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CNTNAP2 is a direct FoxP2 target *in vitro* and *in vivo* in zebra finches: complex regulation by age and activity

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Mutations of *FOXP2* are associated with altered brain structure, including the striatal part of the basal ganglia, and cause a severe speech and language disorder. Songbirds serve as a tractable neurobiological model for speech and language research. Experimental downregulation of FoxP2 in zebra finch Area X, a nucleus of the striatal song control circuitry, affects synaptic transmission and spine densities. It also renders song learning and production inaccurate and imprecise, similar to the speech impairment of patients carrying *FOXP2* mutations. Here we show that experimental downregulation of *FoxP2* in Area X using lentiviral vectors leads to reduced expression of *CNTNAP2*, a FOXP2 target gene in humans. In addition, natural downregulation of *FoxP2* by age or by singing also downregulated *CNTNAP2* expression. Furthermore, we report that FoxP2 binds to and activates the avian *CNTNAP2* promoter *in vitro*. Taken together these data establish *CNTNAP2* as a direct FoxP2 target gene in songbirds, likely affecting synaptic function relevant for song learning and song maintenance.

Keywords: CNTNAP2, EMSA, FoxP2, gene expression, luciferase assay, QPCR, songbird, spiny neurons, striatum, vocal learning, zebra finch

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Mutations of the transcription factor *FOXP2* cause Developmental Verbal Dyspraxia (DVD), a severe speech and language disorder (Lai *et al.* 2001). Patients with *FOXP2* mutations have profound deficits in productive and perceptive aspects of speech and language, and perform particularly poorly in word repetition tasks (Watkins *et al.* 2002). Interestingly, learning of other motor tasks is less (Alcock *et al.* 2000) or not affected (Watkins *et al.* 2002). The disorder is inherited in a monogenic, autosomal dominant way. This makes it possible to study the causality between *FOXP2* mutations and DVD and provides an entry point to start addressing the molecular underpinnings of human language.

Toward this end, over 800 candidate direct Foxp2 target genes have been identified in large scale screens in mice and humans (Mukamel *et al.* 2011; Roll *et al.* 2010; Spiteri *et al.* 2007; Vernes *et al.* 2007, 2008, 2011). Among them is contactin-associated protein-like 2 (*CNTNAP2*), a member of the neurexin protein family (Suedhof 2008; Vernes *et al.* 2008). *CNTNAP2* is implicated in diverse human disorders such as autism, schizophrenia, cortical dysplasia focal epilepsy (CDFE), specific language impairment (SLI), epilepsy, Gilles de la Tourette syndrome and attention deficit hyperactivity disorder (ADHD; Rodenas-Cuadrado *et al.* 2014), some of which affect language (e.g. autism, Tourette and CDFE). How CNTNAP2 function relates to these pathologies is not yet well understood. CNTNAP2 is relevant for the clustering of voltage gated potassium channels at the juxtaparanodes of myelinated axons (Girault *et al.* 2003; Horresh *et al.* 2008; Poliak *et al.* 1999), but also plays important roles in neuronal migration, network formation, and the growth and maintenance of dendritic spines (Anderson *et al.* 2012; Gdalyahu *et al.* 2015; Penagarikano *et al.* 2011; Rodenas-Cuadrado *et al.* 2014). Knockdown of *Cntnap2* in primary neuron cultures leads to a decrease in dendritic branching, spine head width and synaptic strength (Anderson *et al.* 2012). Loss of function in mice (*Cntnap2–/–*) results in fewer striatal interneurons and a lower spine density in cortical layer 5b neurons compared to litter-mate controls (Gdalyahu *et al.* 2015; Penagarikano *et al.* 2011). The decrease in spine density occurs because existing spines are eliminated, not because newly generated spines fail to be stabilized (Gdalyahu *et al.* 2015). Studying *CNTNAP2* as a potential target of FoxP2 in postembryonic and adult songbirds holds the promise to illuminate which of the above functions are relevant for song learning and song maintenance.

Songbirds have shed light on the importance of striatal FoxP2 expression for learned vocalizations (Adam *et al.* 2016; Haesler *et al.* 2007; Heston & White 2015; Teramitsu & White 2006; Thompson *et al.* 2013; Wohlgemuth *et al.* 2014). Experimental alteration of FoxP2 levels in the striatal song nucleus Area X of zebra finches leads to decreased spine density and impairs the learning and production of song (Haesler *et al.* 2007; Heston & White 2015; Murugan *et al.* 2013; Schulz *et al.* 2010). We hypothesized that *CNTNAP2* is a direct FoxP2 target gene in songbirds for the following reasons: (1) *CNTNAP2* is a target of FOXP2 in humans (Vernes *et al.* 2008). (2) CNTNAP2 affects spine density in mice (Gdalyahu *et al.* 2015) as does FoxP2 in zebra finches (Schulz *et al.* 2010), raising the possibility that the observed effect of FoxP2 on spines is mediated via CNTNAP2. (3) Expression levels of *CNTNAP2* and *FoxP2* in adult zebra finches are both higher in the striatum than in Area X (Haesler *et al.* 2004; Panaitof *et al.* 2010; Thompson *et al.* 2013). Here we demonstrate

that FoxP2 can bind to and regulate the *CNTNAP2* promoter *in vitro* and that *CNTNAP2* expression is correlated to *FoxP2* expression in Area X of juvenile and adult males. A *FoxP2* knockdown can lead to a correlated *CNTNAP2* down-regulation, suggesting that the striatal regulation of *CNTNAP2* by FoxP2 is the result of a direct interaction.

Methods

Nomenclature

The FoxP2 nomenclature follows the convention proposed in Kaestner *et al.* (2000): FOXP2 in *Homo sapiens*, Foxp2 in *Mus musculus* and FoxP2 in all other species, including zebra finches (*Taeniopygia guttata*). For all genes we use the italicized letters to refer to the gene or cDNA and roman type to refer to the protein.

Subjects

All experiments were performed in accordance with the guidelines of the governmental law (TierSchG). 39 male zebra finches were used in this study. Prior to the experiments, animals were housed in large free flight aviaries under a 12 h:12 h light:dark-cycle with food and water provided *ad libitum*. Birds were sexed on the day of hatching via polymerase chain reaction (PCR) as previously described (Adam *et al.* 2014). At the beginning of the experiments, all birds (except those that underwent surgery) were isolated in sound attenuated recording boxes overnight to monitor morning vocal activity. Birds that did not sing were either overdosed with isoflurane immediately after the lights went on in the morning or 2 h later. Birds in the singing group were allowed to sing undirected song and were overdosed after they sang more than 500 motifs within 2 h after their first motif of the day. The first motif had to be sung within 2 h after the lights went on. Vocalizations were continuously monitored and recorded using the Sound Analysis Pro software SAP2011 (Tchernichovski *et al.* 2000).

Injection of lentiviral vectors

Injections of lentiviral vectors mediating a *FoxP2*-knockdown were performed as described previously (Adam *et al.* 2016; Haesler *et al.* 2007). In brief, at post hatch day 23 (day 23) male zebra finches were injected into Area X with two different lentiviral vectors, in one hemisphere with the vector carrying one of two *FoxP2*-knockdown constructs (shFoxP2-f or shFoxP2-h), in the other hemisphere with a non-silencing control construct (Haesler *et al.* 2007). Injections (approximately 200 nl per site) were placed into eight sites in each hemisphere. Injection side and order as well as the choice of the *FoxP2*-knockdown construct were randomized. After surgery the animals were transferred to their home cages and allowed to grow up normally in the presence of their biological parents and siblings. At day 35 birds were overdosed, the brain was quickly extracted and cut into acute slices. Subsequently, microbiopsies of Area X were taken and stored individually at −80°C.

Electrophoretic mobility shift assay

HEK293T cells were transfected with pcDNA4-FoxP2-V5-HISB (Haesler *et al.* 2007) or empty vector and lysates were affinity purified via the HIS-V5 tag. One microgram of purified FoxP2 protein and 0.8 ng of DIG labeled CNTNAP2 probe (oligo sequence 5′-TATTAT**TATTTATTTTT**GTACTCTACATTCCTTGT**TATTTGA**TACT-3′, FoxP2 binding sites are indicated by bold letters) were incubated in binding buffer (20 mM Hepes KOH (pH 7.6), 30 mM KCl, 1 mM EDTA (pH 8), 0.2% Tween-20, 10 mM $(NH_4)_2SO_4$, 1 mM DTT) for 15 min at room temperature. For the competition assay 200 ng of unlabeled probe were added to the reaction. For the supershift assay, 1 μg protein was preincubated with 0.5 μg anti-V5 antibody prior to the binding reaction. Separation of protein–DNA complexes was carried out on a 4% polyacrylamide Tris/Glycine/EDTA gel.

Luciferase assays

The zebra finch cell line G266 (Itoh & Arnold 2011) was seeded in a 96-well plate at 2×10^4 cells/well and transfected with 30 ng pGL4.13-SV40-Luc or pGL4.13-CNTNAP2-Luc, 30 ng pGL4.75-CMV-Renilla and either 125 ng of pcDNA3.1-FoxP2-Flag (Mendoza *et al.* 2015) or empty vector. Forty-eight hours after transfection, Luciferase and Renilla activity were measured in a plate reader (Tecan, GENios, Männedorf, Switzerland) using the Dual Glo Luciferase Kit (Promega, Fichtburg, Wisconsin, USA). Mean background from wells not transfected with Luciferase or Renilla expressing vectors was subtracted from all other wells. Luciferase activity was calculated as Relative Luciferase Activity (Luciferase RLU/Renilla RLU).

Microbiopsies, RNA-extraction and cDNA synthesis

All RNA samples were generated for a previous study (Adam *et al.* 2016), in which the *FoxP2* expression data were used in conjunction with another target, the reelin receptor VLDLR.

Microbiopsies were taken as previously described (Adam *et al.* 2016; Olias *et al.* 2014). Briefly, brains were frozen at −80°C in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) and cut sagittaly into 200 μm sections on a cryostat. Microbiopsies (1 mm diameter) of Area X were excised and stored individually at −80°C. Remaining sections were stored in 4 % (w/v) paraformaldehyde solution and inspected under the microscope for proper targeting of the biopsy.

In the case of virus-injected animals, Area X from both hemispheres was sampled, in the case of unmanipulated birds, only one hemisphere per bird was sampled. The side was chosen randomly.

Total RNA from properly targeted microbiopsies of birds with a *FoxP2* knockdown was extracted from individual biopsies. For all other animals, properly targeted microbiopsies of one hemisphere were pooled. In all cases total RNA was extracted using the RNAXS kit (Macherey-Nagel, Düren, Germany). Complementary DNA (cDNA) synthesis was carried out using random hexamer primers and 180 ng or 40 ng total RNA for the pooled or individual microbiopsy samples, respectively. Reverse-transcriptase free reactions were included to control for genomic DNA contamination. All cDNAs were diluted with nuclease free water (10-fold for pooled, fivefold for individual microbiopsies).

Quantitative Reverse Transcription PCR

qRT-PCR reactions were run in duplicates in a total reaction volume of 20 μl. Five microliter of diluted cDNA were added to 15 μl reaction mix containing 10 μl 2x KAPA SYBR FAST Universal QPCR Mix (Peqlab, Erlangen, Germany), 10 pmol of each primer (18 pmol in the case of *FoxP2*) and ROX (50 nM, Peqlab) as a reference dye. Reverse-transcriptase free samples as well as no template controls were included to test for DNA-contamination and contamination of reagents, respectively. An inter-run calibrator (IRC) was always included, if samples were run on different plates. The efficiency of each primer pair was checked prior to all measurements using 10-fold dilution series over six orders of magnitude. The efficiency of all primer pairs ranged within $2 \pm 10\%$. We used the following primer pairs: *FoxP2* (5′-CCTGGCTGTGAAA GCGTTTG-3′/5′-ATTTGCACCCGACACTGAGC-3′) (Haesler *et al.* 2007), *HMBS* (5′-GCAGCATGTTGGCATCACAG-3′/5′-TGCTTTGCTCC CTTGCTCAG-3′) (Haesler *et al.* 2007), *GFP* (5′-AGAACGGCATCAAG GTGAAC-3′/5′-TGCTCAGGTAGTGGTTGTCG-3′) (Adam *et al.* 2016) and *CNTNAP2* (5′-GAGGGCAAGGTCAGTGTCCA-3′/5′-GAATCGAAC TTCATGCCACTGC-3′). Reactions were run on a MX3005P system (Agilent, Santa Clara, California, USA) using the following temperature program: 10 min at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 65°C (64°C for *FoxP2* and 60°C for *HMBS*) and a melting curve to check for amplification specificity.

The mean C_t for each sample was derived from the run data and used to calculate relative gene expression for the gene of interest (GOI; *FoxP2* or *CNTNAP2*). We used *HMBS* as a reference gene, as it is the most stable of all tested potential reference genes for our experiments (Adam *et al.* 2016; Haesler *et al.* 2007). We used the

following formula to calculate the relative expression of our GOIs:

$$\text{Rel. Exp.}_{\text{GOI}} = \frac{E_{\text{GOI}}^{-(C_{t,\text{GOI}} - C_{t,\text{IRC,GOI}})}}{E_{\text{REF}}^{-(C_{t,\text{REF}} - C_{t,\text{IRC,REF}})}}$$

Relative expression values were averaged per animal and hemisphere.

Individual biopsies of virus-injected animals were screened for *GFP* expression as a marker for successful infection prior to running all other assays. Only cDNA from *GFP*-positive biopsies were used to measure the expression of *FoxP2*, *CNTNAP2* and *HMBS*. Eighty-four percent of all injected birds expressed GFP in both hemispheres.

Search for transcription factor binding sites in the CNTNAP2 *promoter*

We utilized the *CNTNAP2* promoter sequence that we used in the Luciferase experiment (genomic location: chr2:31 216 312–31 217 544, WashU taeGut3.2.4/taeGut2) to predict transcription factor binding sites by means of three databases – Jaspar (Mathelier *et al.* 2016), Patch (Matys *et al.* 2006) and MatInspector (Cartharius *et al.* 2005) – using the default settings. We merged all results into one table and then retained only matches of transcription factors with a zebra finch homolog according to the current Ensembl annotation (ENS87, Yates *et al.* 2016). Homologs were identified using Biomart (Smedley *et al.* 2015). We further filtered the list of transcription factors potentially binding to the *CNTNAP2* promoter by keeping only transcription factors that are expressed in adult, but not juvenile Area X and vice versa, using a published microarray dataset (Hilliard *et al.* 2012), and our own unpublished transcriptome data. In a last step we removed transcription factors with mRNA expression values that are strongly correlated with those of *FoxP2* (orange Module in Hilliard *et al.* 2012), because we were interested in finding potential regulators of *CNTNAP2* that act either independently of FoxP2 or counteract FoxP2's downregulation of *CNTNAP2*.

Statistical analysis

All statistical tests were performed using the data analysis software R (R Core Team 2013): Paired *t*-tests (Luciferase assays) were conducted using *t*-test(), Mann Whitney *U*-tests (age difference *FoxP2* and *CNTNAP2*) using wilcox.test(),regressions (dependency of *CNTNAP2* expression on *FoxP2* expression) using lm() and Wilcoxon signed rank tests (reduction of *FoxP2* and *CNTNAP2* after knockdown) using wilcox.test(). Plots were generated using the ggplot2 package (2.0.0) (Wickham 2009).

Results

FoxP2 is directly enhancing CNTNAP2 *expression* in vitro

A prerequisite for *CNTNAP2* to be a FoxP2 target gene is that it has FoxP2 binding sites in its regulatory regions and that FoxP2 can regulate *CNTNAP2* expression by binding to these motifs. To investigate this in zebra finches, we screened for FoxP2 binding sites (Nelson *et al.* 2013) in the regulatory regions of the zebra finch *CNTNAP2* gene (ENSTGUG0 0000001794). We identified several FoxP2 binding sites, in particular in the 5′-UTR-region of the gene. To test whether the FoxP2 protein binds to these sites, we conducted electrophoretic mobility shift assays (EMSAs). We designed a 46 bp probe containing three binding sites approximately 350 bp upstream of the start-codon (genomic location: chr2:31 216 574–31 216 619, WashU taeGut3.2.4/taeGut2). Adding FoxP2 protein to the labeled probe resulted in an upwards shift of the band (Fig. 1a, 'ps'), which was abolished by the addition of unlabeled probe ('competitor'). Pre-incubation with an antibody against FoxP2-V5 led to a supershift of the band ('ss'), confirming that FoxP2-protein caused the initial shift.

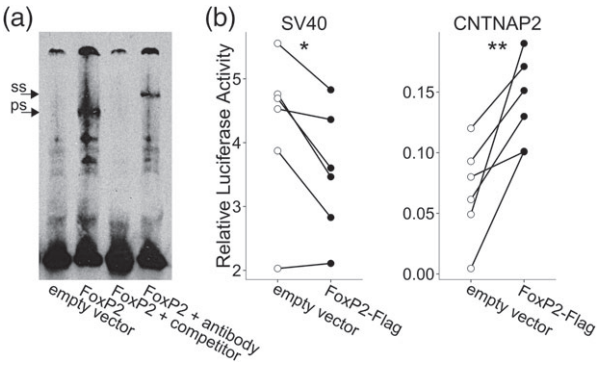

Figure 1: FoxP2 binds to and activates the *CNTNPA2* promoter. (a) Protein lysate from HEK293T cells transfected with pcDNA4-FoxP2-V5-HISB or empty vector was affinity purified and subsequently used in EMSA assays. Presence of FoxP2 in the sample led to an upward protein shift (ps) of the labeled DNA probe (second left lane), which was successfully competed by unlabeled probe (third lane). Preincubation of the purified protein with anti-V5 antibody led to an additional upward shift (supershift, ss) of the labeled probe indicating specific binding of FoxP2 protein to the probe (last lane). (b) Overexpressed FoxP2 protein repressed luciferase transcription in the zebra finch cell line G266 transfected with a pGL4.13 plasmid containing the *SV40* promoter. In contrast, FoxP2 significantly enhanced transcription via the *CNTNAP2* promoter.

We further used luciferase reporter assays to test if binding of FoxP2 to the *CNTNAP2* promoter changed its transcriptional activity. We performed these experiments on a zebra finch cell line (G266) derived from tumor tissue of a male bird (Itoh & Arnold 2011). Choosing the G266 cell line instead of the typically used human cell lines, ensured that not only the promoter and FoxP2-protein were from zebra finches, but also the entire transcriptional machinery. Overexpression of FoxP2 protein in G266 cells reduced the expression of the luciferase reporter gene under the control of the *SV40* promoter (paired *t*-test, $n = 6$, $t = 3.1417$, $\text{df} = 5$, $P = 0.0256$; Fig. 1b, SV40) as it does in HEK293T cells (Adam *et al.* 2016). When we tested the FoxP2-dependent transcription from a 1591 bp region of the *CNTNAP2* promoter including the 5′-UTR we found a significant enhancement of transcription in the presence of FoxP2, but not in the presence of the empty vector control (paired *t*-test, $n = 6$, $t = -4.2861$, $\text{df} = 5$, $P = 0.0078$; Fig. 1b, CNTNAP2). We thus conclude that FoxP2 has the capacity to directly regulate *CNTNAP2* expression in zebra finches.

CNTNAP2 *and* FoxP2 *expression are positively correlated in Area X of juvenile males*

FoxP2 expression is lower in Area X of adult males than in juvenile males (Haesler *et al.* 2004; Thompson *et al.* 2013)

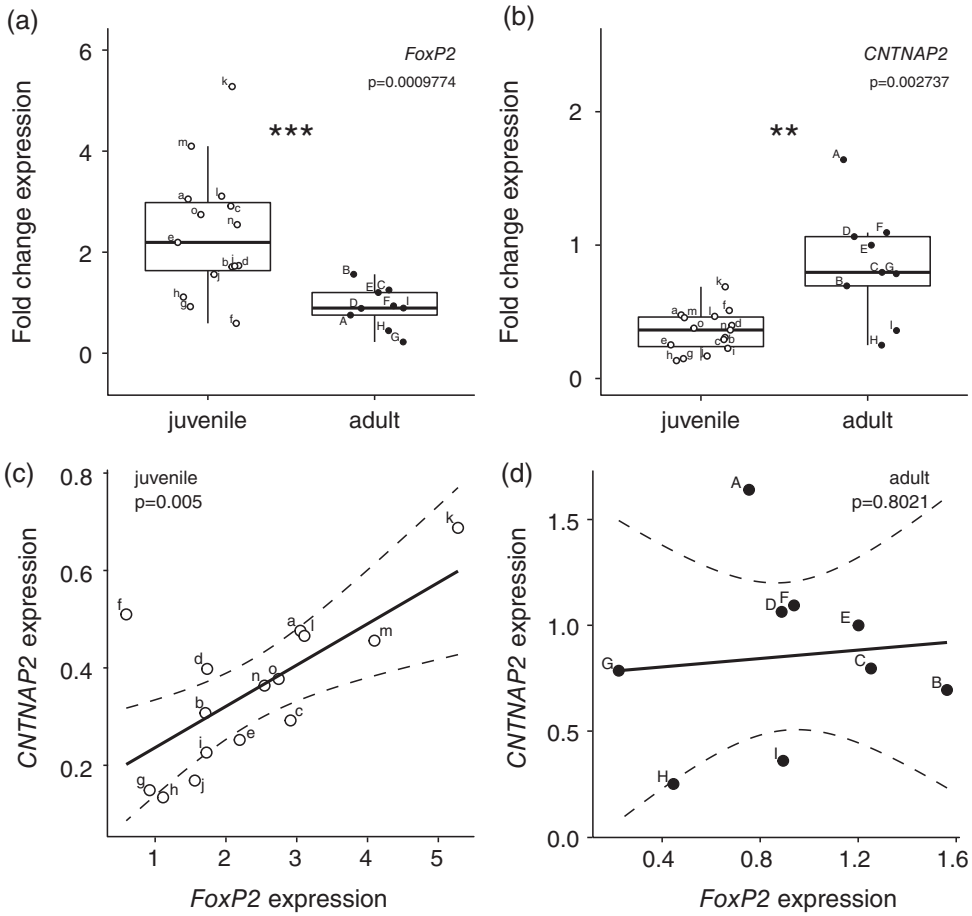

Figure 2: *CNTNAP2* expression is strongly and positively correlated to *FoxP2* expression in Area X of non-singing juveniles. (a) *FoxP2* mRNA expression levels in Area X are significantly higher in juvenile males (50–52 days, mean 50 days, no fill, $n = 15$) than in adult males (>120 days, 123–968 days, mean 297 days, black fill, $n = 9$). Expression levels were measured by qRT-PCR from microbiopsy samples of Area X of juvenile and adult males that did not sing prior to sacrifice. (b) *CNTNAP2* mRNA expression levels are significantly higher in adults than in juveniles. (c) Expression of *CNTNAP2* was significantly and positively correlated with *FoxP2* expression in non-singing juveniles, (d) but not in non-singing adults. Each dot represents one individual; the dashed lines around the fitted line denote the 0.95 confidence interval. Gene expression was normalized to the reference gene *HMBS* and the mean of the adult group was set to 1. Upper case letters denote adult males, lower case letters juvenile males.

and is downregulated by undirected singing in both age groups (Teramitsu & White 2006; Teramitsu *et al.* 2010). If FoxP2 directly regulated *CNTNAP2* in Area X of zebra finches we would expect to see positively correlated changes in *CNTNAP2* mRNA levels. To test our hypothesis, we measured the expression levels of both genes in Area X of juvenile and adult males. To exclude the influence of undirected song on this dataset, we made sure that males did not sing on the day of sacrifice. After taking microbiopsies from Area X we performed qRT-PCR. The results confirmed our previous finding that *FoxP2* levels in Area X are lower in adult birds (>120 days, 123–968 days, mean 297 days, $n = 9$) than in juveniles (50–52 days, mean 50 days, $n = 15$; Mann Whitney *U*-test, $U = 120$, $P = 0.0009774$; Fig. 2a; Haesler *et al.* 2004). The opposite was the case for *CNTNAP2* expression, which was significantly higher in adults compared to juveniles (Mann Whitney *U*-test, $P = 0.002737$; Fig. 2b). This direction of regulation suggested that FoxP2 may be repressing *CNTNAP2* expression in Area X of male zebra finches. If this were the case, we expected to find a negative correlation between the expression levels of *FoxP2* and *CNTNAP2* in individual birds. However, *FoxP2* and *CNTNAP2* levels were strongly positively correlated in juvenile individuals (Linear Model, $R^2 = 0.467$, adjusted $R^2 = 0.4261$, $F_{1,13} = 11.39$, $P = 0.004973$; Fig. 2c). Interestingly, this was not the case in adult birds (Linear Model, $R^2 = 0.009593$, adjusted $R^2 = -0.1319$, $F_{1,7} = 0.0678$, $P = 0.8021$; Fig. 2d). How can we reconcile the finding that *CNTNAP2* expression is enhanced by *FoxP2* in juvenile non-singing males with the fact that levels of *CNTNAP2* expression are not linked to levels of *FoxP2* expression in non-singing adults? Another, unidentified, age-dependent factor might enhance

CNTNAP2 expression in adult Area X and this could cause the significantly higher *CNTNAP2* mRNA levels in adult males in comparison to juvenile males. To address this possibility, we searched *in silico* for transcription factor binding sites on the *CNTNAP2* promoter to narrow down the list of potential transcription factors affecting *CNTNAP2* expression in adult non-singers. We therefore only took transcription factors into account that are expressed in adults but not in juveniles (Hilliard *et al.* 2012, unpublished own data), (see *Methods* for details). We identified 76 transcription factors, which are only expressed in adult Area X and have predicted binding sites in the *CNTNAP2* promoter (Table S1, Supporting information).

CNTNAP2 *and* FoxP2 *expression levels are positively correlated after undirected song*

Undirected song, which leads to a downregulation of FoxP2 expression in adult male zebra finches (Teramitsu & White 2006; Thompson *et al.* 2013) is thought to be associated with enhanced neural plasticity (Brainard & Doupe 2013; Riters *et al.* 2014), which bears resemblance to the juvenile state of song learning. We thus asked whether the singing induced change in *FoxP2* mRNA levels would lead to a correlated downregulation of *CNTNAP2* expression in adult male birds. We hypothesized that the interaction of FoxP2 with *CNTNAP2* might only occur during periods of enhanced plasticity, like song learning or undirected song (Andalman & Fee 2009; Brainard & Doupe 2013; Charlesworth *et al.* 2011; Tumer & Brainard 2007; Woolley *et al.* 2014). To test this, we added an additional group of adult birds that sang more than 500 motifs of undirected song within two hours after the first motif of the day (see *Methods* for more details). Indeed, in contrast to the results in non-singing adults (Fig. 2d) *CNTNAP2* expression was significantly and positively correlated with *FoxP2* expression in singing individuals (Linear Model, $R^2 = 0.3098$, adjusted $R^2 = 0.565$, $F_{1,6} = 10.09$, $P = 0.01916$; Fig. 3).

CNTNAP2 *expression is reduced after* FoxP2 *knockdown*

To causally link our finding that *CNTNAP2* expression is positively correlated with *FoxP2* expression, we experimentally reduced FoxP2 levels in Area X of juvenile males using lentiviral vectors as described previously (Adam *et al.* 2016; Haesler *et al.* 2007; Murugan *et al.* 2013; Schulz *et al.* 2010). We expected to see a downregulation of *CNTNAP2* mRNA levels positively correlated to the decrease of *FoxP2*. Lentiviral vectors mediating a *FoxP2* knockdown were injected into Area X of one hemisphere of juvenile males at day 23, while a control construct was injected into the contralateral hemisphere. After confirming that *FoxP2* was downregulated on the knockdown hemisphere compared to the control hemisphere (Wilcoxon signed rank test, $W = 28$, $P = 0.01563$, $n = 7$; Fig. 4a), we measured the expression of *CNTNAP2* in all hemispheres. *CNTNAP2* levels were reduced in four of seven knockdown hemispheres when compared to the control hemisphere of the same animal (Wilcoxon signed rank test, $W = 18$, $P = 0.1422$; Fig. 4b). Moreover the expression of *CNTNAP2* was positively correlated to the *FoxP2* expression levels in both, the control (Linear Model, $R^2 = 0.6817$, adjusted $R^2 = 0.618$, $F_{1,5} = 10.71$ $P = 0.02215$; Fig. 4c) and the knockdown group (Linear Model, $R^2 = 0.5686$, adjusted $R^2 = 0.4823$, $F_{1,6} = 6.591$ $P = 0.0502$; Fig. 4d) as indicated by the significant positive correlation between the expression of the two genes. We concluded that FoxP2 can indeed regulate *CNTNAP2* expression *in vivo*.

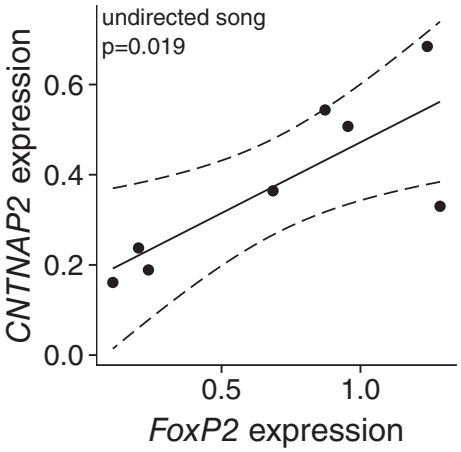

Figure 3: *CNTNAP2* expression is positively correlated with *FoxP2* expression in singing adults. *CNTNAP2* expression in adult males (248–405 days, mean 352 days, $n = 8$) that sang more than 500 motifs of undirected song was strongly and positively correlated with *FoxP2* expression. Each dot represents one individual; dashed lines around the fitted line denote the 0.95 confidence interval.

Discussion

In this study, we investigated whether *CNTNAP2* is a direct FoxP2 target gene in songbirds as reported for humans (Vernes *et al.* 2008). We found that FoxP2 protein directly bound to and regulated the *CNTNAP2* promoter. In non-singing juvenile zebra finches *CNTNAP2* and *FoxP2* expression were positively correlated, which was also the case in adult males when they were singing vigorously. In the same vein, an experimental knockdown of FoxP2 led to lower *CNTNAP2* levels in four of our seven birds, even though this difference did not reach statistical significance. The regulatory relationship between FoxP2 and *CNTNAP2* may contribute to the behavioral and neuroanatomical changes observed after knockdown of FoxP2 in male songbirds, like the previously observed effect on striatal spine density (Schulz *et al.* 2010).

CNTNAP2 *expression in zebra finches can be regulated by FoxP2*

We found *CNTNAP2* expression levels in Area X to be lower in non-singing juveniles than in non-singing adult male zebra finches. While age-related upregulation of *CNTNAP2* was also reported in LMAN of zebra finches (Condro & White 2014; Panaitof *et al.* 2010) and in cultured murine pyramidal

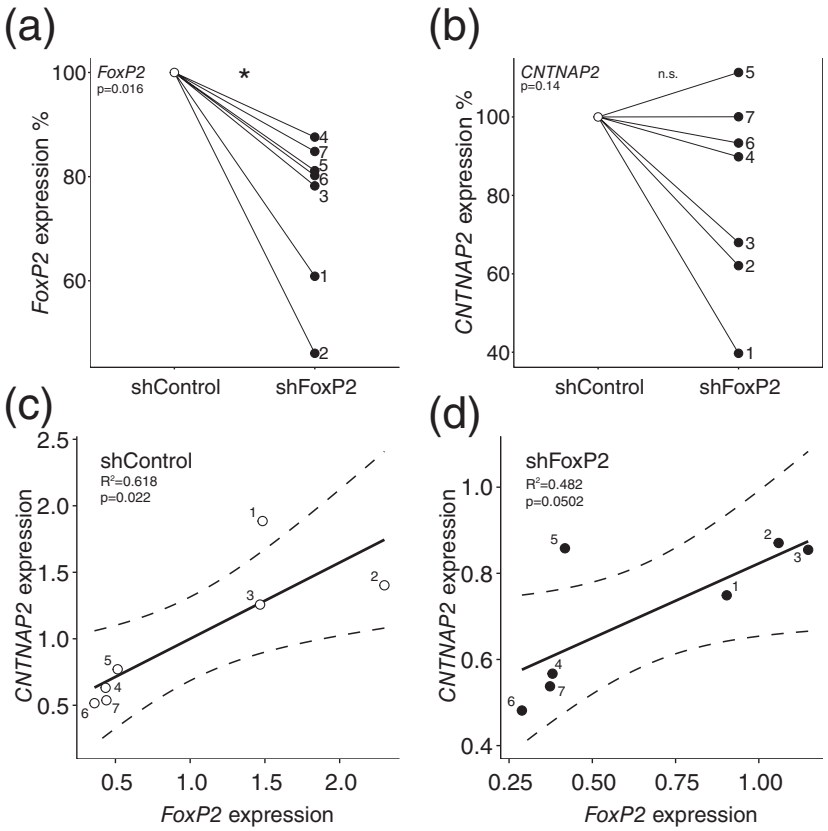

Figure 4: Knockdown of *FoxP2* reduces *CNTNAP2* expression levels. Lentiviral vectors mediating the expression of either a shRNA directed against *FoxP2* or a non-silencing control shRNA were injected into left and right Area X of juvenile (day 23) male zebra finches. Injection order and side were randomized. (a) *FoxP2* expression was significantly decreased on in the knockdown hemisphere (black fill) compared to the control hemisphere (no fill) of the same animal. (b) Expression of *CNTNAP2* was decreased in some animals. (c) *CNTNAP2* expression was significantly and positively correlated with *FoxP2* expression in both, the control and (d) knockdown hemisphere. Each dot represents one individual; the dashed lines around the fitted line denote the 0.95 confidence interval. Numbers denote individual males.

neurons (Varea *et al.* 2015), Panaitof *et al.* (2010) did not find expression differences between juvenile and adult Area X. The discrepancy between our findings and those by Panaitof *et al.* (2010) might be due to the fact that we used qRT-PCR whereas Panaitof *et al.* (2010) performed *in situ* hybridizations, methods with different dynamic ranges. Additionally, Panaitof *et al.* (2010) normalized the *CNTNAP2* expression in Area X to the expression in the surrounding striatum, so that a concomitant upregulation of *CNTNAP2* in the striatum could obscure the upregulation of *CNTNAP2* in Area X.

In our study, *FoxP2* expression was positively correlated with *CNTNAP2* expression in zebra finches. The direction of regulation differs in human neuron-like cell line, where overexpression of FOXP2 leads to downregulation of *CNTNAP2* expression (Vernes *et al.* 2008). Likewise, in the developing human cortex *CNTNAP2* is expressed highest in layers with low *FOXP2* expression and vice versa (Vernes *et al.* 2008). One explanation for these tissue differences could be that transcription factors binding to the same site can repress or enhance a gene depending on the presence of other transcription factors (Diamond *et al.* 1990). Different cofactors exist in different cell lines and tissues, influencing the direction of regulation. Examples for such cofactors could be other FoxP-family members (FoxP1 and FoxP4), Nkx2.1, CtBP1, PIAS1, members of the NuRD chromatin-remodeling complex and members of the FoxO-subfamily, all known to interact with FoxP2 and to modulate its ability to regulate target gene expression (Chokas *et al.* 2010; Estruch *et al.* 2016; Li *et al.* 2004; van Boxtel *et al.* 2013; Zhou *et al.* 2008). Furthermore, all of these cofactors are expressed in Area X of juvenile as well as adult male zebra finches (unpublished own data, Hilliard *et al.* 2012). Especially the FoxO-family members are promising candidates because FoxO3 and FoxP1 are known to bind to the same enhancers. Through this interaction FoxP1 is able to regulate specific FoxO3 target genes (van Boxtel *et al.* 2013). The same kind of interaction might occur between FoxP2 and FoxO4 binding sites, which are very similar to the FoxP1 and FoxO3 binding sites, respectively (Mathelier *et al.* 2016).

Tissue specific differences determining the direction of regulation are also likely since the negative correlation of *FOXP2* and *CNTNAP2* levels in human cortex (Vernes *et al.* 2008) is not mirrored in developing dorsal thalamus and striatum of humans where both genes can be co-expressed strongly (Alarcon *et al.* 2008; Teramitsu *et al.* 2004). Furthermore, in the zebra finch brain *CNTNAP2* and *FoxP2* expression overlap in the striatum, Purkinje cell layer and the optic tectum, whereas in LMAN and nidopallium *CNTNAP2* is expressed but *FoxP2* is not. In general, *CNTNAP2* has a much wider expression pattern in the songbird brain (Condro & White 2014; Panaitof *et al.* 2010) than *FoxP2* (Haesler *et al.* 2004; Teramitsu *et al.* 2004). In areas where both *FoxP2* and *CNTNAP2* are co-expressed, as is the case in Area X, our data show that FoxP2 can positively influence *CNTNAP2* expression (e.g. in juveniles and singing adults); however, FoxP2 expression does not always have this effect, shown by the

absence of a relationship in non-singing adults. In the latter case, FoxP2 might not bind to the *CNTNAP2* promoter or if it does, additional factors prevent its transcriptional regulation of *CNTNAP2*.

In summary, FoxP2 is clearly not the only transcription factor regulating *CNTNAP2* expression. To search for additional ones expressed in Area X we used an *in silico* approach and identified 76 transcription factors with predicted binding sites in the *CNTNAP2* promoter. These transcription factors are good candidates to mediate the age-dependent upregulation of *CNTNAP2* expression. Further experiments are needed to address whether the candidates from this list actually bind to the *CNTNAP2* promoter *in vivo*. The candidate gene with most binding sites in the *CNTNAP2* promoter was SP3, a transcription factor known to repress the SP1 mediated activation of the human D1A receptor gene (Yang *et al.* 2000). Interestingly, *D1A* is regulated by FoxP2 in zebra finches (Murugan *et al.* 2013). Being regulated by FoxP2 and SP3 might thus be common to a subset of FoxP2 target genes.

Taking our findings and published data together, FoxP2 does regulate *CNTNAP2* in Area X of male zebra finches, yet the relationship is more complicated than a simple 'more is more' dependency. We propose that during periods of enhanced plasticity, such as in juvenile males and during undirected singing in adult males, FoxP2 influences *CNTNAP2* expression in a linear manner. However, in the 'default state of the adult motor system' (Brainard & Doupe 2013), when the anterior forebrain pathway is not injecting variability into the motor pathway, the influence of FoxP2 on *CNTNAP2* might be overridden by other factors, e.g. miRNAs or other transcription factors.

References

Adam, I., Scharff, C. & Honarmand, M. (2014) Who is who? Non-invasive methods to individually sex and mark altricial chicks. *J Vis Exp* **87**, e51429.

Adam, I., Mendoza, E., Kobalz, U., Wohlgemuth, S. & Scharff, C. (2016) FoxP2 directly regulates the reelin receptor VLDLR developmentally and by singing. *Mol Cell Neurosci* **74**, 96–105.

Alarcon, M., Abrahams, B.S., Stone, J.L., Duvall, J.A., Perederiy, J.V., Bomar, J.M., Sebat, J., Wigler, M., Martin, C.L., Ledbetter, D.H., Nelson, S.F., Cantor, R.M. & Geschwind, D.H. (2008) Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene. *Am J Hum Genet* **82**, 150–159.

Alcock, K.J., Passingham, R.E., Watkins, K. & Vargha-Khadem, F. (2000) Pitch and timing abilities in inherited speech and language impairment. *Brain Lang* **75**, 34–46.

Andalman, A.S. & Fee, M.S. (2009) A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors. *Proc Natl Acad Sci USA* **106**, 12518–12523.

Anderson, G.R., Galfin, T., Xu, W., Aoto, J., Malenka, R.C. & Suedhof, T.C. (2012) Candidate autism gene screen identifies critical role for cell-adhesion molecule CASPR2 in dendritic arborization and spine development. *Proc Natl Acad Sci USA* **109**, 18120–18125.

van Boxtel, R., Gomez-Puerto, C., Mokry, M., Eijkelenboom, A., van der Vos, K.E., Nieuwenhuis, E.E., Burgering, B.M., Lam, E.W. & Coffer, P.J. (2013) FOXP1 acts through a negative feedback loop to suppress FOXO-induced apoptosis. *Cell Death Differ* **20**, 1219–1229.

Brainard, M.S. & Doupe, A.J. (2013) Translating birdsong: songbirds as a model for basic and applied medical research. *Annu Rev Neurosci* **36**, 489–517.

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M. & Werner, T. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**, 2933–2942.

Charlesworth, J.D., Tumer, E.C., Warren, T.L. & Brainard, M.S. (2011) Learning the microstructure of successful behavior. *Nat Neurosci* **14**, 373–380.

Chokas, A.L., Trivedi, C.M., Lu, M.M., Tucker, P.W., Li, S., Epstein, J.A. & Morrisey, E.E. (2010) Foxp1/2/4-NuRD interactions regulate gene expression and epithelial injury response in the lung via regulation of interleukin-6. *J Biol Chem* **285**, 13304–13313.

Condro, M.C. & White, S.A. (2014) Distribution of language-related Cntnap2 protein in neural circuits critical for vocal learning. *J Comp Neurol* **522**, 169–185.

R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Diamond, M.I., Miner, J.N., Yoshinaga, S.K. & Yamamoto, K.R. (1990) Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**, 1266–1272.

Estruch, S.B., Graham, S.A., Deriziotis, P. & Fisher, S.E. (2016) The language-related transcription factor FOXP2 is post-translationally modified with small ubiquitin-like modifiers. *Sci Rep* **6**, 20911.

Gdalyahu, A., Lazaro, M., Penagarikano, O., Golshani, P., Trachtenberg, J.T. & Geschwind, D.H. (2015) The autism related protein contactin-associated protein-like 2 (CNTNAP2) stabilizes new spines: an in vivo mouse study. *PLoS One* **10**, e0125633.

Girault, J.A., Oguievetskaia, K., Carnaud, M., Denisenko-Nehrbass, N. & Goutebroze, L. (2003) Transmembrane scaffolding proteins in the formation and stability of nodes of Ranvier. *Biol Cell* **95**, 447–452.

Haesler, S., Wada, K., Nshdejan, A., Morrisey, E.E., Lints, T., Jarvis, E.D. & Scharff, C. (2004) FoxP2 expression in avian vocal learners and non-learners. *J Neurosci* **24**, 3164–3175.

Haesler, S., Rochefort, C., Georgi, B., Licznerski, P., Osten, P. & Scharff, C. (2007) Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus Area X. *PLoS Biol* **5**, e321.

Heston, J.B. & White, S.A. (2015) Behavior-linked FoxP2 regulation enables zebra finch vocal learning. *J Neurosci* **35**, 2885–2894.

Hilliard, A.T., Miller, J.E., Fraley, E.R., Horvath, S. & White, S.A. (2012) Molecular microcircuitry underlies functional specification in a basal ganglia circuit dedicated to vocal learning. *Neuron* **73**, 537–552.

Horresh, I., Poliak, S., Grant, S., Bredt, D., Rasband, M.N. & Peles, E. (2008) Multiple molecular interactions determine the clustering of Caspr2 and Kv1 channels in myelinated axons. *J Neurosci* **28**, 14213–14222.

Itoh, Y. & Arnold, A.P. (2011) Zebra finch cell lines from naturally occurring tumors. *In Vitro Cell Dev Biol Anim* **47**, 280–282.

Kaestner, K.H., Knochel, W. & Martinez, D.E. (2000) Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* **14**, 142–146.

Lai, C.S., Fisher, S.E., Hurst, J.A., Vargha-Khadem, F. & Monaco, A.P. (2001) A forkhead-domain gene is mutated in a severe speech and language disorder. *Nature* **413**, 519–523.

Li, S., Weidenfeld, J. & Morrisey, E.E. (2004) Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. *Mol Cell Biol* **24**, 809–822.

Mathelier, A., Fornes, O., Arenillas, D.J., Chen, C.Y., Denay, G., Lee, J., Shi, W., Shyr, C., Tan, G., Worsley-Hunt, R., Zhang, A.W., Parcy, F., Lenhard, B., Sandelin, A. & Wasserman, W.W. (2016) JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res* **44**, D110–D115.

Matys, V., Kel-Margoulis, O.V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., Voss, N., Stegmaier, P., Lewicki-Potapov, B., Saxel, H., Kel, A.E. & Wingender, E. (2006) TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucleic Acids Res* **34**, D108–D110.

Mendoza, E., Tokarev, K., During, D.N., Retamosa, E.C., Weiss, M., Arpenik, N. & Scharff, C. (2015) Differential coexpression of FoxP1, FoxP2, and FoxP4 in the Zebra Finch (*Taeniopygia guttata*) song system. *J Comp Neurol* **523**, 1318–1340.

Mukamel, Z., Konopka, G., Wexler, E., Osborn, G.E., Dong, H., Bergman, M.Y., Levitt, P. & Geschwind, D.H. (2011) Regulation of MET by FOXP2, genes implicated in higher cognitive dysfunction and autism risk. *J Neurosci* **31**, 11437–11442.

Murugan, M., Harward, S., Scharff, C. & Mooney, R. (2013) Diminished FoxP2 levels affect dopaminergic modulation of corticostriatal signaling important to song variability. *Neuron* **80**, 1464–1476.

Nelson, C.S., Fuller, C.K., Fordyce, P.M., Greninger, A.L., Li, H. & DeRisi, J.L. (2013) Microfluidic affinity and ChIP-seq analyses converge on a conserved FOXP2-binding motif in chimp and human, which enables the detection of evolutionarily novel targets. *Nucleic Acids Res* **41**, 5991–6004.

Olias, P., Adam, I., Meyer, A., Scharff, C. & Gruber, A.D. (2014) Reference genes for quantitative gene expression studies in multiple avian species. *PLoS One* **9**, e99678.

Panaitof, S.C., Abrahams, B.S., Dong, H., Geschwind, D.H. & White, S.A. (2010) Language-related Cntnap2 gene is differentially expressed in sexually dimorphic song nuclei essential for vocal learning in songbirds. *J Comp Neurol* **518**, 1995–2018.

Penagarikano, O., Abrahams, B.S., Herman, E.I., Winden, K.D., Gdalyahu, A., Dong, H., Sonnenblick, L.I., Gruver, R., Almajano, J., Bragin, A., Golshani, P., Trachtenberg, J.T., Peles, E. & Geschwind, D.H. (2011) Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell* **147**, 235–246.

Poliak, S., Gollan, L., Martinez, R., Custer, A., Einheber, S., Salzer, J.L., Trimmer, J.S., Shrager, P. & Peles, E. (1999) Caspr2, a new member of the neurexin superfamily, is localized at the juxtaparanodes of myelinated axons and associates with K+ channels. *Neuron* **24**, 1037–1047.

Riters, L.V., Stevenson, S.A., DeVries, M.S. & Cordes, M.A. (2014) Reward associated with singing behavior correlates with opioid-related gene expression in the medial preoptic nucleus in male European starlings. *PLoS One* **9**, e115285.

Rodenas-Cuadrado, P., Ho, J. & Vernes, S.C. (2014) Shining a light on CNTNAP2: complex functions to complex disorders. *Eur J Hum Genet* **22**, 171–178.

Roll, P., Vernes, S.C., Bruneau, N., Cillario, J., Ponsole-Lenfant, M., Massacrier, A., Rudolf, G., Khalife, M., Hirsch, E., Fisher, S.E. & Szepetowski, P. (2010) Molecular networks implicated in speech-related disorders: FOXP2 regulates the SRPX2/uPAR complex. *Hum Mol Genet* **19**, 4848–4860.

Schulz, S.B., Haesler, S., Scharff, C. & Rochefort, C. (2010) Knockdown of FoxP2 alters spine density in Area X of the zebra finch. *Genes Brain Behav* **9**, 732–740.

Smedley, D., Haider, S., Durinck, S. *et al.* (2015) The BioMart community portal: an innovative alternative to large, centralized data repositories. *Nucleic Acids Res* **43**, W589–W598.

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., Vernes, S.C., Fisher, S.E., Ren, B. & Geschwind, D.H. (2007) Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. *Am J Hum Genet* **81**, 1144–1157.

Suedhof, T.C. (2008) Neuroligins and neurexins link synaptic function to cognitive disease. *Nature* **455**, 903–911.

Tchernichovski, O., Nottebohm, F., Ho, C.E., Pesaran, B. & Mitra, P.P. (2000) A procedure for an automated measurement of song similarity. *Anim Behav* **59**, 1167–1176.

Teramitsu, I. & White, S.A. (2006) FoxP2 regulation during undirected singing in adult songbirds. *J Neurosci* **26**, 7390–7394.

Teramitsu, I., Kudo, L.C., London, S.E., Geschwind, D.H. & White, S.A. (2004) Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. *J Neurosci* **24**, 3152–3163.

Teramitsu, I., Poopatanapong, A., Torrisi, S. & White, S.A. (2010) Striatal FoxP2 is actively regulated during songbird sensorimotor learning. *PLoS One* **5**, e8548.

Thompson, C.K., Schwabe, F., Schoof, A., Mendoza, E., Gampe, J., Rochefort, C. & Scharff, C. (2013) Young and intense: FoxP2 immunoreactivity in Area X varies with age, song stereotypy, and singing in male zebra finches. *Front Neural Circuits* **7**, 24.

Tumer, E.C. & Brainard, M.S. (2007) Performance variability enables adaptive plasticity of 'crystallized' adult birdsong. *Nature* **450**, 1240–1244.

Varea, O., Martin-de-Saavedra, M.D., Kopeikina, K.J., Schurmann, B., Fleming, H.J., Fawcett-Patel, J.M., Bach, A., Jang, S., Peles, E., Kim, E. & Penzes, P. (2015) Synaptic abnormalities and cytoplasmic glutamate receptor aggregates in contactin associated protein-like 2/Caspr2 knockout neurons. *Proc Natl Acad Sci USA* **112**, 6176–6181.

Vernes, S.C., Spiteri, E., Nicod, J., Groszer, M., Taylor, J.M., Davies, K.E., Geschwind, D.H. & Fisher, S.E. (2007) High-throughput analysis of promoter occupancy reveals direct neural targets of FOXP2, a gene mutated in speech and language disorders. *Am J Hum Genet* **81**, 1232–1250.

Vernes, S.C., Newbury, D.F., Abrahams, B.S., Winchester, L., Nicod, J., Groszer, M., Alarcon, M., Oliver, P.L., Davies, K.E., Geschwind, D.H., Monaco, A.P. & Fisher, S.E. (2008) A functional genetic link between distinct developmental language disorders. *N Engl J Med* **359**, 2337–2345.

Vernes, S.C., Oliver, P.L., Spiteri, E., Lockstone, H.E., Puliyadi, R., Taylor, J.M., Ho, J., Mombereau, C., Brewer, A., Lowy, E., Nicod, J., Groszer, M., Baban, D., Sahgal, N., Cazier, J.B., Ragoussis, J., Davies, K.E., Geschwind, D.H. & Fisher, S.E. (2011) Foxp2 regulates gene networks implicated in neurite outgrowth in the developing brain. *PLoS Genet* **7**, e1002145.

Watkins, K.E., Dronkers, N.F. & Vargha-Khadem, F. (2002) Behavioural analysis of an inherited speech and language disorder: comparison with acquired aphasia. *Brain* **125**, 452–464.

Wickham, H. (2009) *ggplot2: Elegant Graphics for Data Analysis*. Springer Publishing Company, Incorporated.

Wohlgemuth, S., Adam, I. & Scharff, C. (2014) FoxP2 in songbirds. *Curr Opin Neurobiol* **28**, 86–93.

Woolley, S.C., Rajan, R., Joshua, M. & Doupe, A.J. (2014) Emergence of context-dependent variability across a basal ganglia network. *Neuron* **82**, 208–223.

Yang, Y., Hwang, C.K., Junn, E., Lee, G. & Mouradian, M.M. (2000) ZIC2 and Sp3 repress Sp1-induced activation of the human D1A dopamine receptor gene. *J Biol Chem* **275**, 38863–38869.

Yates, A., Akanni, W., Amode, M.R. *et al.* (2016) Ensembl 2016. *Nucleic Acids Res* **44**, D710–D716.

Zhou, B., Zhong, Q., Minoo, P., Li, C., Ann, D.K., Frenkel, B., Morrisey, E.E., Crandall, E.D. & Borok, Z. (2008) Foxp2 inhibits Nkx2.1-mediated transcription of SP-C via interactions with the Nkx2.1 homeodomain. *Am J Respir Cell Mol Biol* **38**, 750–758.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1 Predicted transcription factor binding sites in the zebra finch CNTNAP2 promoter.