactic apparatus from MyNeurolab (St. Louis, USA). Animals were injected bilaterally. The stereotactic coordinates for targeting Area X were

medial/lateral:	1.4 / 1.6
anterior/posterior:	3.6 / 4.0
dorsal/ventral:	3.8 / 4.0

Injection was carried out with a micromanipulator from Narishige (Tokyo, Japan). Per injection site, 2 slow turns on the mechanic wheel of the micromanipulator were carried out during a time period of 2min. Given all 8 injection sites per hemisphere, the total amount of lentiviral solution injected in one hemisphere corresponded roughly to 0.8µl liquid.

2.9 Behavioral Paradigm and Song Analysis

The general procedure for studying the behavioral consequence of locally reduced FoxP2 levels in Area X was as follows. Young birds from around post hatch day (PHD) 7-14 were sexed to identify the males. All adult males, including the father, were removed from the cage at latest by PHD20 to achieve vocal isolation before the onset of the sensory learning phase. On PHD23 lentiviral injections into the brains of male zebra finches were performed. After 12-18h of recovery from the microsurgery, animals were brought back to their home cages. On PHD30 training of the birds with an adult male as tutor started. Tutors and young animals were kept together in sound-isolated recording boxes. From day 45 on, the tutors were removed from the pupils every 3-4 days, from 9am to 2pm. During that time the vocalization of the pupil was continuously monitored using Sound Analysis Pro [SAP+ (Tchernichovski et al., 2001)]. By day PHD91 or later animals were perfused and their brains dissected for further analysis.

2.10 Histology

Animals were perfused with 4% paraformaldehyde (in 0.1M PB). Brains were taken out and postfixed overnight in 4% paraformaldehyde. Then the brains were cut either sagitally of frontal with a vibratome (Leica, Wetzlar, Germany) at a thickness of 50µm. Brain slices were stored in 0.1M PB at 4°C in the dark.

2.11 Immunohistochemistry

Brain slices were permeabilized with 0.2% Triton X in PBS for 1h. After that they were blocked with 4%BSA in PBS for 1h and then incubated with the first antibody diluted in PBS (for dilutions of antibodies see Table 2.12.5). Next, the slices where washed 3 times with 0.5M PB followed by incubation with the corresponding fluorescently labeled secondary antibody. After another triple wash, the slices were mounted on slides using MOWIOL mounting medium (Calbiochem, San Diego, USA).

2.12 Microscopy

All brain slice preparations were analysed with a Leica DMRE2 fluorescence microscope equipped with band pass filters for red, green and blue fluorescence and a Hamamatsu (Shizuoka, Japan) charge-coupled-device (CCD) camera. Image acquisition and analysis was carried out with the software SimplePCI (Compix, Cranberry Township, USA)

Table 2.12.1 List of primers

Primer name	Sequence (5´to 3´)	Annealing
sexing-for	CTCCCAAGGATGAGAAACTG	55°C
sexing-rev	TCTGCATCGCTAAATCCTTT	55°C
FoxP2-SH1-f	GACACCAGCTCTGAAGTAAGCACA	55°C
FoxP2-SH1-r	GGTAGTCGAGGAGGAATTGTTAGT	55°C
FoxP2-SH2-f	ATGATGCAGGAATCTGCGACA	55°C
FoxP2-SH2-r	TCATTCCAGATCTTCAGATAAAG	55°C
FoxP1-SH1-f	GARTTYTAYAARAAYGCNGANGT	55°C
FoxP1-SH1-r	ATTRTGNCGNACNGCRTTYTTCC	55°C
FoxP2-BamH1-Kozak	CGCGGATCCGCCACCATGATGCAGGAATCTGCGACAG	55°C
FoxP2-EcoRI-V5	GCGGAATTCCGTTCCAGATCTTCAGATAAAG	55°C
Seq pFUGW-f	GGTACAGTGCAGGGGAAAGA	55°C
Seq pFUGW-r	GTCCTGATCCTTCCGCCC	55°C
M13-f	GTAAAACGACGGCCAG	55°C
Gapd-SH2-for	CAAATCGGCCGAGCTCTTTT	60°C
Gapd-SH2-rev	TACCGCTTCGGGATGTTCCT	60°C
Vim-SH2-for	CTGCGGGAGAAGTTGCAAGA	60°C
Vim-SH2-rev	GACGTGCCAGAGAGGCATTG	60°C
Hmbs-SH2-for	GCAGCATGTTGGCATCACAG	60°C
Hmbs-SH2-rev	TGCTTTGCTCCCTTGCTCAG	60°C
Hprt-SH2-for	TGGCTTTGAAGTGCCAGACA	60°C
Hprt-SH2-rev	TCTGCTTCCCCGTCTCACTG	60°C
Pfkp-SH2-for	GGGAATACGGAGACGCAACC	60°C
Pfkp-SH2-rev	CAGCTTCAGCCACCACTGCT	60°C
Pgk1-SH2-for	GCGTCGTCATGAGGGTTGAC	60°C
Pgk1-SH2-rev	CCCCATGGTCCAAGCAGTG	60°C
actin-SH2-for	CGAGCGCAAGTACTCCGTGT	60°C
actin-SH2-rev	GCCGGACTCGTCGTACTCCT	60°C
FoxP2-SH2-for	CCTGGCTGTGAAAGCGTTTG	60°C
FoxP2-SH2-rev	ATTTGCACCCGACACTGAGC	60°C
GFP-SH2-for	GGAGCGCACCATCTTCTTCA	60°C
GFP-SH2-rev	TGAAGTCGATGCCCTTCAGC	60°C

Table 2.12.2 Short hairpin target sequences

shRNA	target sequence in FoxP2	Offset*
shFoxP2-a	AAGCAGTTATGTTGCAGCAGC	100
shFoxP2-b	AAGCTGGCTTAAGTCCTGCTG [1]	424
shFoxP2-c	AACATGGAGGGCTAGACCTCA	505
shFoxP2-d	AATGTGGGAGCCATTCGAAGA [2]	1062
shFoxP2-e	AAGTCCTGCTGAGATTCAGCA [3]	434
shFoxP2-f	AACAGGAAGCCCAACGTTAGT [5]	1415
shFoxP2-g	AAGGCGAGACAGCTCGTCACA [6]	629
shFoxP2-h	AACGCGAACGTCTTCAAGCAA [4,7]	844
shFoxP2-I	AAGTGACTGGAGTTCACAGTA	466
shControl-a	no target sequence	- - - - -
shGFP-a	AAGCAAGCTGACCCTGAAGTTCA	N/D

* This is the distance from start ATG of Isoform **IV** in bp.

[1] Homology to chick EST (accession BU344097), that is homologous to FoxP2

[2] Homology to chick EST´s (accessions BU365655 and BU206574), that are homologous to FoxP2

[3] Homology to chick EST (accession BU352559), that is homologous to FoxP2

[4] The target lies in the Leucine Zipper, but the sequence is really very different from FoxP1 and it is unlikely, that any other FoxP proteins has a Leucine Zipper more similar to that of FoxP2 than FoxP1.

[5] Slight homology (bp1-14) to a chick EST that is **not** homologous to FoxP2

[6] Homology to chick EST (accession BU352559), that is homologous to FoxP2

[7] Slight homology to chick EST (bp7-21; accession BU323516) that has no homology to any human gene.

Table 2.12.3 ssDNA sequences encoding shRNA

Name	Sequence (5′ to 3′, target sequence in FoxP2 shown in purple)
shFoxP2-a-L1	TTTGCAGTTATGTTGCAGCAGCgtgaagccacagatgGCTGCTGCAACATAACTGCTTTTT
shFoxP2-a-L2	GTCAATACAACGTCGTCGcacttcggtgtctacCGACGACGTTGTATTGACGAAAAAGC
shFoxP2-b-L1	TTTGCTGGCTTAAGTCCTGCTGgtgaagccacagatgCAGCAGGACTTAAGCCAGCTTTTT
shFoxP2-b-L2	GACCGAATTCAGGACGACcacttcggtgtctacGTCGTCCTGAATTCGGTCGAAAAAGC
shFoxP2-c-L1	TTTGCATGGAGGGCTAGACCTCAgtgaagccacagatgTGAGGTCTAGCCCTCCATGTTTTT
shFoxP2-c-L2	GTACCTCCCGATCTGGAGTcacttcggtgtctacACTCCAGATCGGGAGGTACAAAAAGC
shFoxP2-d-L1	TTTGTGTGGGAGCCATTCGAAGAgtgaagccacagatgTCTTCGAATGGCTCCCACATTTTT
shFoxP2-d-L2	ACACCCTCGGTAAGCTTCTcacttcggtgtctacAGAAGCTTACCGAGGGTGTAAAAAGC
shFoxP2-e-L1	TTTGTCCTGCTGAGATTCAGCAgtgaagccacagatgTGCTGAATCTCAGCAGGACTTTTT
shFoxP2-e-L2	AGGACGACTCTAAGTCGTcacttcggtgtctacACGACTTAGAGTCGTCCTGAAAAAGC
shFoxP2-f-L1	TTTGCAGGAAGCCCAACGTTAGTgtgaagccacagatgACTAACGTTGGGCTTCCTGTTTTT
shFoxP2-f-L2	GTCCTTCGGGTTGCAATCAcacttcggtgtctacTGATTGCAACCCGAAGGACAAAAAGC
shFoxP2-g-L1	TTTGGCGAGACAGCTCGTCACAgtgaagccacagatgTGTGACGAGCTGTCTCGCCTTTTT
shFoxP2-g-L2	CGCTCTGTCGAGCAGTGTcacttcggtgtctacACACTGCTCGACAGAGCGGAAAAAGC
shFoxP2-h-L1	TTTGCGCGAACGTCTTCAAGCAAgtgaagccacagatgTTGCTTGAAGACGTTCGCGTTTTT
shFoxP2-h-L2	GCGCTTGCAGAAGTTCGTTcacttcggtgtctacAACGAACTTCTGCAAGCGCAAAAAGC
shFoxP2-i-L1	TTTGTGACTGGAGTTCACAGTAgtgaagccacagatgTACTGTGAACTCCAGTCACTTTTT
shFoxP2-i-L2	ACTGACCTCAAGTGTCATcacttcggtgtctacATGACACTTGAGGTCAGTGAAAAAGC
shControl-L1	TTTGTTCTCCGAACGTGTCACGTgtgaagccacagatgACGTGACACGTTCGGAGAATTTTT
shControl-L2	AAGAGGCTTGCACAGTGCAcacttcggtgtctacTGCACTGTGCAAGCCTCTTAAAAAGC
shGFP-a-L1	TTTGCAAGCTGACCCTGAAGTTCAgtgaagccacagatgTGAACTTCAGGGTCAGCTTGCTTTTT
shGFP-a-L2	GTTCGACTGGGACTTCAAGTcacttcggtgtctacACTTGAAGTCCCAGTCGAACGAAAAAGC

Table 2.12.4 Summary of plasmids

Name	Description	Antibiotic resistance
pcDNA-FoxP2V5	mammalian expression vector, based on pCDNA4/V5-His B (Invitrogen). This vector expressed zebra finch FoxP2, tagged with the V5 epitope driven by the CMV promoter	Ampicillin
pBudΔU6-shFoxP2-a	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-a	Zeocin
pBudΔU6-shFoxP2-b	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-b	Zeocin
pBudΔU6-shFoxP2-c	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-c	Zeocin
pBudΔU6-shFoxP2-d	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-d	Zeocin
pBudΔU6-shFoxP2-e	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-e	Zeocin
pBudΔU6-shFoxP2-f	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-f	Zeocin
pBudΔU6-shFoxP2-g	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-g	Zeocin
pBudΔU6-shFoxP2-h	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-h	Zeocin
pBudΔU6-shFoxP2-i	Short hairpin expression construct based on pBudCE4.1 from Invitrogen expressing hairpin shFoxP2-I	Zeocin
pFUGW_linker	viral transfer vector based on pFUGW (Lois et al., 2002). This vector contains a ubiquitin C promoter-driven GFP cassette and the human U6 promoter for expression of short hairpin RNA´s	Ampicillin
pFUGW-shControl	viral transfer vector pFUGW_linker expressing shControl	Ampicillin
pFUGW-shGFP	viral transfer vector pFUGW_linker expressing shGFP	Ampicillin
pFUGW-shFoxP2-f	viral transfer vector pFUGW_linker expressing shFoxP2-f	Ampicillin
pFUGW-shFoxP2-h	viral transfer vector pFUGW_linker expressing shFoxP2-h	Ampicillin
pVsVg	envelope vector expressing the vesicular stomatitis virus glycoprotein (VSVg)	Ampicillin
λ8.9	HIV-1 packaging vector, identical to pCMVdeltaR9 (Naldini et al., 1996)	Ampicillin

Table 2.12.5 Antibodies

Antigen	Manufacturer or reference	Dilution Western blot	Dilution Immunocytochemistry
FoxP2	abcam (Cambridge, UK)	1:300	
FoxP2	(Lu et al., 2002)	1:500	1:500
Actin	Chemicon, (Temecula, USA)	1:500	
V5	Invitrogen (Carlsbad, USA)	1:500	
Parvalbumin	Swant (Bellinzona, Switzerland)		1:500
Calbindin	Swant		1:250
NOS	Zymed, San Francisco,		1:500
ChAT	Chemicon, (Temecula, USA)		1:500
DARPP-32	Santa Cruz Biotechnology (Santa Cruz, USA)		1:500
HU	Molecular Probes (Eugene, USA)		1:1000
TH	Santa Cruz Biotechnology		1: 400
PSA-NCAM	AbCys (Paris, France)		1:250
anti-FITC	Chemicon, (Temecula, USA)		1:300
red Alexa 2nd	Molecular Probes (Eugene, USA)		1:300
green Alexa 2nd	Molecular Probes		1:300