Studies on the evolution and function of FoxP2, a gene implicated in human speech and language, using songbirds as a model

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Sebastian Haesler

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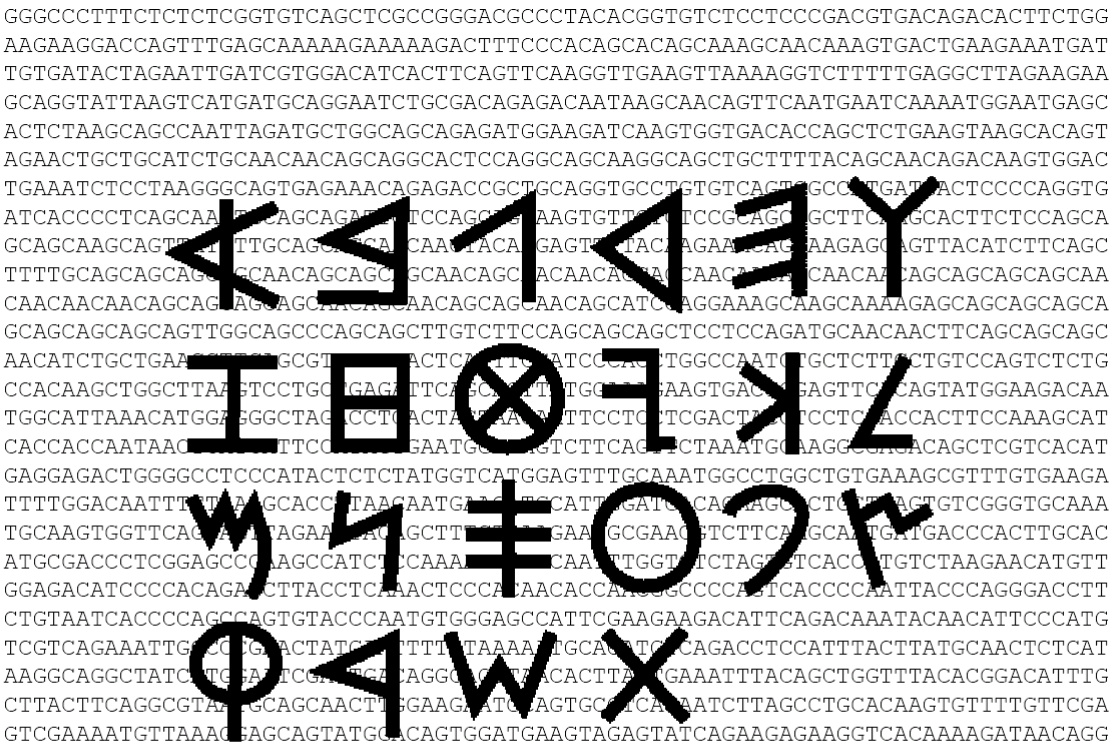
Erstgutachter: Prof. Constance Scharff

Zweitgutachter: Prof. Hans-Joachim Pflüger



Language and Genes can be represented by Letters

The letters of the Phoenician script shown on top of the DNA sequence of the FoxP2 gene from *Taeniopygia guttata* (Zebra finch). The Phoenician script which emanated during the 14th century BC was one of the first truly abstract alphabets. Modern alphabets thought to have descended from Phoenician include Arabic, Greek, Latin (via the Old Italic alphabet), Cyrillic (via the Greek alphabet) and Hebrew (via Aramaic). FoxP2 is the first gene known to be involved in human speech and language.

Contents

1 Introduction

Learning to imitate sounds and the rules of grammar endows humans with the unique ability to communicate infinite meaning with a finite vocal repertoire using language. Although language is learned, a genetic bias towards this learning has already been proposed in Charles Darwin´s "Descent of Man" (Chapter III - Comparison of the Mental Powers of Man and the Lower Animals):

" […] language is an art, like brewing or baking; but writing would have been a better simile. It certainly is not a true instinct, for every language has to be learnt. It differs, however, widely from all ordinary arts, for man has an instinctive tendency to speak, as we see in the babble of our young children; whilst no child has an instinctive tendency to brew, bake, or write."

A modern account of the idea that learning language is not solely based upon experience was put forward by the Linguist Noam Chomsky. He developed the concept of a "universal grammar", which posits the existence of a universal set of rules common to all languages (Chomsky, 1957). This universal grammar shared by all languages suggests that some aspects of how language is learned are determined by intrinsic, genetically defined structural and functional characteristics of the human brain. The first example of a gene possibly contributing to such a genetic predisposition for language was provided by the discovery that disruptions of the FoxP2 gene cause developmental verbal dyspraxia (DVD). Individuals suffering from this speech and language disorder have severe difficulties with articulation and show impaired receptive and cognitive language skills. Although recent theoretical work also puts forward the idea that the universality of certain syntactic rules might just be the by-product of the scale-free network architecture of languages (i Cancho et al., 2005; Nowak et al., 2001), the case of FoxP2 obviously allows to take a closer look on the development and function of neural circuits associated with language from a molecular and cellular perspective.

1.1 FoxP2 and Developmental Verbal Dyspraxia

The causative link between FoxP2 and DVD was established when genomic alterations of FoxP2 were identified in all 16 affected members of the british KE-family and an unrelated

individual with a remarkably similar pathophenotype. Affected KE family members carry a substitution of arginine to histidine (R553H), which most likely renders the protein non-functional (Figure 1.1). This mutation is inherited in a dominant fashion and was found in KE DVD patients across three generations. In the unrelated individual FoxP2 is disrupted by a balanced translocation (Lai et al., 2001). The direct search for FoxP2 mutations in DVD patient panels meanwhile revealed more individuals with a disrupted FoxP2 allele (Feuk et al., 2006; MacDermot et al., 2005).

What is the common behavioral phenotype of individuals with DVD? Affected members of the KE family have severe difficulty in correctly articulating speech. In both word and the non-word repetition tests, where subjects have to repeat words (e.g. *killer*) and non-words (e.g. *rillek*) after hearing them, DVD patients score significantly worse than their unaffected family members (Watkins et al., 2002). The impairment increases gradually with the complexity of the words to be articulated. The DVD family members also have difficulties in the volitional control of skilled non-speech orofacial movements, a symptome called orofacial dyspraxia. Importantly, these difficulties cannot be attributed to a general impairment of motor control, since the patients´ limb praxis performance is indistinguishable from unaffected individuals (Watkins et al., 2002). The patients are also not impaired in their hearing ability. Interestingly, the DVD phenotype resembles that observed in patients with Broca´s aphasia (reviewed in (Damasio and Geschwind, 1984). However, there are important behavioral differences between the two pathologies. Aphasics perform better in the word than the non-word repetition test, whereas affected KE family perform equally bad in both tests. This could indicate that despite their actual problems with articulation, aphasics had learned to associate word articulation patterns with word meanings before the onset of the aphasia, which might help them finding the correct words. In contrast, affected KE family that never learned the correct word articulation patterns would fail in using word meaning to solve the word-repetition task.

In addition to the verbal and orofacial dyspraxia, KE family patients perform significantly worse than their unaffected relatives on tests that assess receptive and grammatical language. The deficit includes the inability to correctly inflect words (i.e. change tense or number) or to match sentences describing subtle relationships between objects with the corresponding pictures. Nevertheless, affected individuals score only slightly, but significantly lower on a non-verbal IQ-test than non-affected individuals and there is

considerable overlap between the groups (Alcock et al., 2000; Vargha-Khadem et al., 1998; Watkins et al., 2002). Taken together, these findings suggest that the primary deficit in the affected KE family members reflects a disruption of the sensorimotor mechanisms mediating the selection, control, and sequencing of learned fine movements of the mouth and face. An open question remains, if the receptive cognitive problems result from the primary articulation problem or if they constitute a second independent core deficit of the disorder. The first possibility would be consistent with the motor theory of speech perception (Liberman and Mattingly, 1985), which posits that decoding speech requires the brain circuitry involved in its production. Although recent human studies support this concept (Fadiga et al., 2002; Watkins et al., 2003), the possibility that aberrations of FoxP2 affect the development of grammatic skills independent of the articulation deficit cannot be ruled out.

First insights into the neural basis of the behavioral abnormalities shown by DVD patients came from the examination of affected and unaffected KE family members with structural and functional brain imaging techniques. Affected KE family members displayed bilateral structural deficits consisting of a reduction in the gray matter density of the caudate nucleus in the basal ganglia (Vargha-Khadem et al., 1998; Watkins et al., 2002) the ventral cerebellum (Belton et al., 2003) and Broca´s area. Abnormally high gray matter density was found in the putamen and Wernicke´s area. Interestingly, the volume of the caudate correlated well with the performance in the test of oral praxis (see above; (Watkins et al., 2002), indicating its involvement in the pathology. Given the well-established role of the basal ganglia in motor planning and sequencing (Graybiel, 1995), the structural abnormalities in the striatal regions of the basal ganglia (caudate and putamen) are generally consistent with an impaired control of orofacial motor function. However, it is less clear how they specifically compromise orofacial movements, without affecting other motor functions.

Functional imaging during the performance in covert (silent) and overt (spoken) tasks revealed lateralized disturbances in language-impaired subjects. In contrast to the typical left-dominant activation pattern involving Broca´s Area that is elicited by a verb generation test in unaffected KE family members, the signal distribution in affected individuals is more bilateral. Extensive bilateralization in the activation pattern was also observed for DVD subjects in the word repetition tasks described above. Consistent with the

morphological findings, an underactivation of Broca´s area and the putamen occurred in the affected family KE members (Liegeois et al., 2003). The observed overactivation of areas normally not involved in language has been interpreted to result from compensatory recruitment of additional brain areas, increased attention or a higher cognitive effort to solve the task. Taken together, the imaging work points to the frontostriatal and frontocerebellar networks as key circuitry affected in impaired KE family members.

1.2 FoxP2 Expression in the Brains of Mice and Men

Mapping the expression of FoxP2 in human and murine brains with *in situ* hybridization and immunohistochemistry has established where mammalian FoxP2 acts (Ferland et al., 2003; Lai et al., 2003; Takahashi et al., 2003). In adulthood, most prominent FoxP2 expression is found in the basal ganglia, in regions of the thalamus that receive input from the basal ganglia, in midbrain visual processing regions and in the inferior olive of the medulla. Further regions expressing high levels of FoxP2 include the cerebellar Purkinje cells, deep cerebellar nuclei, sensory auditory midbrain structures and layer VI neurons of the cerebral cortex (Ferland et al., 2003; Lai et al., 2003). Fetal FoxP2 expression is consistent with the adult expression pattern. In the rodent telencephalon, initial expression of FoxP2 is largely limited to the lateral ganglionic eminence [LGE (Ferland et al., 2003; Takahashi et al., 2003)], the mammalian subpallial germinal zone that gives rise to the striatal projection neurons of the basal ganglia and to the majority of cortical interneurons (Brazel et al., 2003). Within the LGE, FoxP2 is expressed in the subventricular zone and mantle region but not in the proliferative ventricular zone, suggesting that expression is initiated in postmitotic neurons. This interpretation is also compatible with the additional expression site in the non-proliferative cortical plate of the developing cortex (Ferland et al., 2003; Takahashi et al., 2003). Taken together, the FoxP2 expression pattern is consistent with the sites of pathology identified in affected KE family members by brain imaging techniques. However, the question whether the reduction of functional FoxP2 protein affects the function of speech-related neural circuits as a consequence of their improper development, or by means of disturbed neural transmission or both remains unanswered, due to the purely descriptive nature of gene expression mapping.

1.3 Molecular Function of FoxP2

From the molecular perspective, FoxP2 belongs to the large family of winged helix transcription factors that are characterized by a conserved Forkhead box (Fox) DNA-binding domain. The forkhead box binds to distinct sequences in promoter regions of a specific set of target genes, allowing their transcriptional regulation. Fox proteins affect cell fate and differentiation in various tissues, and mutations cause developmental disorders (Lehmann et al., 2003). The common feature in all individuals with speech abnormalities caused by genomic alteration of FoxP2 seems to be a reduction of functional FoxP2 protein by 50%. This haploinsuffiency results from the introduction of a premature stop codon in one patient (MacDermot et al., 2005), the disruption of the gene by a translocation in another patient or a substitution of arginine to histidine (R553H) in the DNA binding domain. All affected members of the KE family in which the speech phenotype was originally described (Lai et al., 2001) carry the R553H mutation. Homology modeling of the FoxP2 forkhead domain structure in conjunction with electrostatic charge calculations predict a net reduction in positive charge on the DNA-binding surface of the R553H mutation, sufficient to disrupt DNA-binding (Banerjee-Basu and Baxevanis, 2004).

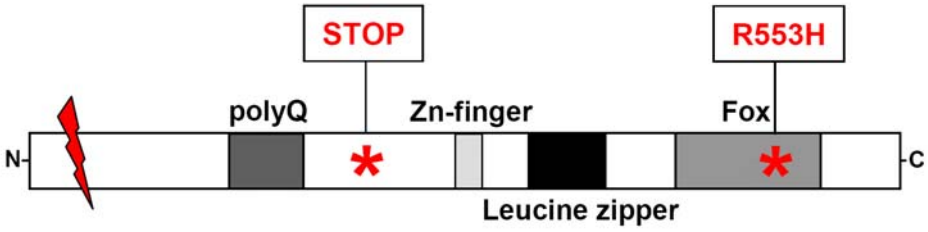


Figure 1.1 Functional domains of the FoxP2 protein. FoxP2 contains a glutamine-repeat region (polyQ), a C2H2 type zinc finger (Zn-finger), a leucine zipper and the forkhead box DNA-binding domain (Fox). All other FoxP family members (FoxP1, FoxP3 and FoxP4) have identical domain architecture with the exception of the polyQ region: in FoxP1, the polyQ stretch is shorter, varies in length among species and lies closer towards the N-terminus of the protein. FoxP3 and FoxP4 do not contain a polyQ region. The positions of the pathogenic alterations of the FoxP2 gene are indicated. In one patient, FoxP2 is disrupted by a balanced translocation (red flash). In another patient, a mutation introduces a stop codon (STOP). In the affected KE family members the mutation of arginine (R) to histidine (H) in position 553 of the amino acis sequence (*) disrupts the DNA-binding capacity of the Forkhead box (R553H).

Murine FoxP2 and the other three members of the FoxP family can act as transcriptional repressors, shown with reporter constructs in different cell lines (Li et al., 2004; Shu et al.,

2001). Thus in patients with FoxP2 mutations reduced levels of functional protein are expected to attenuate transcriptional repression of a specific set of target genes. Their identity is still unknown, in part because the exact DNA sequence to which FoxP2 binds has not been determined experimentally. However the sequence to which FoxP1, the closest homologue of FoxP2, binds is known (Shi et al., 2004; Wang et al., 2003). Interestingly, transcription reporter constructs containing the FoxP1 binding sequence also respond to FoxP2 (Shi et al., 2004), predicting a core motif to which both FoxP2 and FoxP1 can bind. This core motif is very similar to those of the two transcriptional activator families FoxO (Biggs et al., 2001) and FoxC (Saleem et al., 2003). These data suggest that Fox transcription factors are either functionally redundant or require additional protein interactions to specify target gene transcription.

For transcriptional repression to occur FoxP2 needs to dimerize either with itself, with FoxP1 or with FoxP4 (Li et al., 2004). This requirement distinguishes the FoxP family from the other Fox transcription factors. Dimerization depends on a conserved leucine zipper motif (Li et al., 2004). A C2H2 type zinc finger adjacent to the leucine zipper might modulate the specificity of the interaction between FoxP proteins, as reported for FoxP1 (Wang et al., 2003). FoxP1 and FoxP2 but not FoxP4 also interact with the transcriptional co-repressor C-terminal binding protein 1 (CtBP1). CtBP1 binding enhances, but is not essential, for transcriptional repression (Li et al., 2004). A plethora of FoxP2 isoforms including some that lack the forkhead box add further complexity to the system (Bruce and Margolis, 2002).

FoxP2 contains an N-terminal glutamine-repeat that could act as a polar zipper to join other transcription factors bound to separate DNA segments (Perutz et al., 1994), creating a multiprotein transcriptional unit. This hypothesis is consistent with the proximity of a binding site for FoxP1 to a number of other transcription factor binding sites in the *c-fms* promoter, a physiological target of FoxP1 (Shi et al., 2004). Regulation of *c-fms* expression by FoxP1 depends on the polyglutamine-repeat. Interestingly, the only neural sites of *c-fms* expression are the cerebellar Purkinje cells (Murase and Hayashi, 1998), which also strongly express FoxP2 (see below). The presence of a polyglutamine stretch in FoxP2 also prompted the search for pathogenic glutamine repeat extensions implicated in many neurodegenerative disorders (Zoghbi and Orr, 2000). However, the glutamine

region of FoxP2 is neither expanded in the DVD patients studied so far, nor in a set of 142 patients with progressive movement disorders (Bruce and Margolis, 2002).

The molecular factors that regulate FoxP2 expression and the neural target genes of FoxP2 are still unidentified. Analysis of signal transduction pathways relevant for the development of tissues in which FoxP2 is expressed and comparison with molecular interactions of other Fox genes converge on the sonic hedgehog (Shh) pathway as a candidate for interactions with FoxP2. FoxP2 is strongly expressed during lung morphogenesis (Shu et al., 2001), during which FoxA1 and FoxA2 regulate sonic hedgehog (Shh; (Wan et al., 2005). Knockout of FoxP2 (see below) and transgenic overexpression of FoxA2 in mice (Zhou et al., 2001) both disrupt cerebellar morphogenesis which also depends on Shh signaling (Dahmane and Ruiz-i-Altaba, 1999). FoxP2 could also lie downstream of Shh like FoxE1 (Eichberger et al., 2004), FoxM1 (Teh et al., 2002) and FoxF1 (Mahlapuu et al., 2001). In addition, the zinc finger of FoxP2 is highly homologous to those of the major Shh downstream transcriptional effectors Gli1, Gli2 and Gli3 (Shu et al., 2001).

Taken together, dimerization of FoxP proteins and their potential interaction with other transcription factors provide opportunity for complex patterns of target gene repression. In addition, the similarity of the predicted core DNA-motif to which both FoxP1 and FoxP2 bind raises the possibility that they can compensate for each other when co-expressed in the same cells.

1.4 FoxP2 Knockout Mouse

Whereas heart defects in FoxP1 knockout (KO) mice cause embryonic lethality (Wang et al., 2004), mice with disruption of both FoxP2 alleles live for three weeks after birth (Shu et al., 2005). They are developmentally delayed, and are impaired in tests that assay motor function. Heterozygous mice perform only moderately worse than wild-types and catch up by their second week of life. Adult heterozygous FoxP2 knockout mice show no deficits in the Morris water maze, which requires coordinated movement of the limbs and measures spatial learning abilities. Spatial learning depends on the hippocampus, which does not express FoxP2 in mice (Ferland et al., 2003; Lai et al., 2003) and would therefore not be expected to be strongly impaired in FoxP2 knockout mice.

Consistent with the conserved cerebellar FoxP2 expression (Ferland et al., 2003; Lai et al., 2003), FoxP2 knockout mice display cerebellar abnormalities. These include abnormal Bergmann glia and the delayed and incomplete postnatal resolution of the external granular layer, suggesting impaired cell migration. In addition, the molecular layer in heterozygous animals is thinner, the Purkinje cells have underdeveloped dendritic arbors and are misaligned. It is possible that the cerebellum is particularly vulnerable to the absence of FoxP2, because it lacks coexpression of FoxP1 (Tamura et al., 2004). FoxP1 might compensate for the absence of FoxP2 during development in regions that normally express both, e.g. the basal ganglia and the thalamus. The basal ganglia that strongly express FoxP2 and FoxP1 during development do not exhibit gross histological abnormalities in FoxP2 KO mice. Since KE family patients do have structural abnormalities of the basal ganglia (Watkins et al., 2002) it will be interesting to analyze the anatomy and behavioral function of the basal ganglia in FoxP2 KO mice in more detail.

Homozygous FoxP2 knockout pups vocalize less in the sonic range than heterozygous and wild-type animals when separated from their mothers. In the ultrasonic range, both homo- and heterozygous knockout animals utter fewer whistles. Interestingly, the acoustic structure of the vocalizations is preserved in FoxP2 KO pups indicating that the motor areas controlling acoustic features of sound production are intact. Ultrasound communication in adult homozygotes could not be tested because they die too early (Shu et al., 2005). Because FoxP2 is implicated in cellular differentiation of the developing lung, pneumatic function might be compromised in the knockout mice, which could affect vocalizations. In fact, hypoxia strongly decreases the rate of postnatal vocalizations (Blumberg and Alberts, 1991). Given the speech pathophysiology of patients with FoxP2 mutations, it is particularly interesting that vocal behavior in the KO mice is impaired. The recent finding, that adult male mice are capable of vocalizations with a previously unrecognized complexity that shares major characteristics of song (Holy and Guo, 2005), opens the possibility for a more detailed study of vocalizations in FoxP2 knockout mice. In light of the relative ease of genetic manipulation in mice and the large collection of mouse disease models this seems a particularly promising area of future research. However, it is important to bear in mind that whether mouse vocalization, like human speech, is learned has yet to be determined.

1.5 Human Speech and Birdsong

Although language is unique to humans, a few orders like bats (Esser, 1994), cetaceans, e.g. dolphins (Janik, 2000) and three orders of birds (Baptista and Schuchmann, 1990; Hall et al., 1997; Kroodsma and Baylis, 1982) are capable of learning to produce the vocal repertoire required for communicating with their conspecifics. This capacity of auditory-guided, imitative learning has been studied particularly well in the three avian orders: songbirds, parrots and hummingbirds. Collectively, these studies revealed many parallels between human speech and learned birdsong, which are briefly discussed in the following (for review see (Doupe and Kuhl, 1999). Although many of the parallels mentioned below also apply to parrots and hummingbirds, I will refer to songbirds if not stated otherwise to avoid false generalizations.

Birdsong consists of ordered strings of sound, separated by brief silent intervals. The smallest sound unit in the song is the note, that can be defined as a continuous marking on a sound spectrogram. Notes can be grouped together to form syllables. By definition syllables are separated by silent intervals. They can be seen as the basic processing unit of birdsong, as birds interrupted by a light flash or sound while singing still complete the entire syllable (Cynx, 1990). In human speech, syllables are similarly considered to be the phonological building blocks of words. Song syllables are usually assembled to form phrases or motifs, which can be a series of identical or different syllables. Many of the avian song learners sing several motifs in a fixed order. The timing and sequencing of syllables and phrases is not random, but usually follows a set of rules, called syntax. It is important to keep in mind though, that the term syntax in human language refers to the rules of grammar, which allow to create an infinite number of dependencies between words in a sentence. This is not to the case for the syntax of song. Avian vocal learners also do not seem to actively change the syntax of their vocalization to convey a different symbolic content. One exception may be the alarm calls of Black-capped Chickadees, which signal size and threat of a predator by adjusting the frequency of a particular syllable within their mobbing vocalization (Templeton et al., 2005). Although song syllables also lack abstract meaning, it is definitely not meaningless for a bird to vocalize. Birdsong advertises for mating, territorial ownership and fitness and communicates species and individual identity, including “neighbor” and “stranger” (Collins, 2004).

The similarities between birdsong and human speech are evident not only with regard to the acoustic structure of the vocalization and their importance for communication, but also the mechanisms of their perceptual learning. Both song learning and speech acquisition proceeds through several stages. In an initial sensory phase, babies and birds have to build the auditory memory for the sound characteristics of their vocal repertoire. Babies have to memorize the phonetic units and prosodic (pitch and intonation) characteristics that typify the mother tongue, birds store the specific notes, syllables, and prosodic characteristics that typify their species. In the subsequent motor phase, the production of sound is initiated. Babies and birds use the patterns stored in memory to guide motor production through the process of imitation. The motor phase of intensive rehearsing leads to speech in humans and to the adult, crystallized song in songbirds. This adult song is usually very similar to the tutors song. In some songbird species, like the zebra finch, and in humans the sensory and motor phases highly overlap. The ability to learn decreases with age in humans and birds, pointing to the existence of a "critical period" usually before reaching adulthood, where vocal learning is achieved best (Marler, 1970; Vargha-Khadem et al., 1997). The exception of the rule are the so-called "open-learner" species, which continue to modify their song throughout adulthood, e.g. canaries. Another well known example of an open-learner is the budgerigar, a member of the psittaciformes [parrots (Hall et al., 1997)].

For both speech acquisition and song learning auditory input is critical. The absence of exposure to other individuals leads to abnormal vocalizations in humans (Fromkin et al., 1974; Lane, 1976) and in songbirds (Thorpe, 1958). The existence of local dialects (Marler and Tamura, 1964), cross-fostering (Immelmann, 1969) and deafening experiments (Konishi, 1965) have further demonstrated the importance of auditory tutoring in songbirds. Interestingly once vocalizations are learned, both humans and songbirds depend less on hearing their own voice, even though deafness acquired in the adulthood deteriorates speech and song to some degree (Cowie and Douglas-Cowie, 1992; Nordeen and Nordeen, 1993). Another parallel between humans and songbirds exists with respect to the effect of altered or delayed auditory feedback. Experimental manipulation of the auditory feedback negatively influences the stability of the vocalization more severely than the absence of auditory feedback, suggesting that sensory input has some access to the adult vocal system (Howell and Archer, 1984; Leonardo and Konishi, 1999). Finally, since vocal communication is a social behavior, it is maybe not surprising that the social

context is an important component of both song- and speech-learning (Goldstein et al., 2003; Kuhl, 2003).

Parallels between human speech and birdsong not only exist on the behavioral level, but also on the level of the neural circuits mediating these behaviors. The neural pathways for vocal control in both humans and songbirds are hierarchically organized (for an anatomical overview of the songbird brain see Figure 1.2). At the periphery, brainstem and midbrain areas direct the movement of the vocal tract and the respiratory motor neurons (Figure 1.3). Whereas the function of these areas in sound production is not limited to vocally learning animals, higher-level cortical (in humans) and pallial (in birds) control of vocalizations has only been described in animals capable of auditory-guided vocal learning. In the songbird forebrain these telencephalic structures include the nuclei HVC and RA, which form the initial part of the motor pathway (Figure 1.3). HVC initiates a "central motor program" for the song (Vu et al., 1994). It projects to RA, which then connects to all the nuclei involved with vocal motor and respiratory control (Wild, 1997). HVC generates sequences of sparse bursts during song apparently encoding the temporal structure of the syllables (Hahnloser et al., 2002). Interestingly, the sparse bursting patterns are sometimes recapitulated during sleep (Hahnloser et al., 2006). This is reminiscent of earlier findings, that timing and structure of activity elicited by song playback during sleep matches the activity during daytime singing. (Dave and Margoliash, 2000). The songbird forebrain motor pathway has to be intact in order to produce normal song. Lesions in any of the two nuclei HVC and RA disrupt song production at all stages of life of the animal (Nottebohm et al., 1976).

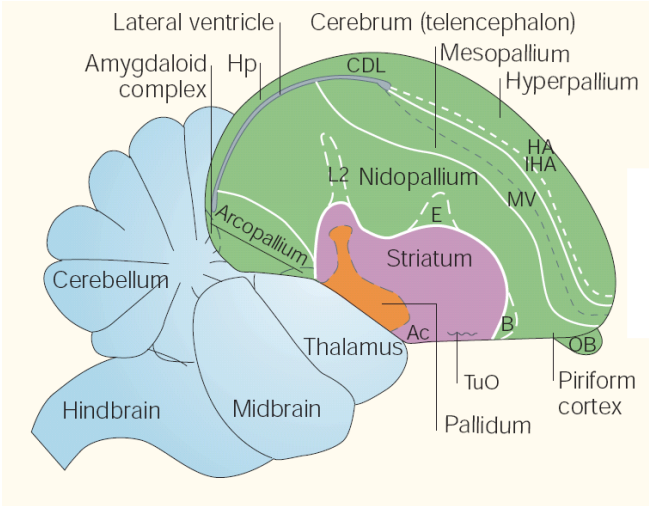


Figure 1.2 **Side view of the avian brain.** Solid white lines are lamina (cell-sparse zones separating brain subdivisions). Dashed grey lines divide regions that differ by cell density or cell size; dashed white lines separate primary sensory neuron populations from adjacent regions. Abbreviations: Ac, accumbens; CDL, dorsal lateral corticoid area; E, entopallium; B, basorostralis; HA, hyperpallium apicale; Hp, hippocampus; IHA, interstitial hyperpallium apicale; L2, field L2; MV, mesopallium ventrale; OB, olfactory bulb; TuO, olfactory tubercle (Figure from Jarvis et al., 2005).

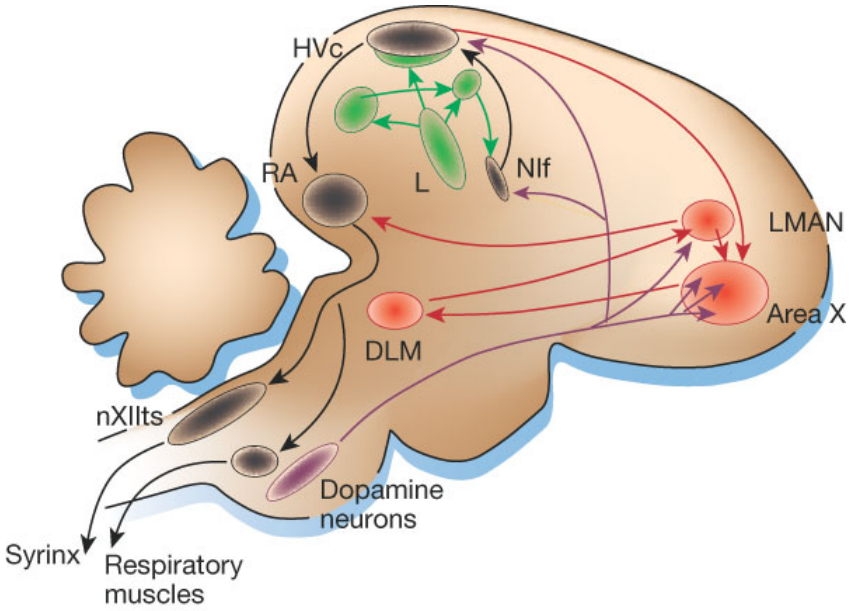


Figure 1.3 **The song system of the zebra finch.** The motor pathway (black) is necessary for normal song production throughout life, and includes HVC (abbreviation used as proper name) and the robust nucleus of the archistriatum (RA). RA projects to the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), which controls the bird's vocal organ or syrinx, and to nuclei involved in control of respiration during song. Additional nuclei afferent to HVC, including the nucleus interfacialis (NIf), are likely to be part of the motor pathway, but their role is less clear. HVC sends a second projection to the anterior forebrain pathway (AFP, red). The AFP includes Area X, which is homologous to mammalian basal ganglia, the medial nucleus of the

dorsolateral thalamus (DLM), and the lateral magnocellular nucleus of the anterior neostriatum (LMAN; a frontal cortex-like nucleus). LMAN sends a projection back into to the motor pathway at the level of RA. Like basal ganglia in other vertebrates, Area X is the target of strong midbrain dopamine projections; LMAN, HVC and NIf also receive dopamine inputs (purple). The Field L complex is the avian primary forebrain auditory area and projects to a complex network of higher auditory areas (green), including the caudomedial neostriatum and caudal portion of the ventral hyperstriatum (not labelled). Auditory inputs likely enter the song system at the level of NIf and possibly HVC (Figure from Brainard and Doupe, 2002)

The higher complexity of the human neocortex and the layered, columnar organization make it more difficult to pinpoint the higher motor areas for speech in the human brain. According to the traditional view, Broca´s area in the posterior frontal inferior cortex is responsible for the production of speech, as patients with lesions in this part of the brain show expressive aphasia. However, investigation of brain activity with non-invasive techniques, like positron emission tomography and (PET), functional magnetic resonance imaging (fMRI), electro- and magnetoencelephalography (EEG, MEG) have revealed additional cortical areas active during speech generation i.e. the motor cortex, supplementary motor areas and the anterior cingulate. This suggests that there is not one single area for speech, but rather a parallel distribution of brain processes subserving different language functions in the brain (Ojemann, 1991).

On the subcortical level, three structures are involved with motor control of vocal output in humans: the cerebellum, the basal ganglia and the thalamus. These structures are generally implicated in the initiation of volitional movements and their modification on a minute-to-minute basis. The cerebellum is important for motor learning, the basal ganglia are critical for the ability to establish habits, procedures and stereotyped behaviors (for reviews see Boyden et al., 2004 and Packard and Knowlton, 2002). Cortical motor areas project to the basal ganglia and to the cerebellum. Both structures project back to the cortex, through the thalamus, building a cortico-basal-ganglia and a cortico-cerebellar loop, respectively. Whereas the output of the cerebellum is mainly excitatory, the output from the basal ganglia is mainly inhibitory. The balance between the two systems allows smooth coordinated movements and disturbances cause movements disorders. More specifically for speech and language, lesions in the cerebellum have been associated with articulatory deficits and slowed speech tempo (Ackermann et al., 1992), lesions in the caudate nucleus of the basal ganglia impair articulation and prosody, but interestingly also language comprehension (Damasio et al., 1982). Some evidence further indicates that parts of the

basal ganglia are activated when optimal performance in processing or production of speech is not (yet) achieved. A PET study revealed that dopamine requirement in the left striatum was negatively correlated with the accuracy and speed of phonological processing (Tettamanti et al., 2005). The left putamen has also been shown to be active when humans are generating words in a second language, but not when performing the same task in their native tongue (Klein et al., 1994). In another fMRI-study of bilingual brains, the left caudate has been identified to play a role in monitoring and controlling the language in use (Crinion et al., 2006).

Whereas the connectivity of the cerebellum within the song circuitry and its role for song production has yet to be established, the importance of the basal ganglia network for learned vocalizations in songbirds is well documented. The pallial nucleus HVC projects to the striatal nucleus Area X. Area X in turn projects to the thalamic nucleus DLM (medial nucleus of the dorsolateral thalamus). The pallial-basal-ganglia loop closes through a projection from DLM to the pallial nucleus lMAN (lateral magnocellular nucleus of the anterior nidopallium) which is connected with the motor pathway at the level of RA (Figure 1.3). The pallial-basal-ganglia loop of songbirds has been termed anterior forebrain pathway (AFP). In contrast to the songbird motor pathway, lesioning lMAN or Area X in most adult birds has no immediate consequence on song production. However, in Bengalese finches, a species in which adult animals rely more on auditory feedback (Okanoya and Yamaguchi, 1997) Area X lesions cause song deficits (Kobayashi et al., 2001). This suggests a role of the AFP in adult song maintenance. A common feature of AFP lesions in all songbirds is that song does not develop properly when they are performed in young birds (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Electrical stimulation of lMAN can direct real-time changes in vocal output in young zebra finches (Brainard, 2004), which might suggest that the AFP corrects vocal output, whenever there is a mismatch between song heard and the song to be produced during phases of song learning or adult song plasticity. However the finding that firing patterns of neurons in lMAN are insensitive to abnormal auditory feedback, has rather promoted the idea that the AFP "injects" variability in the motor output during phases of learning (Leonardo, 2004). This variability is required to reinforce learning of the correct syllables by selecting for the appropriate vocal output. Consistent with this, pharmacological inactivation of lMAN in juvenile zebra finches reduces variability of syllable acoustic features and song syntax, which together with the fact that spiking patterns recorded in lMAN are highly variable

across song renditions, indicates that the AFP may initiate vocal experimentation (Olveczky et al., 2005).

The task of learning song in birds or speech in humans requires auditory input for two reasons. First, it is used for building an internal representation of the tutor song or speech, respectively. Second, auditory input is required to monitor self-produced vocalizations. Recognition of spoken language involves hierarchically organized cortical structures, that perform the acoustic-phonetic, phonological and lexical-semantical processing of language. The main areas involved in speech perception are the primary auditory cortex and additional auditory association areas, in particular the superior temporal gyri, including Wernicke´s area. The analogous structure to the human primary auditory cortex in the songbird brain is the field L, which is connected to a number of reciprocally connected secondary auditory nuclei, some of which process auditory information to HVC. The major source of auditory input to HVC is the nucleus interfacialis (NIf) afferent to HVC (Coleman and Mooney, 2004). Since NIf also shows premotor activity, it has been considered to be part of the motor pathway (McCasland, 1987). But bilateral lesions of NIf do not affect song production, demonstrating that at least in adult zebra finches, an intact NIf is dispensable for motor output (Cardin et al., 2005).

Another characteristic shared by human and songbird brains is the functional lateralization of the neural circuits for learned vocalization, although some important differences exist between the two systems. The avian syrinx is a bilateral vocal organ capable of producing two independent sounds from each syringeal half (Goller and Suthers, 1996). Each side of the syrinx receives input from one hemispheres via ipsilateral connections. In humans, contralateral projections from the two hemispheres converge on a single sound source. As is the case for speech processing in humans, there seems to be a bias towards the left hemisphere in song production in songbirds. Cutting the left tracheosyringeal nerve to the syringeal musculature in canaries results in more severe disruption of song, than when cutting the equivalent nerve on the right side (Nottebohm, 1970). Similarly, unilateral left HVC lesions are more detrimental to song than right HVC lesions (Nottebohm et al., 1976). Interestingly, in zebra finches, the right song system is dominant (Floody and Arnold, 1997).

Taken together, the many parallels between birdsong and humans speech on the functional, behavioral and neural circuit level emphasize the suitability and relevance of songbirds for the study of the basic principles of learned vocalizations, including speech. The songbird model, might also offer insights into the pathology of human speech abnormalities, like DVD. The striking overall consistency between FoxP2 expression pattern in the mammalian brain and the site of pathology in DVD patients, with areas involved with song learning draws the attention to a possible role of FoxP2 in the song system.

1.6 Genes for Vocal Learning ?

In both songbirds and humans, vocal learning is not solely dependant on acoustic cues, but there is evidence for innate mechanisms that govern aspects of how learning proceeds. Birds can discriminate between homo- and heterospecific (from another species) song very early in life (Dooling and Searcy, 1980; Nelson and Marler, 1993). They also show an initial innate predisposition for species-typical signals (Marler and Peters, 1977) and song learning in birds tutored with song from alien species, usually takes longer and is less accurate and less complete, than when tutored with conspecific song (Marler and Peters, 1977). Even in total absence of auditory input due to deafening, a few songbirds still produce some of the normal syntactical rules of the species song (Konishi, 1985). Crossing of two canaries strains with two distinct vocal repertoires yields offspring that develops a mixed repertoire when presented with songs from both repertoire types. Purebred birds tutored accordingly learned songs of their own genetic type (Mundinger, 1995). These data point to a genetic influence on the syllable catalog available to the canaries, suggesting the existence of innate mechanisms that restrict the vocal repertoire prior to any sensory input.

The situation in humans is less clear, in part because the “classical” bird experiments - deprivation of auditory input or tutoring with alien species song - cannot be carried out for obvious reasons. Nevertheless, there is evidence for an inborn perceptual bias for language. Humans can discriminate very early in life between different phonetic units (Kuhl, 1987). Babies born deaf start babbling normally, but their vocalization rapidly becomes distinguishable from hearing babies. Another subject of investigation has been the “spontaneous” development of sign language among deaf people from different cultures. The gestures of naturally evolving sign languages are assembled according to

rules that follow the general rules of human grammar (Goldin-Meadow and Mylander, 1998; Sandler et al., 2005; Senghas et al., 2004). Deaf babies who are exposed to sign language also babble using their hands (Petitto and Marentette, 1991). Moreover, simple "pidgin" languages can develop from a crude mixture of different languages into discrete, more complex languages in relatively short time, as children improve the grammar within every generation without external instruction (Pinker, 1994). If these examples are indicative of an innate predisposition specific to language or just reflect the generalized human capacity to learn to segment and group complex sensory inputs is still a matter of discussion (Fitch et al., 2005; Pinker and Jackendoff, 2005). Given that both aspects can be regarded as two sides of the same coin, this discussion however appears to be of rather semantic nature.

The innateness of certain aspects of learned vocalizations point to a genetically encoded neural circuitry that can later be shaped by perceptual learning in a species-specific way. Following this concept, it is important to point out that even though only a certain predisposition is genetically determined, the behavioral outcome of vocal learning is influenced by the action of genes at all levels - from building the brain, establishing the appropriate connections to their adjustment by experience. The genes involved in these different steps during the dynamic process of vocal learning are largely unknown. In songbirds, a few candidate genes have been identified, based on either their striking expression patterns in song nuclei or a known involvement in learning and memory in mammals (Scharff and White, 2004). Among those genes are IGFII, which is strongly expressed in the Area X-projecting neurons in HVC (Holzenberger et al., 1997), a gene for a yet-to-be-identified antigen which is expressed almost exclusively in RA (Akutagawa and Konishi, 2001) and α-synuclein, which is best known for its role in human Parkinson´s and Alzheimer disease, but is also differentially regulated during song learning (George et al., 1995). In addition, the immediate-early genes *c-Fos,* ZENK and Arc, are responsive to neural activity, and have provided much insight into the different activation patterns involved in song behavior (Jarvis et al., 1998; Kimpo and Doupe, 1997). Many glutamate receptor subtypes also show differential expression in songbird vocal nuclei (Wada et al., 2004) and haven been linked to forms of synaptic plasticity underlying learning and memory.

None of the above mentioned bird genes has directly been shown to be essential for vocal learning, in part because of the difficulty manipulating genes in avians (see below). Similarly, none of these genes have been shown to be specifically implicated in vocal learning, for some it is known they are not. In humans, while a number of genes have been found to impair cognitive abilities when disrupted (Ropers and Hamel, 2005), FoxP2 is the only gene known to be both essential and relatively specific for speech and language.

1.7 Analysis of Gene Function by Genetic Manipulation in Songbirds

The identification of FoxP2 as the cause of DVD, begs the question whether the parallels between human speech and birdsong also exist at the genetic level. In view of the FoxP2 haploinsufficiency in patients with DVD one could imagine generating homo- an heterozygous knockout-birds and subsequently assaying their capacity for song learning and -production. However, to date, no genetic modification of an avian vocal learner has been reported, mostly because of technical difficulties in the development of efficient methods for genetic modification of birds (Zajchowski and Etches, 2000). Recent success in the generation of transgenic chicken (McGrew et al., 2004) and quails (Scott and Lois, 2005) by use of lentiviral vectors have brought transgenic songbirds into close reach. Nevertheless, with these approaches it would still not be possible to target specific genes by homologous recombination, such that a gene can be “knocked out” or replaced with an expression reporter (“knock-in”). Another problem exists with respect to the temporal and spatial control of the genetic manipulation. To date no songbird promoters have been characterized, and although some already described mouse or chicken promoters might be of use, they would require intensive testing to confirm correct gene expression.

One method to circumvent both problems is to inject a lentiviral vector that induces RNA interference (RNAi) into defined brains areas at a defined time. RNAi is a mechanism of posttranscriptional gene silencing through sequence specific degradation of mRNA (Figure 1.4). In mammalians and chicken (Pekarik et al., 2003) it can be induced by double-stranded RNA of 21-23 nucleotide length (short interfering RNA or siRNA) that direct ribonucleases to homologous mRNA targets, thus leading to their cleavage (reviewed in Dykxhoorn et al., 2003). The triggering agent of RNAi, the siRNA, can also be expressed from vectors using promoters, originally derived from mammalian small nuclear RNA genes. These promoters are particularly suitable for the expression of small RNA´s

because they have a very precise transcription initiation start and recruit polymerase III (polIII) for transcription. In contrast to the more common polymerase II, which transcribes most mRNA´s, polIII does not add poly-A tails to the RNA transcripts. The "double-strandedness" of the expressed siRNA is achieved by designing the expression construct to encode the sense siRNA sequence, followed by a loop sequence and the antisense siRNA sequence, such that the linear transcript folds back to build a hairpin structure. Hairpin siRNA is also referred to as short hairpin RNA (shRNA). These hairpin structures have been shown to induce RNAi efficiently *in vitro* and *in vivo* (Krichevsky and Kosik, 2002; Rubinson et al., 2003). For the delivery of the shRNA, lentiviruses have become one of the most powerful genetic tools available. They readily infect non-dividing cells, escape transgene silencing effectively, usually integrate into transcriptionally active regions of the host genome and do not elicit an immune response in the host (Lois et al., 2002). The injection of a lentivirus encoding shRNA into brain regions of interest has been used successfully to alter neural gene expression and behavior in mice (Hovatta et al., 2005; Rumpel et al., 2005).

Figure 1.4 Theoretical model of RNAi induction by shRNA. Short hairpin RNA is processed by a protein complex including the RNAse III nuclease DICER. This generates double stranded RNA (dsRNA) molecules which are structurally similar to siRNA. The dsRNA mediates the recognition of the homologous mRNA target by the RNA-induced silencing complex (RISC). Argonaute, the catalytic component of the RISC then degrades the target mRNA by endonucleolytic cleaveage (after Dykxhoorn and Lieberman, 2005).

Given the rich knowledge about the neurobiology of birdsong, it seems desirable to adapt methods for the functional analysis of genes contributing to learning and production of song. A suitable songbird for establishing the above described method is the zebra finch, because is readily breeds in captivity and has already been studied extensively. The stereotypy of the zebra finch song as well as the availability of appropriate software for automated recording and quantitative analysis ease the investigation of song learning behavior with and without genetic manipulation (Tchernichovski et al., 2001).

1.8 The emergence of Vocal Learning and the Molecular Evolution of FoxP2

Why vocal learning evolved in some avian species and not others, is a matter of debate. What compensates for the cost of learning the vocal repertoire over using an innate, genetically encoded vocalization system? An advantage of learned song could be that unlike strict genetic transmission, socially transmitted behaviors can spread very quickly through a population (Freeberg, 2000). By creating mating boundaries in relative short time, vocal learning could thus have also increased speciation (Lachlan and Servedio, 2004). The developmental stress hypothesis relates the emergence of vocal learning in birds to its predictability of fitness. The quality of song learning success during juvenile life constitutes an honest trait which females can assess when choosing a mating partner (Nowicki and Searcy, 2004). Learned songs might also be used to identify individuals that are adapted to a particular habitat or social environment (Baker et al., 1981). Related to this, vocal learning could also have developed to maintain individual-specific bonds within changing social groupings. In cooperatively breeding birds, learned "calls" can function in a kin-recognition system, such that only the subset of kin within the population with whom the altruistic animal had direct association benefits from the cooperative behavior (Sharp et al., 2005). Learned songs could also be used to maximize outbreeding, by identifying the most distantly related mating partner. At least in Darwin's finches this does not seem to be the case (Millington and Price, 1984). Given the multitude of different vocally learning bird species, that differ in their ecological environment , their social structures etc. it seems unlikely, that a single, exclusive cause exists, to explain the emergence of avian vocal learning. It might have rather developed for many of the above described reasons, each contributing to varying degree, depending on the species studied.

Given the apparent selective advantage of the open-ended expressive power of modern language, it is surprising why it did <u>not</u> evolve in our closest relatives, the great apes, However, it is still a matter of debate, which selective advantage gave rise to the emergence of language. Suggestions have ranged from enhanced communication of information (Pinker and Bloom, 1990) to improved organization of internal thought (Dennett, 1995), sexual selection (Miller, 2000) and increased social cohesion (Dunbar, 2003). It is also not clear what came first - a means for the fine articulation of the vocal tract, a prerequisite for speech, or a means for combining individual communicative elements and coordinating them with meaning, a prerequisite for language. Alternatively

the two co-evolved (Liberman and Whalen, 2000). The origins of human language date to ~6 million years ago (MYA) and proficient language first appeared between 30,000 and 200,000 years ago in the species *Homo sapiens* concomitant with or subsequent to the emergence of anatomically modern humans (Klein, 1989; Wall and Przeworski, 2000). The invention of modern human language probably coincided with the explosive expansion of modern humans around the globe. The dynamic of this invention process was most likely gradual and involved morphological remodeling on different levels (MacWhinney, 2002). The evolution of bipedalism 7-10 MYA freed the hands, maybe allowing increased use of gestures. This could also have promoted the restructuring of the vocal tract, with the descent of the larynx as a result. The lower position of the larynx produces a larger pharyngeal cavity that is useful in making a wide variety of vowel sounds. On the level of the brain, a two to three-fold increase in size in the period between 2 MYA and 100,000 years ago probably increased cognitive abilities dramatically (MacWhinney, 2002). If he faculty of language inside these bigger brains emerged by a gradual extension of pre-existing communication schemes or by "high-jacking" already adapted systems like spatial or numerical reasoning remains unresolved (Fisher and Marcus, 2006; Hauser et al., 2002).

Since the disruption of FoxP2 impairs human speech, this gene might have constituted a genetic constraint during language evolution in humans. But, vertebrate species ranging from mice to chimpanzees also carry a FoxP2 gene in their genomes and all of these FoxP2 genes show an extraordinarily high degree of sequence conservation. This rather speaks for a general importance of FoxP2 for vertebrate fitness. However, the involvement of FoxP2 in speech and language is clearly unique to humans. This apparent discontinuity led to an analysis of the differences in the exact protein and genomic sequence of FoxP2 across mammalian species. A comparison of synonymous mutations (i.e. base substitutions that do not alter the amino acid sequence) and non-synonymous mutations (i.e. base substitutions that alter the amino acid sequence) in the FoxP2 sequences of mice, great apes and humans revealed that the gene must have been under selection pressure during recent human evolution (Clark et al., 2003; Enard et al., 2002). After divergence from the great apes, two non-synonymous but no synonymous substitutions occurred. However, one of the two previously presumed human-specific amino acids exists also in non-human carnivores (Zhang et al., 2002). The functional significance of the amino acid that remains unique to humans is unclear as it lies in an uncharacterized protein domain.

The pattern of FoxP2 sequence variation among humans further suggest that the human-specific allele was fixed in the population as a result of positive selection rather than relaxation of negative selection. Fixation is assumed to have occurred within the last 200,0000 years during which proficient language also appeared (see above). Taken together these findings indicate that FoxP2 might have been pivotal for the development of human language.

It is not known, which aspect of language might have been influenced through the evolution of the human unique FoxP2 allele, but the answer to this question is certainly intimately connected to FoxP2 function. If the FoxP2 allele proved to be necessary for the development of generative grammar, then also the selective sweep on FoxP2 should have been unique to human evolution. However, given the pathophenotype of affected KE family members, it rather seems plausible that the evolution of FoxP2 in humans improved their ability to learn and execute sequenced, orofacial motor behaviors. In this case, selection on FoxP2 might also have occurred in other species, particularly those capable of auditory-guided vocal imitation. The parallels between the neural circuits associated with the pathology of affected KE family subjects and the neural circuits involved in song learning in songbirds (Jarvis, 2004), emphasize the possibility that FoxP2 was under selection during the evolution of vocal learning in birds too. It has been proposed that vocal learning was gained three times independently in three distantly related groups of birds (hummingbirds, parrots, songbirds) during the evolution of the avian family, as a parallel loss of vocal learning from the last common ancestor in the 4 remaining groups of birds is considered rather unlikely (Sibley and Ahlquist, 1990). If this assumption is correct, evolution might also have left its mark on the FoxP2 sequences from songbirds, hummingbirds and parrots, as was the case for human FoxP2

1.9 Aims of this Study

It is well established, that the basic principles of acquired vocalization, including human speech can be studied in songbirds. Thus songbirds might also prove useful as a model for human speech pathologies, like DVD which is caused by mutations in the gene FoxP2. The overall consistency between the FoxP2 expression pattern in the mammalian brain, the site of pathology in DVD patients, and areas involved with song learning draws the attention to a possible role of FoxP2 in the song system. Therefore in the first part of this

study, I identify and characterize the zebra finch FoxP2 gene. Because of the functional interaction of the FoxP2 protein with its closest homolog the FoxP1 protein, the FoxP1 gene from the zebra finch is also cloned and sequenced. FoxP1 and FoxP2 expression patterns in the brains of songbirds are compared to the expression patterns in mammals. Is FoxP2 expressed in any of the well-characterized song nuclei of the zebra finch? Are FoxP2 expressing areas analogous to those involved in the human DVD pathophysiology? The strong conservation of the FoxP2 gene among vertebrates, most of which are not capable of auditory-guided vocal imitation leads to the question if there is something special to FoxP2 function in vocally learning species. Hence, FoxP2 brain expression is compared between birds that learn to vocalize, like the zebra finch and those that do not need to learn their vocalizations, like pigeons or doves. Taken together these experiments aim to answer the question if FoxP2 expression in the songbird brain is consistent with an involvement in learning and/or production of song.

To test if FoxP2 has a direct influence on song learning, I use a lentiviral expression system that induces gene knockdown by RNAi in the zebra finch brain *in vivo*. Stereotactic injection of pseudoviruses into defined brain areas of young zebra finches delivers expression constructs encoding shRNA in a temporally and spatially confined manner. Expressed shRNA target FoxP2 mRNA by RNAi, resulting in reduced FoxP2 levels in the brain. This is in analogy to the FoxP2 haploinsufficiency observed in KE family members. It is important to mention that the post-hatch genetic manipulation of FoxP2 allows to study FoxP2 function in the neural circuits for learning, isolated from its involvement in the development of the brain. All genetically manipulated animals are tutored and their songs recorded. Using software for the quantitative analysis of song the consequence of FoxP2 knockdown on song learning success was evaluated.

Analysis of the molecular evolution of human FoxP2 has revealed that the gene contains changes in amino-acid coding and a pattern of nucleotide polymorphisms which suggest it has been the target of selection during recent human evolution. This might indicate that FoxP2 was pivotal for the development of human language. If so, FoxP2 could also have been critical to the evolution of vocal learning in birds. To address the question of whether the FoxP2 gene has evolved differently in birds that learn their song from those whose song is not learned, I compare the FoxP2 sequences from avian vocal learners, non-learners and the evolutionary closest non-avian relative, the crocodile. The FoxP2 genes

from *Taeniopygia guttata* (zebra finch), *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) are cloned and sequenced. The resulting DNA and amino acid sequences are analyzed for their phylogenetic relationship to test if a particular variant of FoxP2 segregates with the ability for vocal learning.

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

3 Results

3.1 Expression Pattern of FoxP2 and FoxP1

3.1.1 Cloning of the zebra finch FoxP2 and FoxP1 genes

Initially, an 845bp fragment of zebra finch FoxP2 was amplified from adult male zebra finch brain cDNA using primers designed on the basis of the mouse FoxP2 (mFoxP2) sequence. With subsequent 5´ and 3´ RACE (Rapid Amplification of cDNA Ends), we assembled 2830bp of FoxP2 mRNA that included 296 bp of the 5´ untranslated region (UTR), the entire ORF of 2207 bp, and 327 bp of the 3´ UTR (GenBank accession numbers AY549148, AY549149, AY549150, and AY549151). To further confirm the FoxP2 sequence, we sequenced 12 independent clones carrying the entire ORF amplified from adult male zebra finch brain cDNA. We found that two DNA segments, called splice1 (71bp) and splice2 (60bp), were either present or absent in these clones, suggesting the existence of four FoxP2 mRNA isoforms, each different at the 5´ end of the gene (Figure 3.1). Splice1 introduces a stop codon at position 261 (relative to the first start codon), resulting in predicted protein isoforms III or IV that miss the first 92 amino acids. In human but not mouse, the splice1 fragment also exists (Bruce and Margolis, 2002). Splice2 introduces 20 additional amino acids in-frame into the predicted protein isoforms I and III. When the splice2 fragment is absent, it results in isoforms II and IV. In human and mouse, splice2 is apparently never spliced out.

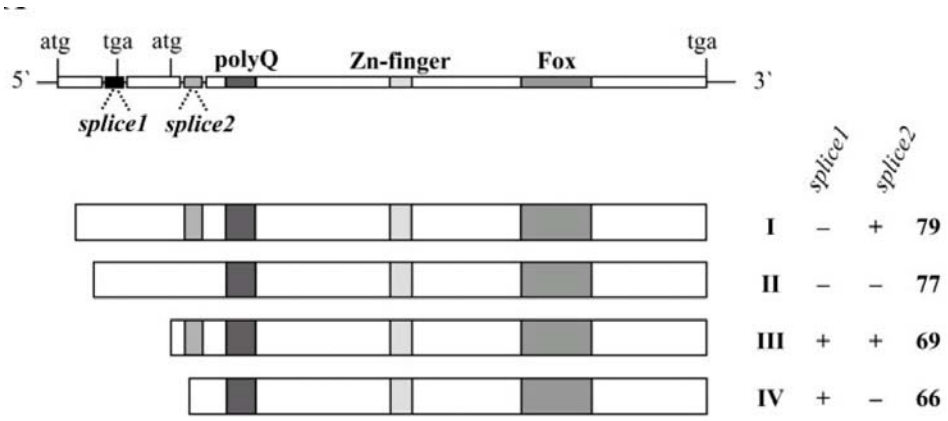


Figure 3.1 FoxP2 isoforms from the zebra finch. Identification of the zebra finch FoxP2 mRNA. Schematic representation of the zebra finch FoxP2 mRNA structure and its four predicted protein isoforms (I-IV). Positions of start (atg) and stop (tga) codons, the polyglutamine tract (polyQ), zinc finger (Zn-finger), and forkhead box (Fox) DNA-binding domains are shown. Two mRNA segments (splice1 and splice2) are subject to alternative splicing. The presence (+) or absence (-) of splice1 and splice2 leads to variation in the

length of ORFs. Splice1 contains a stop codon that shifts the frame so that the ORF begins at the second atg, splice2 inserts 60bp in-frame into the coding region. The four predicted protein isoforms are shown. For the calculation of their theoretical molecular weight, we used Peptide Mass (http://www.expasy.org/tools/peptide-mass.html).

Reverse transcription (RT)-PCR with RNA from a variety of zebra finch tissues using primers at both ends of the alternatively spliced region generated products that matched the sizes expected for the isoforms (Figure 3.2). There were, however, differences between tissues, with isoform IV being predominant in muscle, II-IV in lung, and all four in brain and liver (Figure 3.2).

isoform	length of ORF (bp / AA)	length of RT-PCR product spanning alternatively spliced region*
I	2464 / 711	774
II	2404 / 691	714
III	1860 / 619	845
IV	1800 / 599	785

* bp 114-959 relative to first start codon

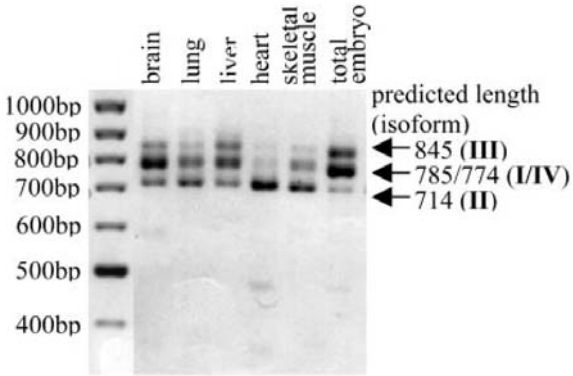


Figure 3.2. Length [in base pairs (bp) and amino acid (AA)] **of the zebra finch FoxP2 isoforms (I-IV) and the length of the RT-PCR products spanning the alternatively spliced region.** RT-PCR on RNA of different zebra finch tissues spanning the alternatively spliced region, but not the entire ORF, yields DNA fragments of the expected sizes.

Northern hybridization on adult zebra finch brain and lung total RNA revealed four transcripts of 9.0, 6.5, 3.5, and 2.5kb, respectively (Figure 3.3). The 9.0, 3.5, and 2.5kb transcripts corresponded in size to the transcripts found in mouse (Shu et al., 2001), whereas the 6.5kb transcript matched the size of the human transcript (Lai et al., 2001). The size of the two most abundant FoxP2 transcripts of 9.0 and 6.5kb suggests that they contain large amounts of regulatory sequence, perhaps to precisely regulate FoxP2 translation, mRNA location, and mRNA stability. To determine which protein isoforms are found in the zebra finch brain, we probed juvenile zebra finch brain extracts with an antibody raised against amino acids 613-715 of mouse FoxP2 (Lu et al., 2002) by Western blot. This antibody should recognize all four isoforms. We could exclude the existence of abundant levels of the short isoforms III and IV, because no protein corresponding to their

predicted molecular weight (Figure 3.1) was detected (Figure 3.3). Thus, isoforms III and IV are present only in a small population of cells or at low levels across most cells. In zebra finch brain, one or both of the long isoforms (I and II) predominate, although we could not distinguish between their similar molecular weights of 77 and 79kDa, respectively (Figure 3.3). For the mouse FoxP2 protein, a molecular weight in this range has been observed (Lu et al., 2002).

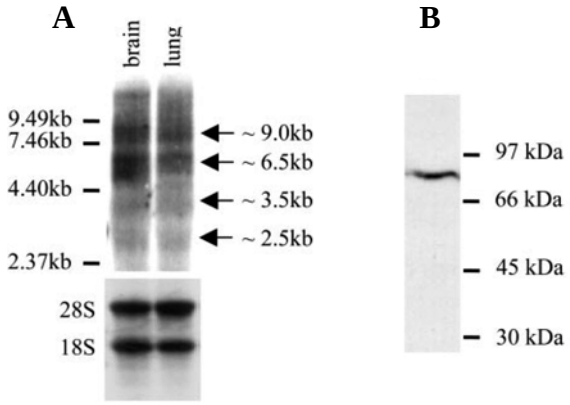


Figure 3.3 Transcript and protein analysis of zebra finch FoxP2. (A) Northern blot analysis of 20 µg of total RNA from adult zebra finch brain and lung was performed with a ^{32}P-labeled DNA fragment spanning bp 114 -959 (relative to the first start codon of isoform III). Ethidium bromide staining of 18S and 28S ribosomal bands demonstrates equal RNA loading. The different FoxP2 transcripts are indicated with arrows. **(B)** Western blot analysis of 50µg of brain nuclear protein extract from a 40-day old male zebra finch reveals a FoxP2 protein corresponding in size to either isoform I or II, recognized by a polyclonal antibody raised against amino acids 613-715 of mouse FoxP2 (Lu et al., 2002).

The zebra finch FoxP2 protein (isoform I) shares 98.2% identity with the human FoxP2 protein and 98.7% identity with mouse FoxP2 protein (Figure 3.4). This underscores the extreme degree of conservation of the FoxP2 gene (Enard et al., 2002; Zhang et al., 2002), because 320 MYA is the latest time at which modern mammals and birds shared a common ancestor (Evans, 2000). At five amino acid positions that are identical in mice and men, FoxP2 differs. At three additional positions, the mouse and zebra finch sequence are identical but the human sequence diverges. Of these three amino acids (Figure 3.4), one also exists in carnivores (amino acid framed by circle), one is common to primates (boxed amino acid), one is unique to humans [amino acid framed by triangle (Zhang et al., 2002)].

```
ZebraFinch  MMQESATETISNSSMNQNGMSTLSSQLDAGSRDGRSSGDTSTEVSTVELLHLQQQQALQAARQLLLQQQTSGLKSPKGSEKQRPLQVPVSVAMMTPQVIT  100
mouse       ----------------------------------------S---------------------------------S----------------------  100
human       ----------------------------------------S---------------------------------S-D--------------------  100

ZebraFinch  PQQMQQILQQQVLSPQQLQALLQQQQAVMLQQQQLQEFYKKQQEQLHLQLL..QQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQHPGKQAKE.  197
mouse       ---------------------------------------------------QQ-----------------------------------------.  199
human       ---------------------------------------------------QQ-----------------------------------------Q  200

ZebraFinch  .QQQQQQQQLAAQQLVFQQQLLQMQQLQQQQHLLNLQRQGLISIPPGQSALPVQSLPQAGLSPAEIQQLWKEVTGVHSMEDNGIKHGGLDLTTNNSSSTT  296
mouse       Q---------------------------------S-------------A-------------------------------------------------  299
human       Q---------------------------------S-------------A-------------------------------------------------  300

ZebraFinch  SSTTSKASPPITHHSIVNGQSSVLNARRDSSSHEETGASHTLYGHGVCKWPGCESVCEDFGQFLKHLNNEHALDDRSTAQCRVQMQVVQQLEIQLSKERE  396
mouse       ---------------------------------------------------------------I---------------------------------  399
human       --N-------------------S---------------------------------------I---------------------------------  400

ZebraFinch  RLQAMMTHLHMRPSEPKPSPKPLNLVSSVTMSKNMLETSPQSLPQTPTTPTAPVTPITQGPSVITPASVPNVGAIRRRHSDKYNIPMSSEIAPNYEFYKN  496
mouse       ----------------------------------------------------------------------------------------------------  499
human       ----------------------------------------------------------------------------------------------------  500

ZebraFinch  ADVRPPFTYATLIRQAIMESSDRQLTLNEIYSWFTRTFAYFRRNAATWKNAVRHNLSLHKCFVRVENVKGAVWTVDEVEYQKRRSQKITGSPTLVKNIPT  596
mouse       ----------------------------------------------------------------------------------------------------  599
human       ----------------------------------------------------------------------------------------------------  600

ZebraFinch  SLGYGAALNASLQAALAESSLPLLSNPGLINNASSGLLQAVHEDLNGSLDHIDSNGNSSPGCSPQPHIHSIHVKEEPVIAEDEDCPMSLVTTANHSPELE  696
mouse       ----------------------------------------------------------------------------------------------------  699
human       ----------------------------------------------------------------------------------------------------  700

ZebraFinch  DDREIEEEPLSEDLE  711
mouse       --------------  714
human       --------------  715
```

Figure 3.4 Comparison of zebra finch FoxP2 with human and mouse FoxP2. In the human sequence the R553H mutation that is associated with developmental verbal dyspraxia is marked with an asterisk (*), a primate-specific amino acid is boxed, a carnivore-specific amino acid is circled and the unique human-specific amino acid is highlighted by a triangle. The polyQ region, the zinc finger domain and the forkhead box DNA Binding domain are boxed in yellow, blue and green respectively.

In addition to FoxP2, we cloned its closest homolog FoxP1 from the zebra finch. With 5´ and 3´ RACE, 2412bp of FoxP1 mRNA covering the ORF and 164bp of the 3´ UTR (GenBank accession number AY54952) were assembled. FoxP2 and FoxP1 amino acid sequences are highly similar (Figure 3.5), with the largest differences being that FoxP1 misses the poly-glutamine stretch and 100 amino acids on the N terminus. For human FoxP1, an isoform that lacks the first 100 amino acids is reported (Banham et al., 1999), suggesting that we found a short FoxP1 isoform. The strong similarity between FoxP2 and FoxP1 is consistent with their reported synergistic molecular function.

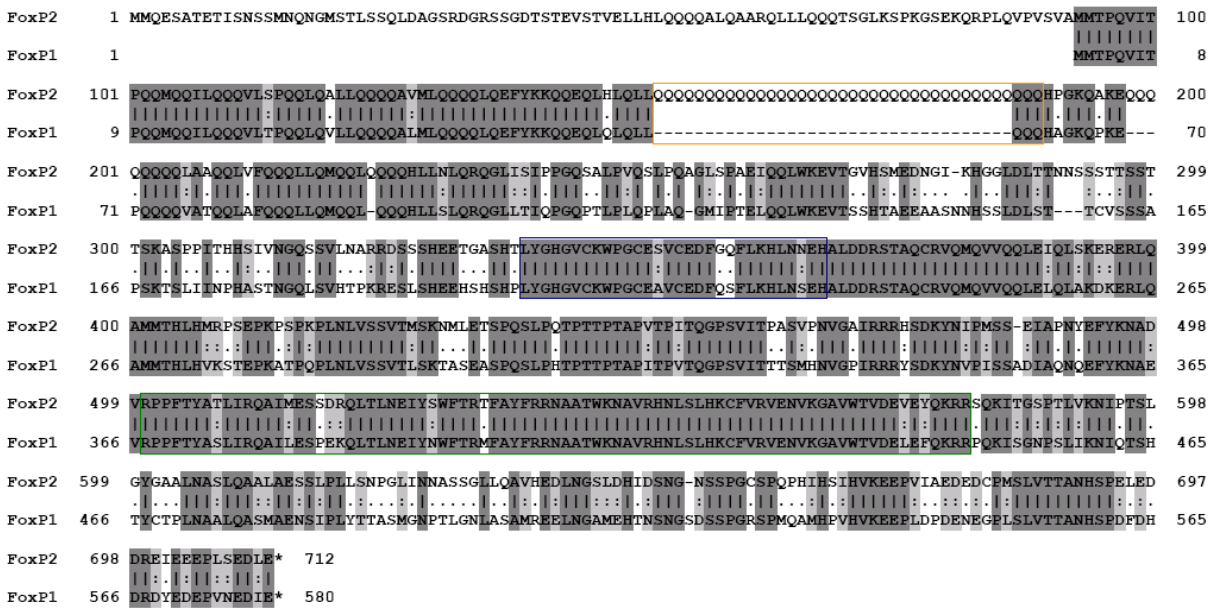


Figure 3.5. Comparison between FoxP2 and FoxP1 from the zebra finch. Alignment of zebra finch FoxP1 and FoxP2 protein sequences. Identical amino acid are shaded in dark grey, similar amino acids are shaded in light grey and non-similar comparisons remain white.

3.1.2 Embryonic FoxP2 expression

Consistent with the reports from developing human and mouse brain (Lai et al., 2003; Shu et al., 2001; Takahashi et al., 2003) we detected FoxP2 expression in the embryonic zebra finch brain as early as stage 26 [Butler and Juurlink, 1987; Hamburger and Hamilton, 1951 (Figure 3.6 A)]. The highest expression was in the striatum and dorsal thalamus. This expression persisted throughout development (Figure 3.6 B) and was not restricted to vocal learners, because chickens also showed strong expression in the embryonic striatum (Figure 3.6 C). Closer examination at stage 34 revealed that the basal plate of the telencephalic vesicle, part of which gives rise to dorsal striatal areas in the adult, expressed FoxP2 (Figure 3.6 D), as did the region that develops into the dorsal thalamus (data not shown). In the ventral midline of the mesencephalic vesicle, labeled cells appear to invade the laterally adjacent neuroepithelium (Figure 3.6 E). At limb levels of the spinal cord, cells that appear to be departing the roof plate and migrating to ventromedial regions expressed FoxP2 (Figure 3.6 F). Expression was strong in the floor plate at this level, extending rostrally into the mesencephalon (Figure 3.6 F). The lateral margins of the hindbrain neuroepithelium and the region of the metencephalic/mesencephalic isthmus also strongly expressed FoxP2.

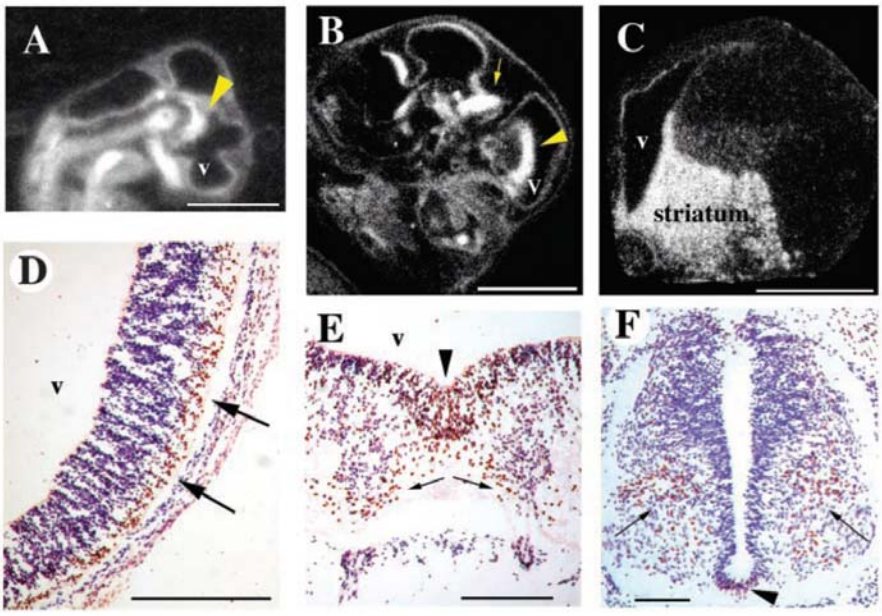


Figure 3.6 FoxP2 expression in the developing embryo. Embryonic FoxP2mRNA **(A-C)** and protein **(D-F)** expression. Sagittal sections through stage 26 **(A)** and 34 **(B)** zebra finch embryos show expression in presumptive striatum (arrowheads) and presumptive dorsal thalamus (arrow). The heads face toward the right. **(C)** Embryonic chicken brain (embryonic day 13) had strong expression in the developing striatum and also in the pallial and subpallial germinal ventricular zone, shown in a frontal right hemisection. The FoxP2 mRNA label appears white in dark-field illumination in **(A-C)**. **(D-F)**, FoxP2 expression in a stage 26 zebra finch embryo frontal sections. FoxP2 immunoreactivity is brown, and cresyl violet-stained cells are purple/blue. **(D)** A prominent band of FoxP2-positive cells is visible among cresyl violet-stained neurons in the ventrolateral telencephalic vesicle. The floor plate at the rostral end of the mesencephalic vesicle **(E, arrowhead)** has many FoxP2-expressing cells that seem to disperse laterally **(E, arrows)**. At limb levels of the spinal cord, floor plate neurons expressed FoxP2 **(F, arrowhead)**, as did a population of neurons in ventral cord **(F, arrows)**. Scale bars: **(A-C)** 2 mm; **(D-F)** 100 µm.

3.1.3 Subtelencephalic FoxP2 expression in the adult zebra finch

For identification of subtelencephalic brain regions expressing FoxP2, we analyzed serial frontal and sagittal sections through the entire brain of male zebra finches and used the region-specific parvalbumin (Braun et al., 1991; Braun et al., 1985; Wild et al., 2001) and ChAT (Medina and Reiner, 1994) staining in adjacent series of sections as landmarks to ascertain the identity of brain regions that expressed FoxP2 (Figure 3.7 B, C, H and I). Table 3.1.1 lists subtelencephalic structures that did or did not express FoxP2.

Table 3.1.1 Expression pattern of FoxP2 in suptelencephalic brain regions of adult zebra finches

Abbreviation	Subtelencephalic region	FoxP2
AN	Nucleus angularis	+
DM	Dorsomedial nucleus of the midbrain	−
DT	Dorsal thalamus (posterior nuclei)	+++
nIII	Cranial nucleus III (Edinger–Westphal)	−
Cn	Cuneate nucleus	+
Gn	Gracile nucleus	+
GCt	Substantia grisea centralis	+
Imc	Nucleus isthmi, pars magnocellularis	−
Ipc	Nucleus isthmi, pars parvocellularis	−
IO	Nucleus isthmo-opticus	+
La	Nucleus lateralis anterior thalami	+
LLi	Nucleus lemnisci lateralis	+
MC	Nucleus magnocellularis	+
MLd	Nucleus mesencephalicus lateralis, pars dorsalis	++
MnV	Motor part of trigeminal nucleus or V nucleus	−
MnX	Dorsal motor part of the vagus nucleus or X nucleus	−
nBOR	Nucleus of the basal optic root	−
nIX	Glossopharyngial nucleus or IX nucleus	−
nVI	Abducens nucleus or VI nucleus	−
nXII	Hypoglossal nucleus or XII nucleus	−
OI	Nucleus olivaris inferior	+++
Omd	Nucleus nervi oculomotorii, pars dorsalis	−
OMdm,OMv	Nucleus nervi oculomotorii, pars dorsalis/ventralis	−
OMv	Nucleus nervi oculomotorii, pars ventralis	
Ov	Nucleus ovoidalis	+
PAG	Periaqueductal gray	+
PMH	Nucleus medialis hypothalami posterior	+
PT	Pretectal nucleus	−
PTD	Nucleus pretectalis diffusus	−
PTM	Nucleus pretectalis medialis	++
PVN	Paraventricular nucleus	−
Rt	Nucleus rotundus	++
RPC	Nucleus reticularis pontis caudalis	+
R	Red nucleus	+
ST	Nucleus of the solitary tract	+
SNc	Substantia nigra, pars	+
SP	Nucleus subpretectalis	−
SpL	Nucleus spiriformis lateralis	−
T	Nucleus triangularis	++
VeD	Nucleus vestibularis descendens	+
VeL	Nucleus vestibularis lateralis	+
VTA	Ventral tegmental area	+

FoxP2 was expressed in many regions that are involved in relaying and integrating ascending sensory information, including auditory regions [e.g., midbrain nucleus MLd (dorsal part of the lateral mesencephalic nucleus, Figure 3.7 A and B) and thalamic nucleus ovoidalis (data not shown)], visual regions [e.g., afferent upper layers of midbrain optic tectum (Figure 3.7 A and F) and thalamic nucleus rotundus (Figure 3.7 D)], multimodal regions [e.g., layers 10 and 11 of the optic tectum (Figure 3.7 F)], and somatosensory regions [e.g., sensory trigeminal (data not shown)]. Prominent FoxP2 expression was observed in the inferior olive (Figure 3.7 G), which gives rise to all the climbing fibers

innervating the Purkinje cells of the cerebellum. Consistent with this, FoxP2 expression was also found in the Purkinje cells (Figure 3.7 E, Figure 3.8 A-I, Figure 3.10 E and F). All species tested, including males and females, regardless of whether they learn their vocalization or not, expressed FoxP2 in these regions. In contrast, FoxP2 expression was not found in midbrain and brainstem motor control areas, such as the vocal nucleus DM [dorsomedial motor nucleus of the intercollicular region (Figure 3.7 B and C)], the hypoglossal vocal and tongue nucleus, nXII (Figure 3.7 H and I), and most other motor cranial motor nuclei.

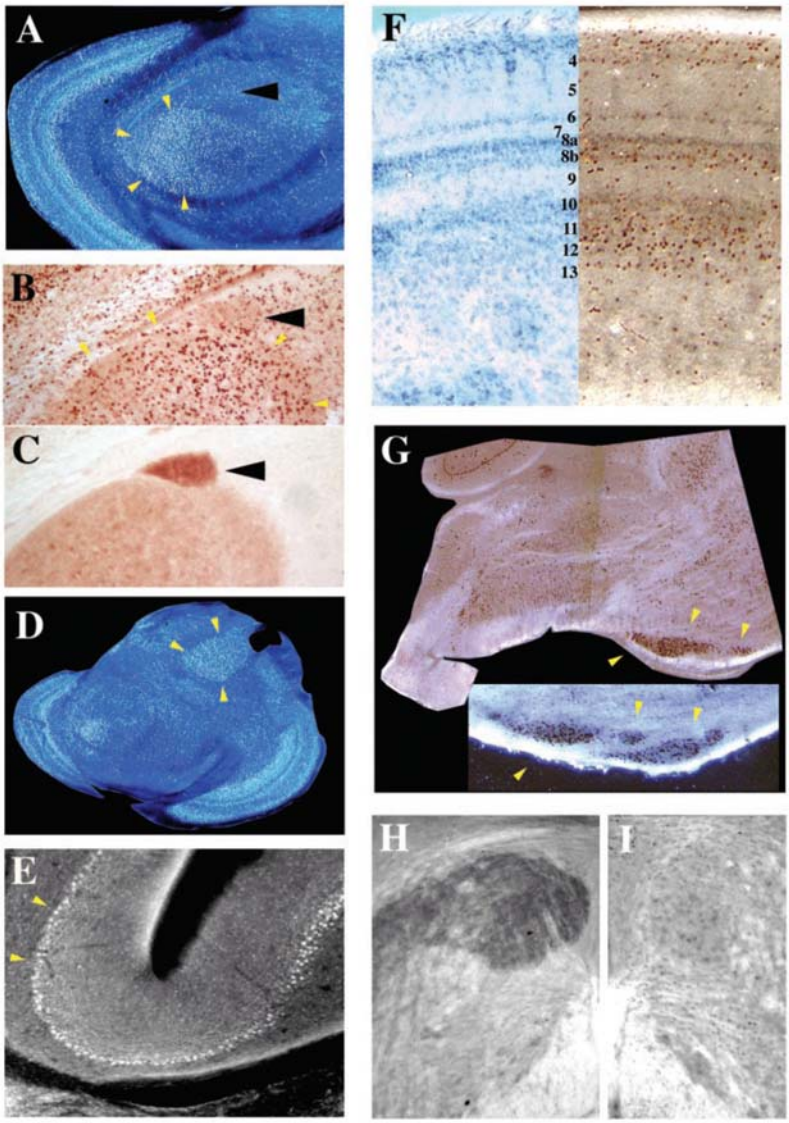


Figure 3.7 Subtelencephalic FoxP2 epxression in the adult zebra finch. (A-H) FoxP2 expression in subtelencephalic regions was associated more with afferent sensory or multimodal areas rather than with pure motor areas. Auditory nucleus MLd (dorsal part of the lateral mesencephalic nucleus) expressed FoxP2 (white dark-field label in **(A)** and brown label in **(B)**; both surrounded by yellow arrowheads). In contrast, the dorsomedial motor nucleus of the intercollicular region (DM), which controls vocalizations, showed little

mRNA and immunoreactivity for FoxP2 **(A, B, black arrowheads)** but strong parvalbumin immunoreactivity **(C)** (Braun et al., 1985). Also, FoxP2-immunoreactive cells were seen in the visual thalamic nucleus rotundus **(D)**, cerebellar Purkinje cells **(E)**, specific layers of the optic tectum in the midbrain **(F)**, and brainstem nucleus inferior olive **(G)** but not in the tracheosyringeal portion of the nucleus of the hypoglossal nerve nXIItx **(I)**. We took advantage of the strong parvalbumin immunoreactivity of nXII to unambiguously identify this nucleus (adjacent section to **(I)** stained with parvalbumin in **(H)** (Wild et al., 2001). Immunoreactivity in dark-field images appears white, and in bright-field photomicrographs brown. **(A, D and E-G)** are sagittal sections, rostral is to the right, and **(B, C, H, and I)** are frontal sections. Dorsal is up in both orientations.

3.1.4 Expression of FoxP2 in the adult telencephalon

In adult avians, FoxP2 was highly expressed in the cerebellar Purkinje cells, the striatum and nuclei in posterior portions of the dorsal thalamus. This pattern was predominant in all species investigated and in both genders, regardless of whether they are vocal learners or not, and even in a crocodile (Figure 3.8 A-G), the closest non-avian relative. In vocal learners, the dorsal striatum contains a nucleus that is part of the specialized song system, called Area X in songbirds, vocal nucleus of the anterior striatum [VAS; previously called VAP (Jarvis and Mello, 2000)] in hummingbirds, and magnocellular nucleus of the medial striatum [MMSt; previously called LPOm (Striedter, 1994)] in parrots. This structure is part of a basal ganglia loop, the anterior forebrain pathway (Bottjer and Johnson, 1997; Durand et al., 1997; Farries and Perkel, 2002) and is essential for vocal learning (Scharff and Nottebohm, 1991; Sohrabji et al., 1990). FoxP2 expression in Area X of adults of four songbird species and in the corresponding region VAS in hummingbirds differed relative to the surrounding striatum (Figure 3.8). In chickadees and strawberry finches, both seasonal breeders (Langham, 1987; Smith, 1991), FoxP2 expression was higher in Area X than in the surrounding striatum (Figure 3.8 A and B, J). In song sparrows and Bengalese finches, FoxP2 expression was lower than the surrounding striatum (Figure 3.8 D and E, J). The chickadees were caught during the fall months (October and November), whereas the song sparrows were caught during late spring [April and May (Jarvis et al., 1997)], when song sparrows sing fewer variations of song types and song is more stereotyped than in the fall (Smith et al., 1997). Bengalese finches are not strongly seasonal birds and breed opportunistically (Seiler et al., 1992), as do zebra finches, although the latter are also sensitive to photoperiod (Bentley et al., 2000). Rufous-breasted hermit hummingbirds, captured near the end of their breeding season (Jarvis et al., 2000), showed slightly

elevated levels of FoxP2 in the hummingbird striatal vocal nucleus VAS (Figure 3.8 F). We did not find differential expression in MMSt of parrots (Figure 3.8 G). In adult zebra finches, there was variability in FoxP2 expression in Area X. Of 10 adult male zebra finches examined, 7 had expression levels in Area X similar to the region surrounding it, two slightly lower and one slightly higher (data not shown).

To address the source of the differences in FoxP2 expression in Area X/VAS/MMSt among different species, we checked whether they might be related to differences in overall vocal syntax complexity, using the equations of (Scharff and Nottebohm, 1991). Scores of vocal syntax complexity are low when song elements are mostly rendered in an unvarying, stereotyped manner. When songs consist of elements that are rendered in highly variable sequences, scores of syntax complexity are high. Vocal syntax complexity is low in strawberry finch, zebra finch, and somber hummingbird; intermediate in Bengalese finch, canary, and song sparrow; and high in rufous-breasted hermit hummingbird and budgerigar (K. Wada and E.D. Jarvis, unpublished observation). Thus, vocal syntax complexity cannot account for the observed FoxP2 expression differences among the species (data not shown). Instead, the FoxP2 expression pattern in chickadee, strawberry finch, and song sparrow are more consistent with the notion that during times of increased song stereotypy, as is usually observed during the breeding season, FoxP2 is not upregulated in Area X, whereas outside of the breeding season, when song tends to be more plastic, FoxP2 expression in Area X tends to be higher. Hummingbirds and parrot differed with respect to pallial expression from the six songbird species investigated. In the hummingbird, the differential higher expression of FoxP2 in the striatum relative to the pallium was less pronounced than in the other species. In the parrot, FoxP2 expression in mesopallium was much higher relative to other pallial regions than it was in the other species tested. However, the AFP mesopallial song nucleus [MO; previously called HVo (Jarvis and Mello, 2000)] had low FoxP2 expression (Figure 3.8 G). None of the other pallial vocal nuclei of the parrot, songbird, or hummingbird AFP (songbird lMAN-like) or vocal nuclei of their motor pathways (songbird HVC-like, used as a proper name, and RA-like) expressed high levels of FoxP2.

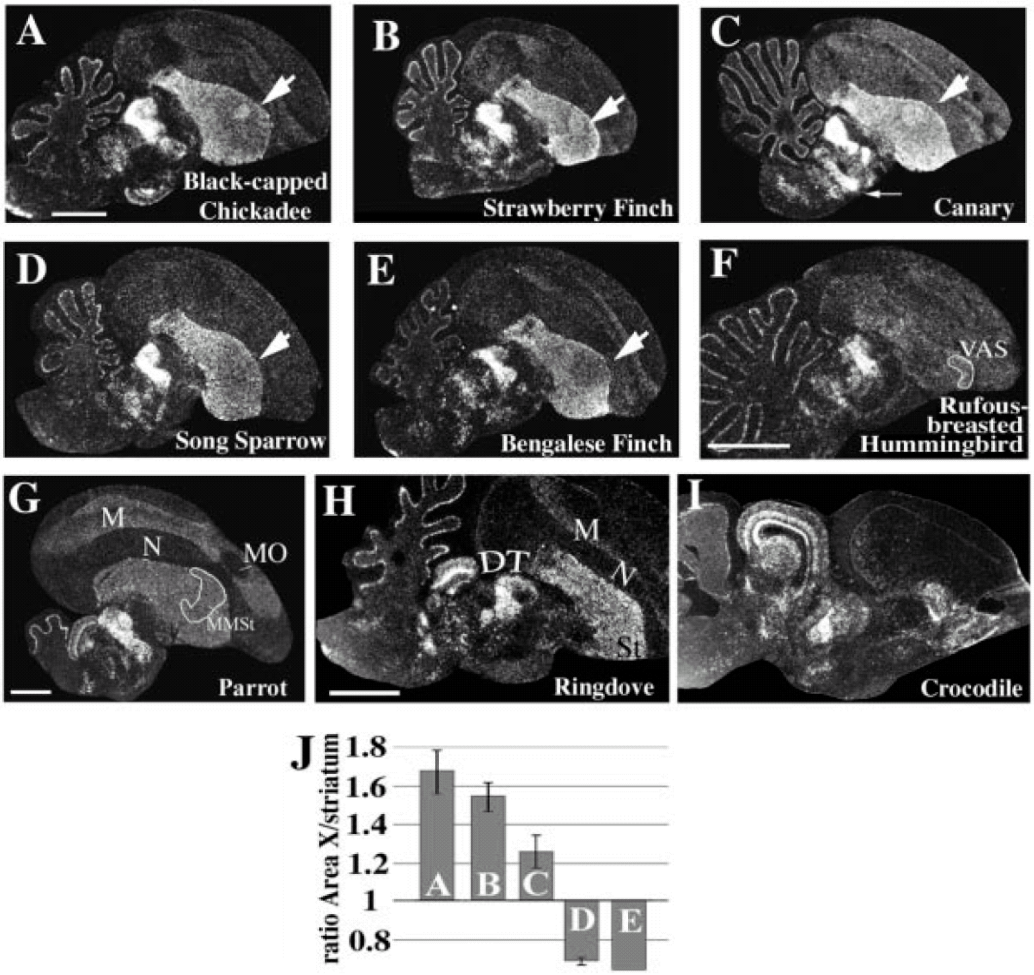


Figure 3.8 FoxP2 expression in adult vocal learners, vocal non-learners and the crocodile. FoxP2 expression in the striatum and dorsal thalamus was conserved among vocal learners, non-learners, and crocodile species. It was exclusive however to the striatal vocal control nucleus of vocal learners (Area X/VAS/MMSt). Area X of chickadees (sampled in the fall), strawberry finches (sampled on long day photoperiod), and canaries (sampled in July) expressed more FoxP2 in Area X than in the surrounding striatum **(A-C)**, reflected in higher expression ratios **(bars A-C in J)**. **(D and E)** Area X of song sparrows (sampled in spring) expressed slightly less FoxP2 than the surrounding striatum **(bar D in J)**, as did Bengalese finch **(bar E in J)**. The rufous-breasted hermit hummingbird **(F)** had slightly higher expression in the VAS, and the parrot **(G)** did not show a difference between vocal nucleus MMSt and the surrounding striatum. The adult ringdove **(H)**, a bird that does not exhibit vocal learning and lacks telencephalic vocal nuclei, expressed high levels of FoxP2 mRNA in the striatum and dorsal thalamus (DT), as did a crocodile **(I)**. The arrow in **(C)** points to the high levels of FoxP2 expression in the substantia nigra pars compacta. M, Mesopallium; MO, oval nucleus of the mesopallium; N, nidopallium; St, striatum; VAS, vocal nucleus of the anterior striatum; MMSt, magnocellular nucleus of the medial striatum. Scale bars (in A for A-E; in H for H, I), 2 mm.

3.1.5 FoxP1 expression

Similar to FoxP2, FoxP1 was expressed at high levels in the striatum and in the dorsal thalamus of zebra finches and other birds (Figure 3.9 A-F). Unlike FoxP2, FoxP1 expression in the striatal vocal nuclei (Area X or MMSt) was similar across development and season, across all songbirds tested, and in parrots [i.e. higher expression in the striatal vocal nucleus relative to the immediate surrounding striatum (Figure 3.9 A-D, F)]. Also unlike FoxP2, within the pallium, FoxP1 was consistently and prominently expressed in the mesopallium in all avian species tested (Figure 3.9 A-F). Interestingly, for the three main songbird pallial vocal nuclei (lMAN, HVC, and RA), FoxP1 expression differed notably from the expression of the subdivisions in which these nuclei are embedded. HVC and RA strongly expressed FoxP1, whereas the surrounding territories did not. The reverse was true for lMAN, which did not express FoxP1, while the region around it did (Figure 3.9 A-D). This was consistent across songbird species. The parrot pallial analog of HVC, the central nucleus of the nidopallium, had noticeably higher levels than the surrounding nidopallium (Figure 3.9 F). In contrast to FoxP2, FoxP1 was never expressed in the Purkinje cells of the cerebellum. FoxP1 expression in the ring dove brain was similar to that of the songbirds and parrot, with the exception that there was no differential expression in the striatum and pallium, where vocal nuclei are found in vocal learners (Figure 3.9 E). A telencephalic expression pattern remarkably similar to that of the avian brain was found in crocodile (Figure 3.9 G), including high expression in striatal-like and mesopallium-like regions. This suggests that the general FoxP1 and FoxP2 expression patterns in vocally learning and non-learning birds were inherited from their common reptilian ancestor.

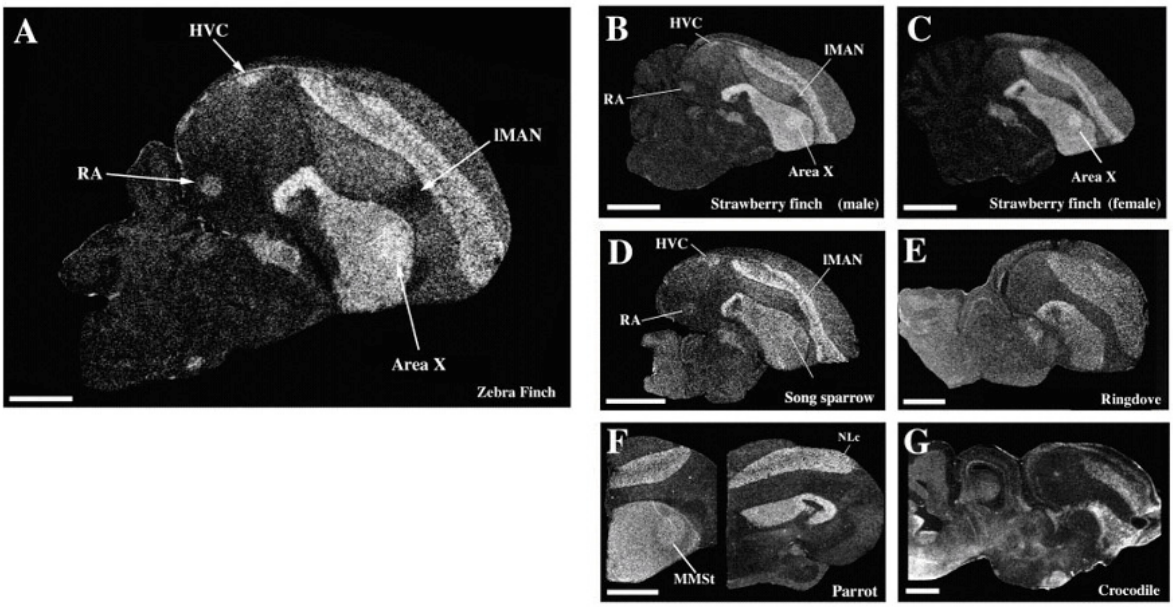


Figure 3.9 FoxP1 expression in the adult brain. (A) Expression pattern of FoxP1 was distinct from but partially overlapping with that of FoxP2. FoxP1, like FoxP2, was expressed in the dorsal thalamus and striatum in adult zebra finches. In addition, it was expressed in vocal nuclei HVC, RA, and Area X (but not lMAN) at higher levels than their surrounding regions and in the mesopallium. Both male **(B)** and female **(C)** strawberry finches, male song sparrow **(D)**, as well as the parrot **(F)** expressed more FoxP1 mRNA in Area X (MMSt in parrot) than in the surrounding striatum. **(E)** The ring dove, a vocal non-learner, also expressed FoxP1 mRNA in the subpalllial and pallial areas. **(G)** The crocodile had a telencephalic pattern very similar to that of birds. All sections are sagittal, except the parrot sections in **(F)**, which are frontal. Scale bars: A-D, 1 mm; E-G, 2 mm.

3.1.6 FoxP2 expression during times of song plasticity

Throughout zebra finch post-hatch development and into adulthood the striatum and nuclei in posterior portions of the dorsal thalamus dominated FoxP2 expression (Figure 3.10 A-F). Expression levels in the striatum decreased slightly with age (Figure 3.10 H). Expression levels in pallial regions (i.e., those dorsal to the striatum) remained low throughout development and into adulthood (Figure 3.10 H). During song development, Area X in male zebra finches expressed more FoxP2 mRNA than the surrounding striatum only at PHD35 and 50, the age at which zebra finches actively learn how to imitate song [Figure 3.10 C and D (Tchernichovski et al., 2001)]. Before this period (at PHD15 and 25) and afterward, when birds crystallized their songs (PHD75) and became adults (more than PHD120), FoxP2 expression in Area X did not differ from expression in the surrounding striatum.

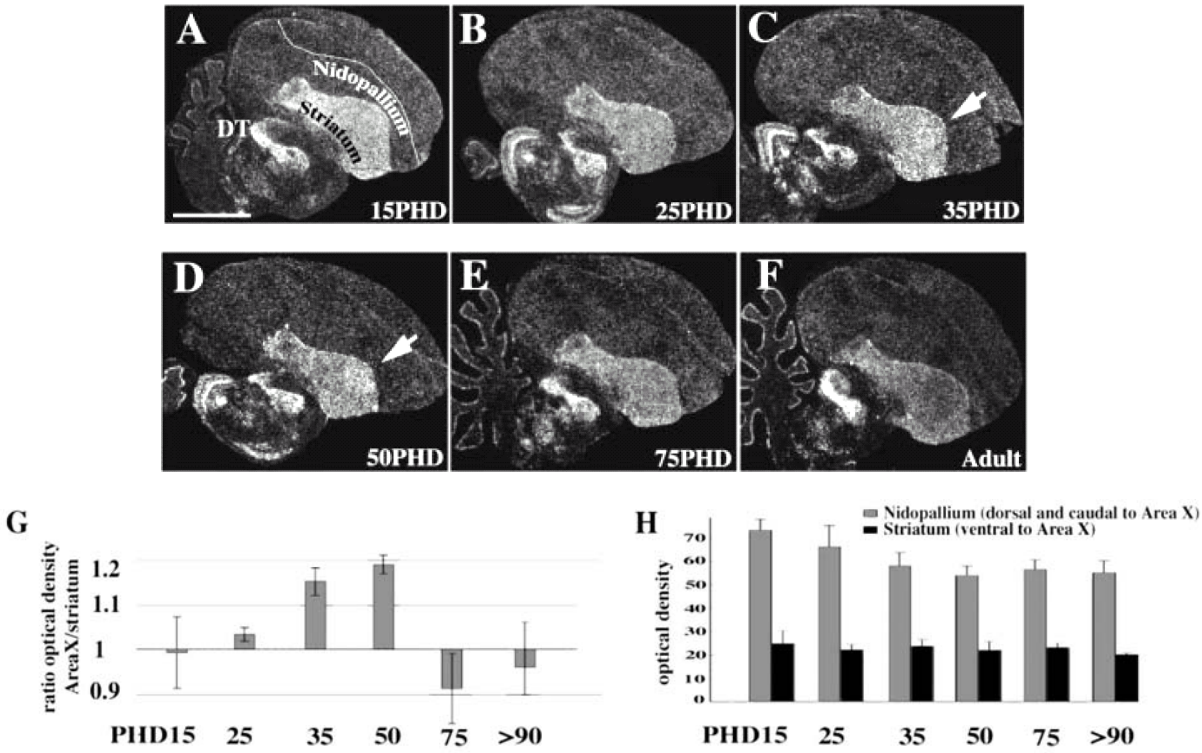


Figure 3.10 Differential FoxP2 expression in Area X during post-hatch zebra finch development. (A-F) Area X expressed more FoxP2 than the surrounding striatum only at PHD35 and 50 **(C, D, arrowheads),** which is the time when zebra finches learn to imitate song. **(G and H)** show the results of autoradiographic densitometric quantification of expression levels at the different ages (n=3 for each age). The ratio of expression between Area X and the surrounding striatum increased during the phase when song imitation occurs on PHD35 and 50 **(G)**. Absolute levels of FoxP2 expression in the nidopallium did not change throughout development, whereas in the striatum (outside of Area X) they decreased slightly from PHD15 to 25 and reached adult levels by PHD 35 **(H)**. Scale bar (in A): A-F, 2 mm.

We also examined FoxP2 expression in adult male canaries during different seasons of the year using a collection of canary brain sections described by (Jarvis and Nottebohm, 1997). In July, August, and September, canaries expressed more FoxP2 mRNA in Area X than in the region surrounding it (Figure 3.11). These are the months when birds add new syllables into their song repertoire and song is more variable (Leitner et al., 2001; Nottebohm et al., 1986) than in the preceding breading season, when song is stable. Breeding occurs in spring and can last through the end of June, and FoxP2 expression during this time (sampled in April and May) did not differ from the surrounding region. This was also the case in October and January (Figure 3.11).

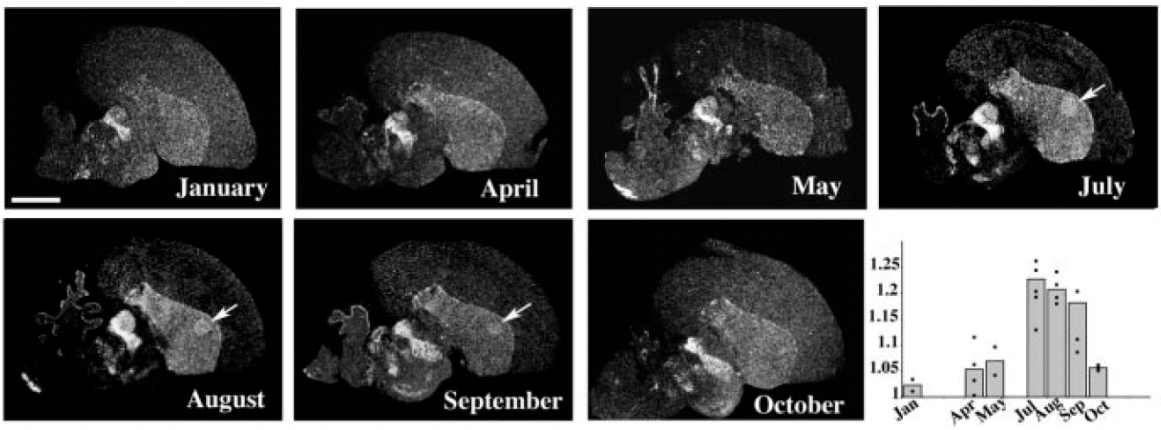


Figure 3.11 FoxP2 expression in canaries varies seasonally. FoxP2 expression in Area X of adult canaries varied seasonally. Area X expressed noticeably more FoxP2 than the surrounding striatum only during the months of July, August, and September, resulting in higher ratios of Area X to striatum expression (bar graphs show mean ratios for each month, superimposed points represent the values for individual birds).

To rule out that the developmental and seasonal changes in Area X FoxP2 expression were the result of a generic feature of gene expression in this region, we compared the zebra finch glutamate receptor subunits NR2B and mGluR2 (Wada et al., 2004) on adjacent sections to those that were probed with FoxP2 (Figure 3.12). We found no differences in mGluR2. There were some developmental changes in NR2B expression in zebra finch Area X at PHD25, as expected from a previous report (Basham et al., 1999). However, the ratio of NR2B expression levels between Area X and the surrounding striatum remained similar at PHD35-75 (Figure 3.12), the time when the FoxP2 expression ratio was higher. In canaries, we observed no seasonal changes of NR2B expression in Area X, as was also shown previously (Singh et al., 2003).

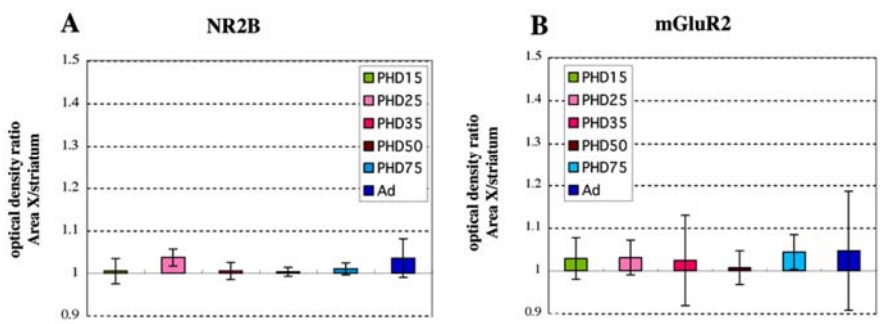


Figure 3.12 Gene expression in Area X is not globally increased during song learning. The expression of the zebra finch glutamate receptor subunit NR2B and subtype mGluR2 was measured in adjacent sections to those in **Figure 3.10**. In contrast to developmental differences in the ratio of FoxP2 mRNA expression in

Area X to the surrounding striatum there were no differences during development of zebra finches (n=3 animals per data point).

3.1.7 Zebra finch FoxP2 expression and singing

We tested whether some of the differential FoxP2 expression in Area X of zebra finches and canaries could be accounted for by singing activity. Singing strongly induces the expression of the immediate early gene ZENK (the avian homolog of mammalian zif268/EGR-1/ NGFI-A/krox24 gene) in Area X (Jarvis and Nottebohm, 1997). Moreover, the 5′ flanking region of human FoxP2 contains three predicted EGR-1 (i.e. ZENK) binding sites (Bruce and Margolis, 2002). We found that for birds of similar age or season there were no significant differences in FoxP2 mRNA expression between quiet control animals (quiet for at least 12hr overnight) and animals that sang spontaneously [for 30 or 60 min for zebra finches (n=3 each) and 1, 15, 30, or 60min or 2, 4, or 6hr for canaries (n=3 each)], whereas ZENK was induced dramatically in zebra finches at PHD 65 or 150 by singing during the last 30min before sacrifice [Jarvis and Nottebohm, 1997 (Figure 3.13). Finally, we could not find any other variable (song complexity, amount of singing, or age at sacrifice) that could account for differential FoxP2 expression in Area X of zebra finches and canaries.

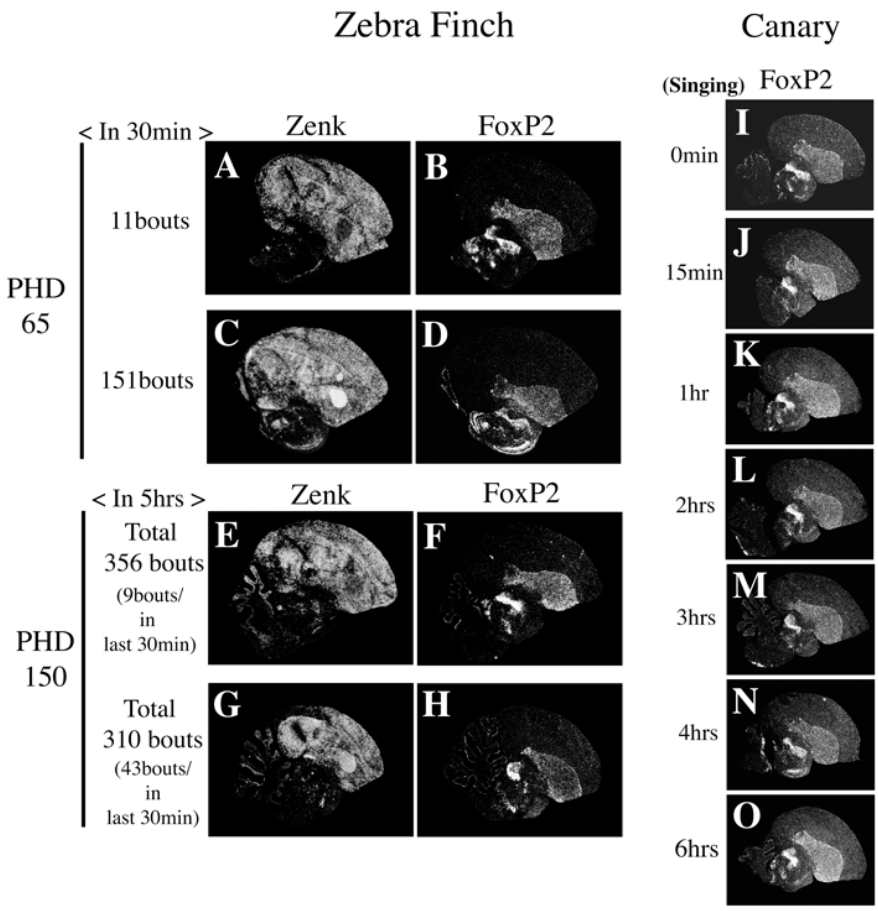


Figure 3.13 FoxP2 expression in Area X is not induced by singing. **(A-H)** In both young (PHD 65, B, D) and adult (PHD 150, F, H) zebra finches, singing undirected song does not induce FoxP2 expression in Area X, whereas in adjacent sections of the same animals, the amount of ZENK expressed reflects the singing activity during the last 30 min before the birds were killed (A, C, E, G). **(I-O)** In adult canaries, there was also no relationship between the amount the bird sang before being killed and the amount of FoxP2 expression in Area X.

3.1.8 Cellular identity of FoxP2 expressing cells

In adult zebra finch striatum, FoxP2 immunoreactivity was characteristically seen in medium or small cells that were uniformly distributed throughout, except for one peculiarity. Small FoxP2-positive cells formed distinct, evenly spaced clusters in the part of the lateral striatum that abuts the pallial-subpallial lamina (PSL; previously called LMD), which separates the pallium from subpallium (Figure 3.14 A and B). More medially in the striatum these clusters formed a thin, continuous band (data not shown), matching the high levels of mRNA seen at the striatum side of the PSL (Figure 3.10 F). In pigeon striatum, similarly arranged patches contain dense ChAT-immunoreactive fibers (Medina and Reiner, 1994). In zebra finch, these FoxP2-immunoreactive cell clusters

were, likewise, innervated by ChAT (Figure 3.14 C). The clusters were also visible in Nissl-stained material (Figure 3.14 D). A Hu antibody, which binds to an RNA-binding protein specifically present in young postmitotic and adult neurons (Barami et al., 1995), revealed that all FoxP2-immunoreactive brain cells were neurons, including the clusters at the PSL in the striatum (Figure 3.14 E and F). Some of the latter also expressed PSA-NCAM, a marker for cellular plasticity and migration [Durbec and Cremer, 2001 (Figure 3.14 G)]. To determine whether the FoxP2-expressing neurons in the striatum belonged to a particular population of neurons, we used markers for the three classes of striatal interneurons (Reiner et al., 2004; Reiner et al., 1998) in conjunction with FoxP2 immunohistochemistry: ChAT to detect the large aspiny cholinergic interneurons, nitric oxide synthase (nNOS) to detect the medium-sized aspiny interneurons that also contain somatostatin and NPY, and the calcium binding protein parvalbumin to detect another population of mediumsized aspiny interneurons that also contain GABA and the neurotensin-related hexapeptide LANT6 (Reiner et al., 2004; Reiner et al., 1998). Neither ChAT (Figure 3.14 J) nor nNOS (Figure 3.14 K) nor parvalbumin (Figure 3.14 L) were detected in the same neurons as FoxP2, suggesting that the striatal neurons that express FoxP2 are projection neurons rather than interneurons. It is known that the striatal neurons that project to the pallidum in birds, as in mammals, and striatal neurons that project to pallidal-like cells in Area X are the site of convergent nigral dopaminergic and cortical (i.e., pallial) glutamatergic input (Reiner et al., 2004; Reiner et al., 1998). DARPP-32 is thought to serve as a critical integrator of these two inputs onto the striatal projection neurons (Hemmings et al., 1995). Concordant with our expectation that FoxP2 is expressed in striatal projection neurons, we found two indicators of dopaminergic innervation. FoxP2-immunoreactive striatal neurons coexpressed DARPP-32 (Figure 3.14 H), which is indicative of the presence of dopamine D1 receptors (Snyder et al., 1998), and immunoreactivity for TH, the synthetic enzyme for biogenic amines, was present in fibers around perikarya of neurons with FoxP2- immunoreactive nuclei (Figure 3.14 I).

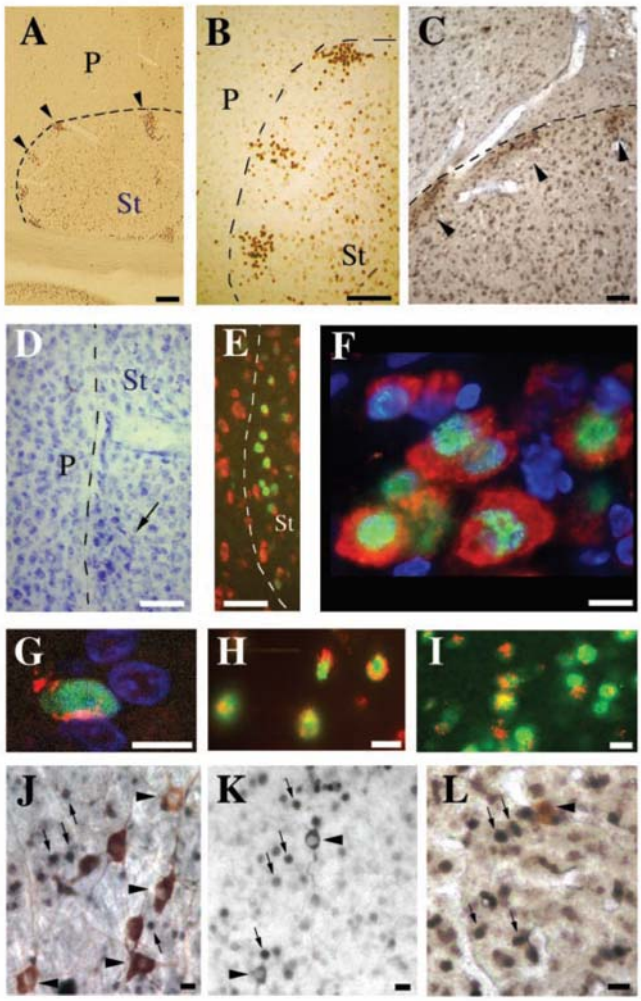


Figure 3.14 FoxP2 expression in distinct populations of neurons in adult zebra finches. Low **(A)** and high **(B)** magnification of a sagittal section showing the dorsolateral extent of the subpallial-pallial (P) border with the striatum (St; black dashed line), where clusters of cells in the dorsal and lateral striatum express FoxP2 (arrowheads; brown immunoreactivity). Dorsal is up, and rostral is to the right. **(C)** These clusters (arrowheads; black-brown immunoreactivity) are characterized by dense ChAT fiber staining (lighter brown immunoreactivity). **(D)** Clusters visualized with cresyl violet stain. **(E)** FoxP2-immunoreactive cells within the clusters are neurons as shown by double labeling with fluorescent anti-Hu (red) and anti-FoxP2 (green). **(F)** Higher magnification in the dorsal thalamus shows that the cytoplasmic neuronal anti-Hu antibody (red) colocalizes with nuclear FoxP2 antibody staining (green). FoxP2-negative nuclei can been seen in blue, stained with nuclear 4′,6-diamidino-2-phenylindole DNA stain. **(G)** Some FoxP2- positive cells are recognized by anti-PSA-NCAM antibody, a cell adhesion protein (PSA-NCAM, red; FoxP2, green; TOPRO3 nuclei, blue). **(H)** Striatal neurons also coexpress DARPP-32 (red) and FoxP2 (green) and appear to be innervated by TH-positive (red) terminals **(I)**. Colabeling with neurochemical markers for three different striatal interneuron populations [ChAT **(J)**, nNOS **(K)**, or parvalbumin **(L)** (brown cytoplasmatic labeling; arrowheads)] revealed that FoxP2 (black nuclear labeling; arrows) was not expressed in these cell types. Scale bars: A, B, 100 µm; C-E, 50 µm; F-L, 10 µm.

3.2 Knockdown of FoxP2 *in vivo*

3.2.1 Establishing lentivirus-mediated RNAi in the zebra finch

To test whether FoxP2 contributes directly to song learning in zebra finches we reduced the levels of FoxP2 expression in Area X *in vivo*, using lentivirus-mediated RNA interference (RNAi). In this approach short interfering hairpin RNA (shRNA) containing sense and antisense sequences from the target gene connected by a hairpin loop are expressed from the viral vector. On PHD23, the beginning of the sensory learning phase, we injected the virus stereotactically into Area X to achieve spatial control of knockdown. Starting on PHD30, each pupil was kept in a sound isolation chamber, together with an adult male zebra finch as tutor. At the end of the learning phase at PHD90, the birds´ vocalization was recorded for subsequent song analysis (for timeline of experiments see Figure 3.15).

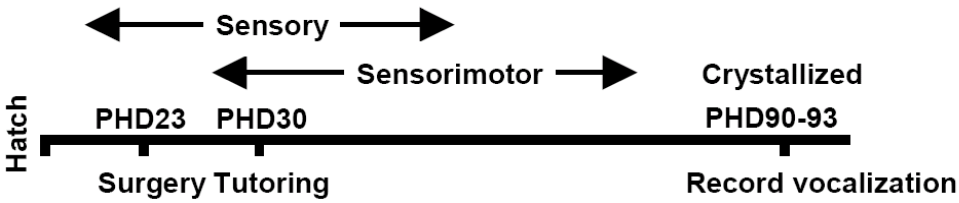


Figure 3.15 Timeline of experiments. By PHD20, fathers and older male siblings were removed from family cages to prevent experimental zebra finches from instructive auditory experience prior to the onset of tutoring. At the beginning of the sensory learning period on PHD23, virus was injected into Area X. From PHD30 on, injected birds were housed in sound-recording chambers together with an adult male zebra finch as tutor. We recorded the song of adult pupils on PHD90 to 93 using an automated recording system.

After song recording, brains were histologically analyzed for correct targeting of the virus to Area X. The lentivirus expressed the green fluorescent protein (GFP) reporter gene, allowing the detection of infected brain areas by fluorescence microscopy (Figure 3.16 H). Animals without GFP signal in Area X were excluded from further analysis (see supplementary information for detailed methods). On average 20.3% ± 9.9% (mean ± SE; n=24 hemispheres from 12 animals) of the total volume of Area X was infected. Within the injected region of Area X the virus targets ~ 90% of all neurons (Wada et al., 2006), among them the medium spiny neurons that express FoxP2 [Figure 3.16 C-E (Haesler et al., 2004)].

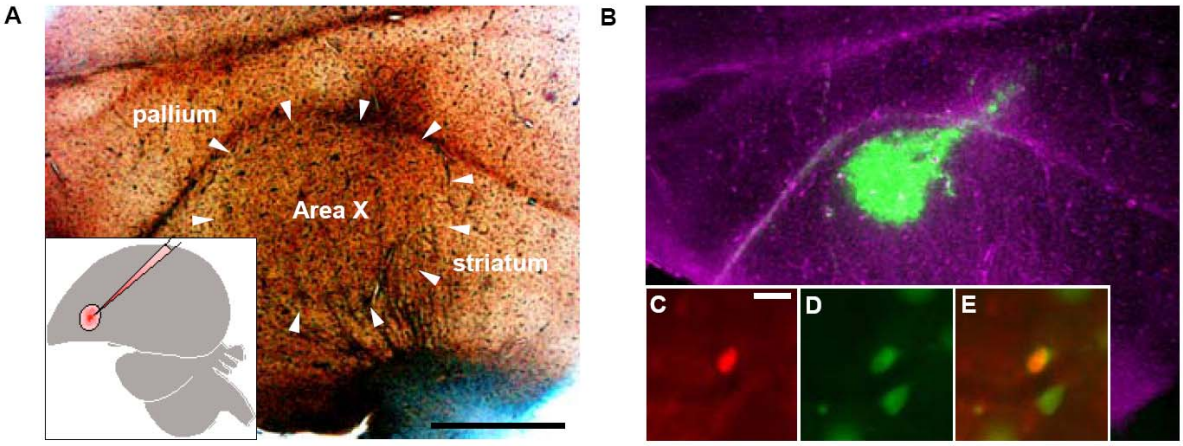


Figure 3.16 Targeting of virus into Area X. (A) Phase contrast image of a male zebra finch 50µm thick sagittal brain slice. Area X is outlined by white arrows (►; scale bar 1mm). The position of Area X within the brain is represented in the inset. **(B)** Fluorescent microscopy image of **(A)**. Virus infected cells expressed GFP (green). **(C)** FoxP2 immunostaining of a medium spiny neuron in Area X (red; scale bar 10µm). **(D)** The neuron shown in **(C)** also expressed viral GFP from injection with shControl. **(E)** Overlay picture of **(C)** and **(D)**.

To demonstrate that RNAi-mediated gene knockdown persists *in vivo* throughout the entire song learning phase, we used a virus expressing shRNA against the viral reporter GFP (shGFP) in conjunction with a virus expressing an shRNA, which does not have a target gene (shControl). We injected young zebra finches on PHD23 with equal amounts of shGFP and the non-targeting shControl virus in the left and right hemisphere, respectively. Analysis of GFP expression on PHD130 revealed that the shGFP-injected hemisphere had markedly reduced GFP signal compared to the shControl-injected hemisphere even more than 3 month post injection (Figure 3.17).

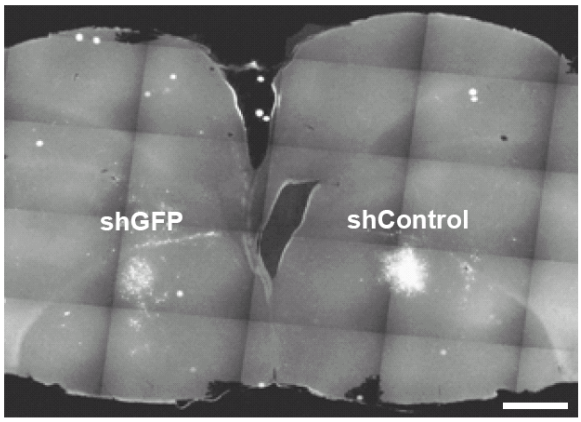


Figure 3.17 Lentivirus-mediated gene knockdown can persist long term. RNAi-mediated knockdown persisted for at least 3 month. Frontal 50µm thick brain slice of zebra finch injected with the indicated virus 105 days prior to perfusion. The intensity of GFP expression, visible as white signal, was reduced in the left

hemisphere injected with the virus targeting GFP, compared to the hemisphere which was injected with shControl (scale bar 1mm).

We identified two short hairpin RNA´s with different target sequences in the FoxP2 mRNA (shFoxP2-f and shFoxP2-h) that strongly reduced FoxP2 levels *in vitro* (Figure 3.18).

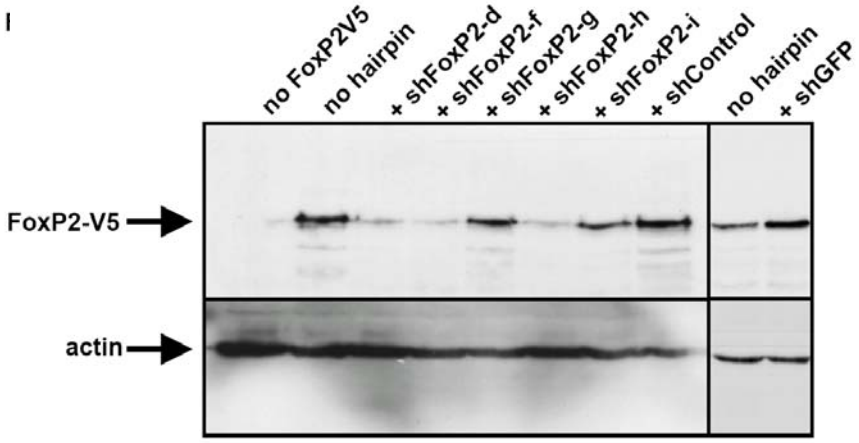


Figure 3.18 Identification of functional shRNA targeting FoxP2 *in vitro*. Hairpin expression constructs were tested for their knockdown efficiency in HEK293-T cells by simultaneous overexpression of zebra finch FoxP2, tagged with the V5 epitope and one of different hairpin constructs (shFoxP2-d - shFoxP2-i). Subsequent Western Blot analysis using a V5 antibody revealed three hairpins (shFoxP2-d, shFoxP2-f and shFoxP2-h) that reduced FoxP2 levels. Neither, the non-targeting control hairpin (shControl) nor the hairpin targeting GFP (shGFP) reduced FoxP2 overexpression. Immunostaining with an actin antibody revealed equal loading of protein samples.

To quantify *in vivo* knockdown efficiency, we determined FoxP2 expression levels on PHD50, the time of peak FoxP2 expression (Haesler et al., 2004), by Real-Time PCR in birds injected on PHD23 with shFoxP2 in one hemisphere and shControl into the contralateral hemisphere. For each hemisphere, FoxP2 mRNA levels were normalized to two independent RNAs coding for the housekeeping genes Hmbs and Pfkp. FoxP2 mRNA was reduced by ~70% in shFoxP2-injected compared to shControl-injected Area X (Figure 3.19). Taken together, these data demonstrate that virus-mediated RNAi can induce robust, long-lasting knockdown of gene expression in Area X.

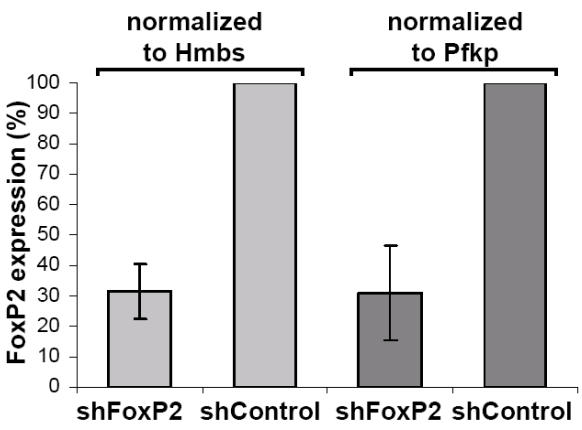


Figure 3.19 Quantification of *in vivo* knockdown efficiency. Real-time PCR quantification of FoxP2 expression in Area X on PHD50. Animals were injected with shControl in one hemisphere and shFoxP2 virus in the contralateral hemisphere, on PHD23. Bars represent relative gene expression between shControl and shFoxP2-injected hemispheres, normalized to either Hmbs or Pfkp as indicated [± standard deviation (STDEV); n=5 animals].

To rule out possible side effects of FoxP2 knockdown on cellular survival in Area X, we investigated apoptosis in Area X 6 days post surgery with terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL). The TUNEL method detects genomic DNA double strand breaks characteristic of apoptotic cells. Apoptotic cells were successfully detected (Figure 3.20), however, from 1149 GFP-positive cells counted in 6 hemispheres from 3 animals, only 5 were TUNEL-positive. ShControl-injected and uninjected animals had similar low levels of apoptotic cells suggesting that cell viability was not affected by knockdown of FoxP2 or virus injection.

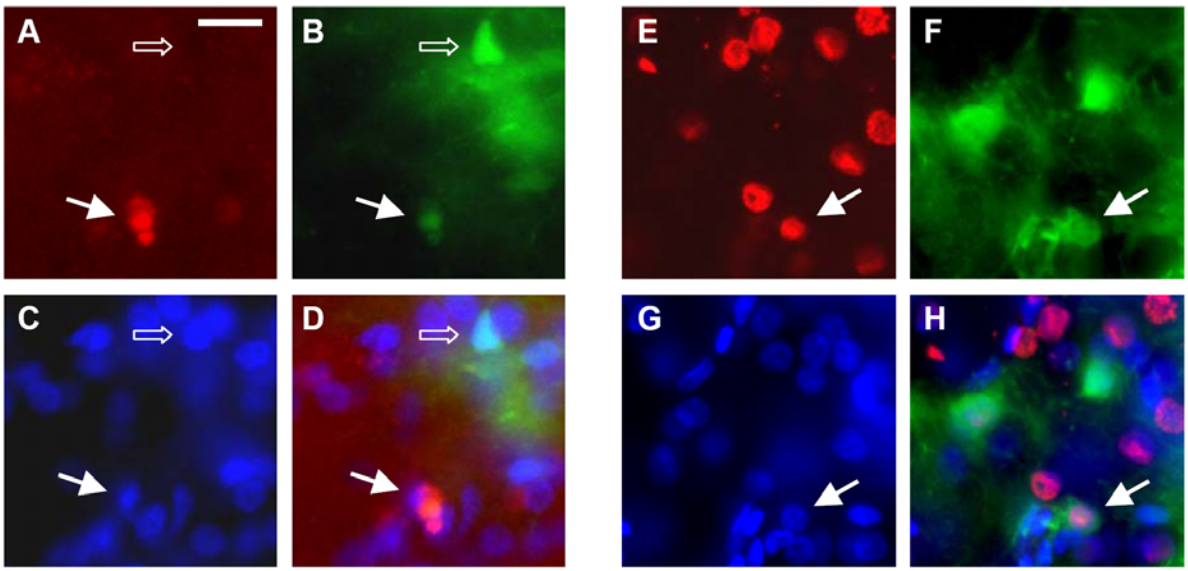


Figure 3.20 Infection with shFoxP2-virus does not induce apoptosis. (A) We labeled apoptotic cells in 50µmsagittalsections from PHD29 male zebra finch brains, injected with shFoxP2 virus on PHD23. DNA double strand breaks characteristic of apoptotic cells were detected using the TUNEL method withFluorescein(FITC)-marked nucleotides (green). To increase the signal intensity, we subsequently stained the sections by fluorescent immunohistochemistry with an anti-FITC antibody (red). The filled white arrow points to TUNEL-labeled cells not infected by shFoxP2. **(B)** The cells shown in **(A)** are also weakly FITC-labeled(green). The light white arrow points to a shFoxP2-infected cell, expressing the viral reporter GFP but showing no sign of TUNEL-labeling **(A)**. **(C)** DAPI staining identifies cellular nuclei. The apoptotic cells (white arrow) contain condensed DNA typical of apoptosis. **(D)** overlay picture of **(A-C)**. **(E)** As positive control for the TUNEL method we treated a section adjacent to that shown in **(A-D)** for 10min with DNAse to artificially induce DNA double strands breaks. **(E-H)** Numerous TUNEL positive cells were now detected, among them a virally infected cell expressing GFP **(white arrow in (E-H)**. Colors as in **(A-D)**. Scalebar in (A) 10µm.

3.2.2 Behavioral consequence of FoxP2 knockdown

We analyzed the behavioral consequences of FoxP2 knockdown in Area X during song learning. Adult zebra finch song is composed of different sound elements, also called syllables that are separated by silent intervals. Syllables are rendered in a stereotyped sequential order, constituting a motif. During a song bout, a variable number of motifs are sung in short succession. When a juvenile male finch is tutored individually by one adult male, the pupil learns to produce a song that strongly resembles that of the tutor (Tchernichovski and Nottebohm, 1998). We therefore determined learning success by the degree of acoustic similarity between pupil and tutor songs. Animals with reduced FoxP2 levels in Area X imitated tutor songs with less fidelity than control animals. The

comparison of sonograms from shControl (Figure 3.21 A) and shFoxP2-injected pupils (Figure 3.21 B and C) with their respective tutors shows the characteristic song abnormalities caused by reduction of FoxP2. Typical features of FoxP2 knockdown birds included syllable omissions (Figure 3.21 B, syllable B; Figure 3.21 C, syllables C, D, F, G), imprecise copying of syllable duration (Figure 3.21 B, syllable D shortened; Figure 3.21 C syllable E longer) and inaccurate imitation of spectral characteristics (Figure 3.21 B, syllable D; Figure 3.21 C, syllable E). In 4 out of 7 knockdown animals the motif contained repetitions of individual syllables or syllable pairs (e.g. Figure 3.21 B and C). In contrast, none of the control or tutor motifs contained repeated elements. Disregarding omitted and repeated syllables, the sequential order of the syllables in the motif followed the order of the syllables in the tutor.

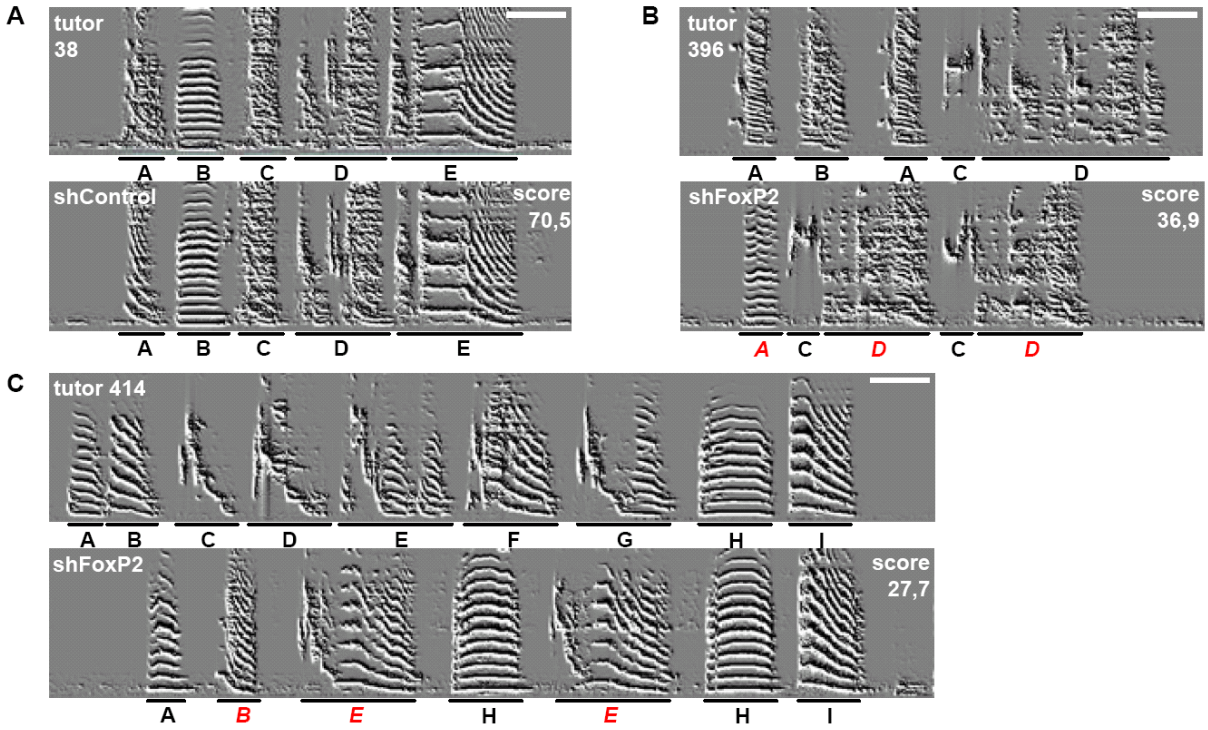


Figure 3.21 Song learning in FoxP2 knockdown birds. Sonograms from FoxP2 knockdown and control birds. Each sonogram depicts a representative motif of one animal (scale bars 100ms, frequency range 0-8600Hz). Tutor syllables are underlined with black bars and identified by letters. The identity of pupil syllables was determined by similarity comparison to tutor syllables using SAP software. Imprecisely copied pupil syllables are designated with red, italic letters. **(A)** tutor #38 and shControl-injected zebra finch. **(B)** tutor #396 and shFoxP2-injected animal **(C)** tutor #414 and shFoxP2-injected animal. The motif imitation scores from each pupil to the respective tutor are indicated in the right upper corner of the sonograms.

We quantified song learning success with Sound Analysis Pro [SAP+ (Tchernichovski et al., 2001)]. Similarity between pupil and tutor song was measured by pairwise comparison of pupil and tutor motifs. SAP provides a similarity score that indicates how much of the tutor sound material was copied by the pupil. This similarity score was significantly lower in knockdown compared to control animals (Figure 3.22). Counting the number of visually identified syllables copied from the tutors confirmed that knockdown animals imitated fewer syllables (Figure 3.23). Of note, there was no difference in the volume of Area X targeted with the different shFoxP2 and shControl viruses (Figure 3.24).

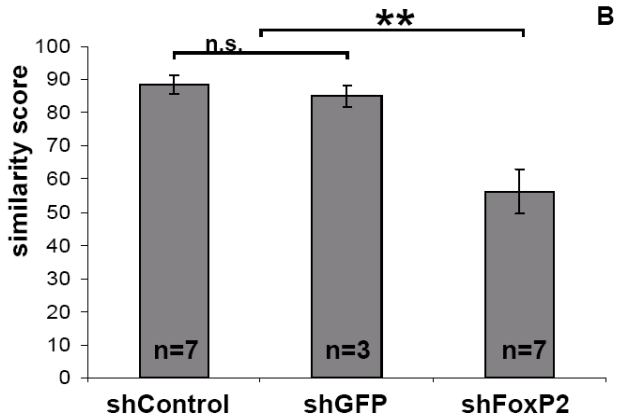


Figure 3.22 Knockdown of FoxP2 reduces motif similarity. The mean similarity between pupil and tutor motifs was significantly lower in shFoxP2 injected animals than in shControl and shGFP-injected birds, indicating that knockdown animals copied less acoustic material from their tutors [± standard error of the mean (SEM); two-tailed t-test, ***P*<0.001, Bonferroni-corrected α-level]. There was no significant difference between shGFP and shControl injected animals [not significant (n.s.), *P*>0.5].

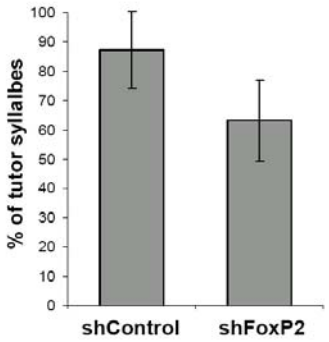


Figure 3.23 Manual counting of syllables copied by knockdown and control animals. All syllables that matched a tutor syllable by visual inspection on a sonogram, were counted for shFoxP2 and shControl-injected animals. Bars represent the mean percentage of tutor syllables copied by the pupils (±STDEV, two-tailed t-test *P*<0.001).

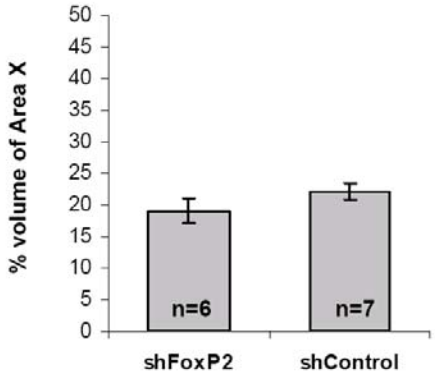


Figure 3.24 The volume of Area X targeted by virus injection was not significantly different in FoxP2 knockdown (shFoxP2) and control animals (shControl; two-tailed t-test *P*>0.5). Bars represent the percentage of total Area X volume, averaged across hemispheres, expressing the viral reporter GFP (±SEM).

Even though knockdown animals were able to copy tutor syllables, imitation appeared to be less precise than in control animals. We therefore obtained motif accuracy values in SAP from pairwise motif comparisons between pupil and tutor. The average accuracy per motif was lower in knockdown animals than in shControl and shGFP-injected zebra finches (Figure 3.25).

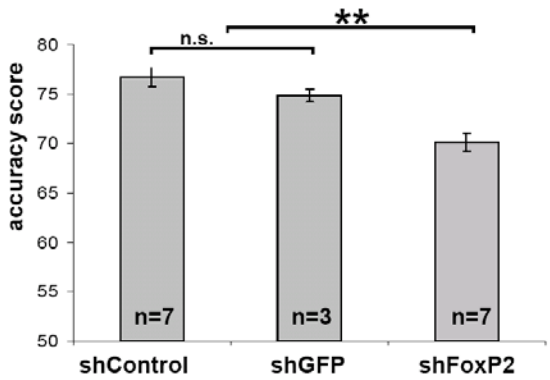


Figure 3.25 Knockdown of FoxP2 reduces motif accuracy. Average motif accuracy was significantly reduced in shFoxP2 knockdown animals compared to control animals, indicating that they imitated their tutors less exactly (± SEM; two-tailed t-test, ***P*<0.001, Bonferroni-corrected α-level). shControl and shGFP-injected birds copied their tutors with similar precision (n.s., *P*>0.3).

To reveal the contribution of individual syllables to the average motif accuracy, we also compared syllable pairs between tutors and pupils using a syllable identity score. The reduced precision of syllable imitation was not skewed towards particular syllables or syllable types (Figure 3.26), pointing to a generalized problem with copying accuracy.

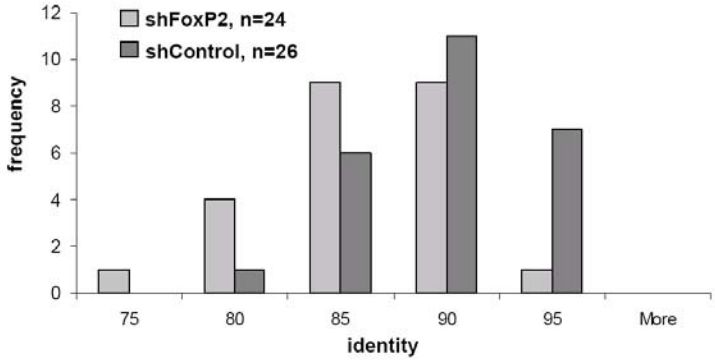


Figure 3.26 Knockdown zebra finches copied all syllables less precisely. Histogram of syllable identity scores obtained from pairwise comparison of visually identified pupil/tutor syllable pairs. In FoxP2 knockdown animals the distribution of the scores was shifted towards lower values indicating that all syllable types were affected.

To get a comprehensive view on how well pupil and tutor motifs matched acoustically, we calculated an overall motif imitation score by multiplying motif similarity and motif accuracy scores. Knockdown animals scored significantly lower than control animals (Figure 3.27).

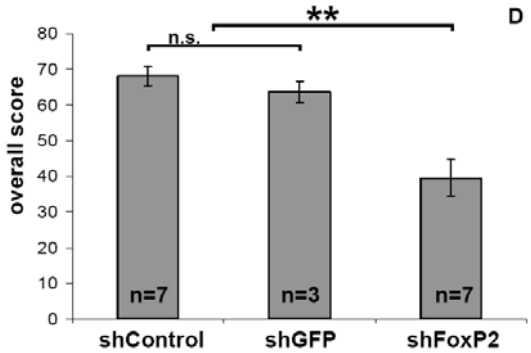


Figure 3.27 FoxP2 knockdown birds have reduced overall imitation scores. The overall imitation score was significantly lower in shFoxP2-injected birds, than in the control group (± SEM; two-tailed t-test, $**P<0.001$, Bonferroni-corrected α-level). Birds injected with shControl or shGFP virus respectively did not score significantly different (n.s., $P>0.3$).

Furthermore, both shFoxP2 hairpins (shFoxP2-f and shFoxP2-h) affected the motif imitation score to a similar degree, which is consistent with their comparable efficiency in reducing FoxP2 mRNA *in vitro* (Figure 3.28).

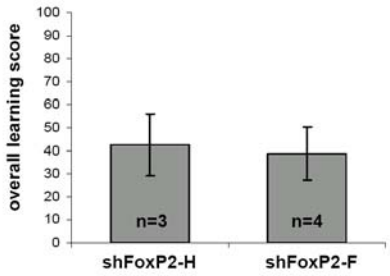


Figure 3.28 Both hairpin constructs targeting FoxP2 impaired song-learning to a similar degree. Bars indicate the overall imitation score of zebra finches injected with either shFoxP2-F or shFoxP2-H (±STDEV).

To rule out that the reduced learning success of knockdown animals was related to specific song characteristics of the tutors or their lacking aptitude for tutoring, we also used tutors of knockdown birds to train control animals. Direct comparison of the motif imitation scores from control and knockdown pupils tutored by the same male revealed that knockdown birds learned on average 22.6% ± 5.4% (mean ± standard error of the mean, SEM; n=8) worse than control animals.

Since the shControl hairpin, in contrast to shFoxP2, has no target gene, it might not stably activate the RNA-induced silencing complex (RISC) essential for knockdown of gene expression. However, recent work suggests an involvement of the RISC in the formation of long-term memory in the fruitfly (Ashraf et al., 2006). To address a possible influence of RISC activation on song learning we compared song imitation in shGFP-virus injected birds, where virally expressed GFP is lastingly knocked down (Figure 3.17) and shControl injected animals. Motif similarity and motif accuracy scores did not differ significantly between shGFP-injected and shControl-injected animals, ruling out an effect of RISC activation on song learning (Figure 3.22 and Figure 3.25).

To investigate the accuracy of syllable imitation on the level of individual acoustic features, we extracted the mean pitch, mean frequency modulation (FM, change of frequency in time), mean entropy and mean pitch goodness (PG, stability of pitch in time) as well as the mean duration from all syllables and compared the values between corresponding pupil-tutor syllable pairs. For each pupil syllable we calculated the absolute deviation of the features from those of the respective tutor syllable. In all features, the syllables produced by knockdown animals deviated more from the tutor syllables than the

syllables of control animals; significant differences were observed for the duration and mean entropy (Figure 3.29).

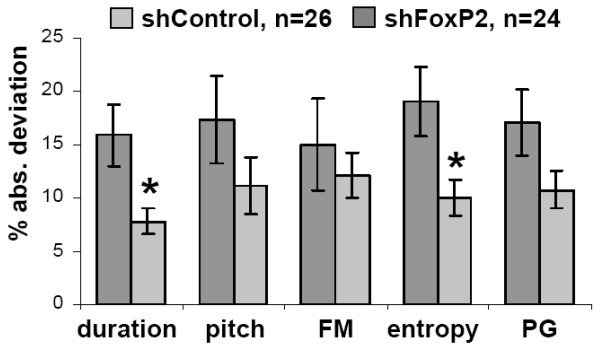


Figure 3.29 Analysis of individual syllable features. Comparison of mean acoustic feature values between pupil syllables and their respective tutor syllables revealed that the absolute deviation in % from the tutor mean values tended to be higher for shFoxP2-injected zebra finches than for shControl-injected animals. The mean values for syllable duration and entropy deviated significantly more from the corresponding tutor values in shFoxP2 than in shControl-injected birds (FM, frequency modulation; PG, goodness of pitch; ± SEM; two-tailed t-test, *P<0.05 Bonferroni-corrected α-level).

Area X is part of a basal ganglia-forebrain circuit, termed AFP (anterior forebrain pathway), which is homologous to mammalian cortical-basal ganglia loops (Doupe et al., 2005). The pallial (i.e. cortical) target of the AFP, the nucleus lMAN (lateral magnocellular nucleus of the nidopallium), may act as a neural source for vocal variability in juvenile zebra finches (Olveczky et al., 2005; Scharff and Nottebohm, 1991). In adult zebra finches neural variability in AFP outflow is similarly associated with the variability of song (Kao et al., 2005) and experimental manipulations inducing adult song variability require an intact AFP (Brainard and Doupe, 2000; Williams and Mehta, 1999). To explore AFP function in FoxP2 knockdown and control zebra finches, we investigated the variability of their songs. First we quantified the variability of syllable duration between different renditions of the same syllable. The coefficient of variation of syllable duration was significantly higher in knockdown than in control birds and tutors, suggesting difficulties with the precise motor coordination on short temporal scales (Figure 3.30). Notably, the timing of syllables in control animals (shControl and shGFP) was as stable as in tutors (Figure 3.30). Syllable duration in tutor and control birds varied in the same range as reported previously (Glaze and Troyer, 2006), emphasizing how tightly adult zebra finches control syllable length.

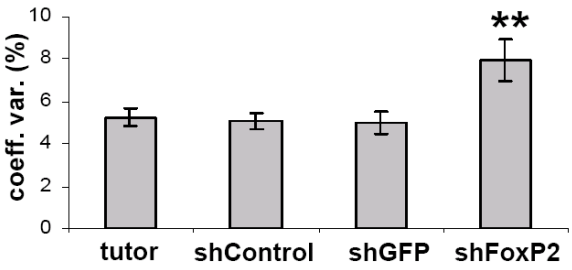


Figure 3.30 Syllable length varied more from rendition to rendition in knockdown birds (shFoxP2) than in controls (shControl and shGFP) and tutors, as indicated by a higher mean coefficient of variation of syllable duration (± STDEV, ANOVA, LSD post hoc test; shFoxP2 versus control: $P<0.002$; shFoxP2 versus tutors: $P<0.001$; no difference between control and tutors: $P>0.3$).

In contrast to the variability of syllable duration, the mean duration of syllables from the repertoire of knockdown and control birds was undistinguishable (Figure 3.31).

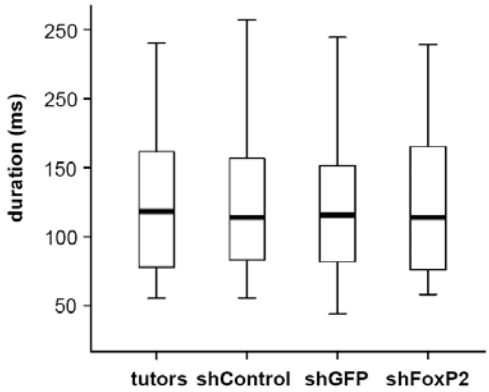


Figure 3.31 Syllables from knockdowns and control zebra finches were similar in their duration. Box plots represent the mean duration of all syllables from tutors and each experimental group (shControl, shGFP and shFoxP2-injected zebra finches). Boxes indicate the interquartile range of the distribution.

Next, we explored the variability of acoustic features using the syllable identity score mentioned above. Pairwise comparison between different renditions of the same syllable revealed significantly higher syllable variability in shFoxP2-injected animals than in control animals and tutors (Figure 3.32). As expected, shControl and shGFP-injected animals and tutors performed their syllables with equal stability (Figure 3.32).

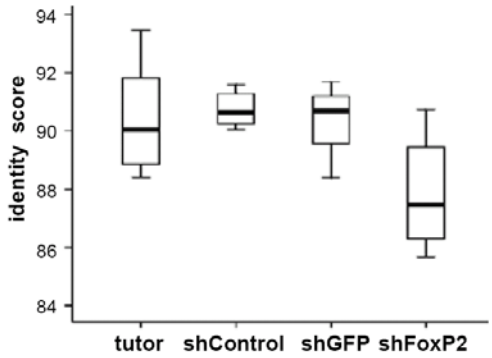


Figure 3.32 Variable song performance in FoxP2 knockdown zebra finches. Acoustic variability of syllables from rendition to rendition was higher in shFoxP2-injected than in control animals (shGFP and shControl injections), as indicated by significantly lower syllable identity scores (ANOVA, LSD post hoc test; shFoxP2 versus control: $P<0.009$; shFoxP2 versus tutors: $P<0.018$). Control birds sang as variable as the tutors (ANOVA, LSD post hoc test; no difference between control and tutors: $P>0.99$). Boxes indicate the interquartile range of the distribution.

Finally, we analyzed the sequential syllable order, also referred to as syntax, over the course of many motifs, using a syntax consistency score. The mean syntax consistency was similar in shControl and shFoxP2 animals (Figure 3.33).

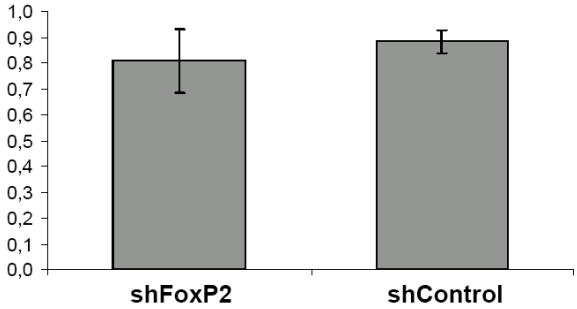


Figure 3.33 The consistency of motif performance was similar in shFoxP2 and shControl-injected birds (two tailed t-test, $P>0.4$). The syntax consistency score was calculated based on the entropy of 300 successive syllables (1-entropy).

3.3 Comparison of FoxP2 Sequences from Birds and the Crocodile

To address the question of whether the FoxP2 sequence of birds that learn their song differed from those whose song is not aquired, we cloned and sequenced the FoxP2 sequences from 7 avian vocal learners, 2 non-learners. The group of vocal learners

included the Zebra Finch (*Taeniopygia guttata)*, the song sparrow (*Melospiza melodia*), parrot (*Melopsittacus undulatus*), the rufous-breasted hermit hummingbird (*Glaucis hirsuta*), the Ruby-throated or North Carolina Hummingbird (*Archilochus colubris*), Sombre Hummingbird *(Aphantochroa cirrhochloris*) and the canary (*Serinus canaria*). Vocal non-learners were represented by the phoebe *(Sayornis phoebe*), the pigeon or rock dove (*Columbia livia*) and the chicken (*Gallus gallus*). The FoxP2 sequence from the latter was obtained from Genbank (accession number AAW28117). To investigate if the FoxP2 sequence diverged specifically in the avian lineage we further cloned and sequenced FoxP2 from a reptilian, the American alligator (*Alligator mississippiensis*).

All cloned FoxP2 transcripts were unambiguously identified by their strong homology to FoxP2 from other vertebrates. Moreover, we obtained several isoforms of FoxP2 transcripts for each species. To maximize sequence coverage we used the information from all transcripts from each species to assemble one single full-length cDNA and protein sequence per species. This approach yielded an average coverage of approximately 14 times. The phylogenetic relationship between the FoxP2 DNA sequences replicates the known relationship among these species (Figure 3.34) and (Sibley and Ahlquist, 1990).

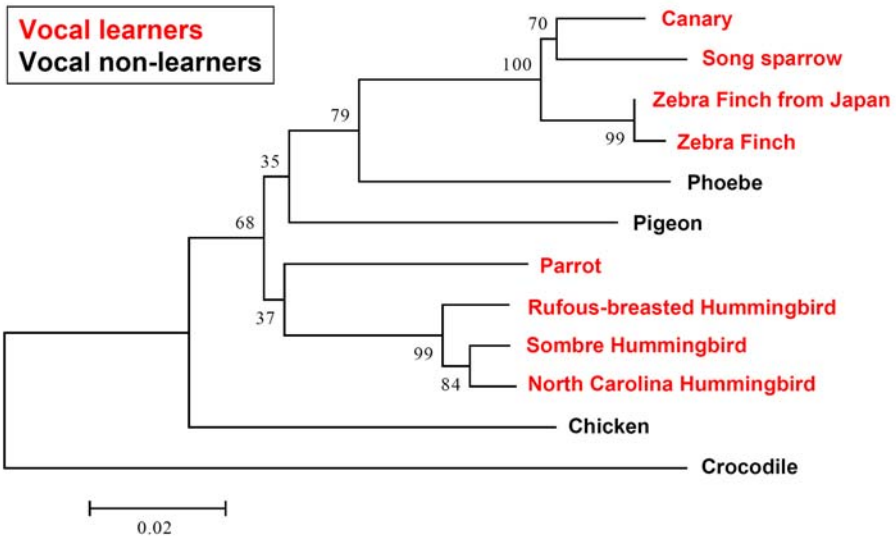


Figure 3.34 Phylogenetic relationship between FoxP2 genes from birds and the crocodile. Species capable of vocal learning are marked in red, non-vocal learners are marked in black. The phylogenetic tree was calculated according to the method of (Li et al., 1985). Bootstrap values on each branching point indicate in percent how well branching of the tree is supported by the experimental data.

The deduced FoxP2 protein sequence was variable at only 10 amino acid positions across the FoxP2 sequences from all species (Figure 3.35). Of note, in none of the species studied the human-specific amino acid was found (Figure 3.35).

```
Positions in FoxP2:
   6,42,78,79,80,236,250,304,326,357

Sombre Hummingbirds           A T G S D N S T N V
Canary                        - - - - E - - - - -
Chicken                       - - - T - - - - - -
Crocodile                     - - - - - - - - - I
Rufous-breasted Hummingbird   - - - - - - - - - -
North Carolina Hummingbird    - - - - - - - - - -
Parrot                        - - - - - - - - - -
Pheobee                       - - - - E - - - - -
Pigeon                        - - - - - - - - - -
Song sparrow                  - - - - E - - - - -
Japanese zebra finch          - - - - E - - - - -
Zebra finch                   - S - - E - - - - -
Mouse                         - S S - E S A - - I
Gorilla                       - S S - - S A - - I
Chimp                         - S S - - S A - - I
Rhesus monkey                 - S S - - S A - - I
Orang utan                    V S S - - S A - - I
Human                         - S S - - S A N S I
```

Figure 3.35 Summary of variable amino acids in the FoxP2 sequences from different species. The position of variable amino acids are indicated by numbers with respect to the first amino acid in the human FoxP2 sequence. All amino acids not shown here are identical between the species studied.

To test if a particular variant of FoxP2 segregates with the ability for vocal learning we mapped all amino acid substitutions onto the phylogenetic tree constructed from the FoxP2 DNA sequences. There was no correlation between a particular variant of FoxP2 and the ability of vocal learning (Figure 3.36).

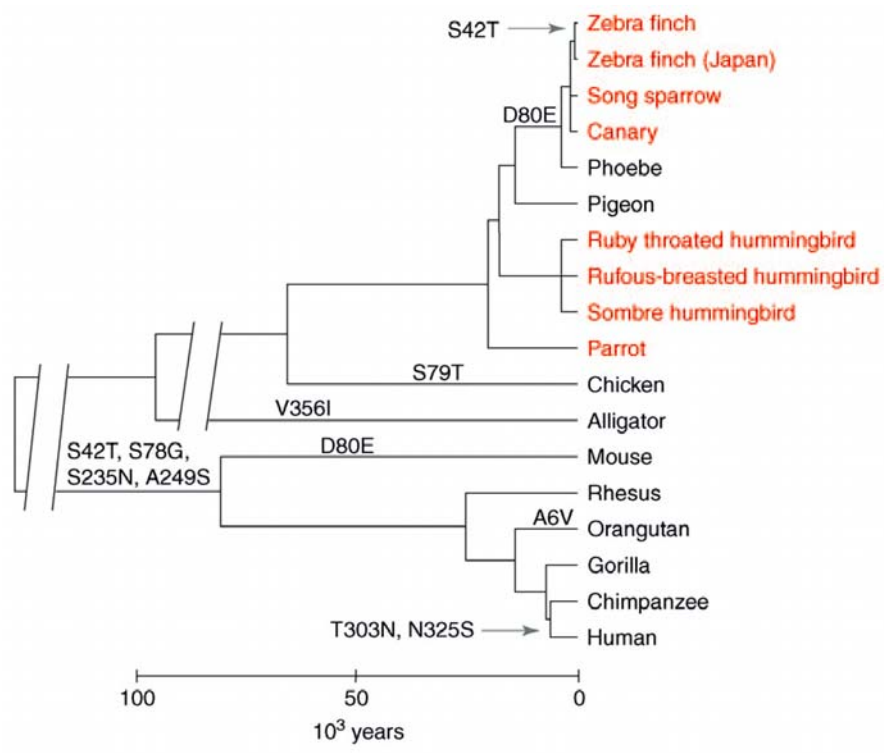


Figure 3.36 FoxP2 amino acid changes mapped on the phylogenetic tree of the species indicated. The seven song-learning avian species are marked in red, all other species, including the three non-song-learning birds, appear in black. Amino acid changes were inferred by parsimony and the phylogenetic tree of the birds is based on that of Wada et al. (Wada et al., 2004). The topology of the tree inferred from silent substitutions in FoxP2 agrees overall with the tree shown here. Note that two amino acid changes have occurred two times independently (D80E and S42T) and that the direction of the four changes on the base of the tree cannot be inferred without an additional outgroup. Sequence positions are based on the human protein sequence. The timescale (in 10^3 years) offers a rough estimate for most divergence times.

4 Discussion

4.1 FoxP2 Expression in Avian Vocal Learners and Non-learners

In order to study the function of FoxP2 in the songbird model system we first identified the FoxP2 homolog of a commonly studied songbird, the zebra finch. Since *in vitro* the FoxP2 protein can heterodimerize with its closest homolog FoxP1 to regulate transcription (Li et al., 2004) we also identified the FoxP1 gene from the zebra finch. This allowed the investigation of the putative sites of action of FoxP2/FoxP1 heterodimers. The protein sequences of both genes were extraordinarily similar to those of other vertebrate FoxP2 proteins which confirmed the successful identification of functional homologues from the zebra finch. By investigating the brain expression pattern of FoxP2 and FoxP1 we inferred information about the function of FoxP2 in the songbird brain. Given that only a few species are capable of acquiring their vocal repertoire through imitation learning, we contrasted the expression patterns of songbirds with those of other birds not capable of song learning. In total, FoxP2 brain expression was investigated in 11 different bird species.

Both vocal learners and vocal non-learners had similar developmental onset of FoxP2 expression in comparable brain regions and equivalent expression patterns in adults. The strongest signal was consistently observed in the striatum of the basal ganglia, song nuclei of the dorsal thalamus and midbrain, the inferior olive, and the Purkinje cells of the cerebellum. Less intense, but consistent, expression was observed in various nuclei connected to these regions. This expression pattern was also found by (Teramitsu et al., 2004) in the zebra finch brain. We also noted a similar expression pattern in a closely related reptilian species, the crocodile. The striatal and subtelencephalic sites of FoxP2 expression in birds and crocodile are homologous to those found in the human, rat, and mouse brain (Ferland et al., 2003; Lai et al., 2003; Takahashi et al., 2003). In the pallium all birds expressed relatively little FoxP2. Only the mesopallium of some species expressed higher levels of FoxP2. Mammals also express little FoxP2 in pallial regions, with the exception of cortical layer 6 (Ferland et al., 2003). These differences in cortical/pallial FoxP2 expression between mammals and birds are difficult to interpret because direct homologies between most avian and mammalian pallial areas remain unresolved (Reiner et al., 2004). The pallium of the avian telencephalon possesses a

nuclear organization, whereas that of mammals shows a layered organization. There are no obvious homologies between the avian mesopallium and cortical layer 6. The main projection of the mesopallial vocal nuclei and other mesopallial areas are to arcopallial, nidopallial, and striatal areas (Brauth et al., 2001; Csillag, 1999; Durand et al., 1997), whereas those of layer 6 in mammals are to the dorsal thalamus in addition to other cortical (pallial) layers (Ferland et al., 2003). These results suggest that strong striatal and subtelenphalic FoxP2 expression in birds and mammals was inherited from a common stem-amniote ancestor (Evans, 2000). In contrast, the species-specific pallial expression patterns might have rather been gained or lost independently in each species.

FoxP1, the closest homolog of FoxP2 is highly expressed in striatal and subtelencephalic sites of the bird brain, which is concordant with the mammalian FoxP1 expression pattern (Ferland et al., 2003; Teramitsu et al., 2004). Therefore it seems reasonable to conclude that the FoxP2 and FoxP1 proteins can act in concert to regulate gene expression in regions of the bird brain which have overlapping expression patterns. In pallial regions birds expressed FoxP1 more widespread relative to FoxP2. The highest FoxP1 expression occurred in the mesopallium and in vocal nuclei HVC and RA of songbirds, but notably low levels in the tissue surrounding HVC and RA. Mammals also express widespread FoxP1 levels in the pallium, cortical layers 3-5 during development, and also in layer 6 during adulthood (Ferland et al., 2003).

The striking conservation of the FoxP1 and FoxP2 sequence and expression pattern in avian, reptilian, and mammalian brains, regardless of whether they learn to vocalize or not, suggests that FoxP2 has a more general role than to enable vocal learning. The evolutionary ancient transcription factor FoxP2 (Mazet et al., 2003) might be implicated in shaping cerebral architecture, perhaps via restriction of certain neuronal lineages, as reported recently for the forkhead box transcription factor FoxG1 (Hanashima et al., 2004). If FoxP2 was involved in the development and/or function of subtelencephalic and striatal sensory and sensory-motor circuits, this could create a permissive environment on which vocal learning can evolve if other factors come into play. Given the prominent role of many other forkhead transcription factors in early development, this is a not an unlikely scenario (Carlsson and Mahlapuu, 2002). Support of this notion also stems from the fact that regions of early FoxP2 expression in the avian embryo are sources of inductive signals that organize adjacent neuroepithelium and neuronal migration during early development.

The analysis of FoxP2 expression during different stages of song ontogeny revealed that FoxP2 expression was elevated in Area X at the time when young zebra finches learn to imitate song and during the time when adult canaries remodel their songs. In addition, in adults of six different species, Area X (and in the equivalent structure VAS in the hummingbird) showed consistent differences in FoxP2 expression, being either higher or lower than the surrounding striatum, in a pattern consistent with periods of change in vocal behavior. Lesions of Area X in zebra finches during vocal learning result in adult song production that is more plastic than when Area X is intact (Scharff and Nottebohm, 1991; Sohrabji et al., 1990), suggesting that Area X helps generate song stability. If FoxP2 acts as a transcriptional repressor in the brain, as it does in the lung (Li et al., 2004; Lu et al., 2002; Shu et al., 2001) then the higher levels found during periods of vocal plasticity might suggest that FoxP2 represses genes involved in neural stability in Area X, thus acting as a plasticity promoting factor. Alternatively it could also restrict plasticity by repressing gene expression initiated by recurrent neuronal activation.

In contrast to a recent report showing down-regulation of FoxP2 in Area X of adult zebra finches during undirected song (Teramitsu and White, 2006), we did not find evidence for online-regulation of FoxP2 expression by undirected singing. This apparent discrepancy might in part be explained by the different experimental conditions used in the two studies. The zebra finches in (Teramitsu and White, 2006) sang in a range of 300 and 900 motifs in 2h before being sacrificed, whereas the birds in this study sang 300 bouts, which usually consisted of several motifs, in 5h, with 43 bouts in the last 30min before being sacrificed. Moreover, we observed relatively low FoxP2 expression levels in adult Area X of non-singing birds, as indicated by an almost negative ratio between Area X and surrounding striatum. (Teramitsu and White, 2006) find higher FoxP2 expression levels in non-singing birds as indicated by a positive ratio of approximately 1.2. These different results might originate from the different experimental procedures used to ensure that the birds were not singing. Whereas the birds of this study were kept in the dark for a whole night and sacrificied immediately in the morning, the silent birds from (Teramitsu and White, 2006) were kept from singing by the experimenter sitting nearby. Finally, we found adult FoxP2 expression levels to be relatively variable. Of 10 adult silent male zebra finches examined, 7 had expression levels in Area X similar to the region surrounding it, two slightly lower and one slightly higher (data not shown). Due to this experimental variability, we might

have missed to observe a downregulation of FoxP2 by singing. It will be interesting to reinvestigate juvenile zebra finches for an activity-dependant regulation of FoxP2 with different experimental paradigms.

How can the FoxP2 expression pattern in avian vocal learners be interpreted in the context of the speech pathology in humans? It has been suggested that the speech and language pathology in humans with FoxP2 mutations consists of an orofacial dyspraxia core deficit (Marcus and Fisher, 2003). This could be primarily attributable to a lack of muscle control over the speech apparatus. However, the expression data suggests that FoxP2 is for the most part expressed in afferent sensory pathways and in the striatal projection neurons, which are the site of convergence for both pallial and subpallial projections. Learning to imitate acoustic signals requires the integration of sensory information with motor output. The basal ganglia as well as the cerebellum in all vertebrates integrate afferent sensory information with descending motor commands and thus participate in the precise control of temporally sequenced muscle movements (Doyon et al., 2003). Although in humans the basal ganglia and the cerebellum have attracted far less attention than the cortical speech and language areas, there is increasing awareness that the basal ganglia and cerebellum are not only essential for the execution but might also be required for the acquisition of human vocal behavior (Lieberman, 2001; Marien et al., 2001). In addition, many sites of FoxP2 expression, such as the inferior olive-Purkinje cell pathway, the optic tectum, and the striatum, are known substrates for experience-dependent plasticity (Doyon et al., 2003; Hyde and Knudsen, 2000; Krupa and Thompson, 1997). Given that the striatum is also the site of functional and structural abnormalities in individuals with DVD, it seems possible that FoxP2 is involved in the acquisition of motor programs for moment-to-moment control over orofacial muscles during speech.

In summary, FoxP2 has a characteristic expression pattern in a brain structure uniquely associated with learned vocal communication, Area X in songbirds. Moreover, the upregulation of FoxP2 in Area X during times of vocal plasiticity is compatible with a direct involvement of FoxP2 in song learning. FoxP2 expression in the rest of the brain of birds that learn to sing and those that do not, predominates in sensory and sensory-motor circuits. These latter regions also express FoxP2 in mammals and reptiles. FoxP2 is virtually absent in nuclei of the songbird motor pathway. Taken together, this suggests that

FoxP2 may be important for the development and/or function of brain pathways including, but not limited to, those essential for learned vocal communication.

4.2 Analysis of FoxP2 Function *in vivo*

Since FoxP2 expression in Area X correlated with vocal plasticity we aimed to test whether FoxP2 was causally related to song learning in zebra finches. We used the method of lentivirus-mediated RNA interference (RNAi) to reduce the FoxP2 expression levels in Area X *in vivo* during the learning period of young zebra finches. This method allows spatially and temporally restricted genetic manipulations in the songbird brain *in vivo*. Of note, the lentiviral injection method is not limited for use with RNAi constructs, it also allows heterologous gene expression in the zebra finch brain. Given that many mammalian or chicken promoters are functional across species, this approach can be used in a broad range of experiments for gene function analysis in songbirds. A limitation of the viral injection technique is the experimental variability in the volume and the exact position of the targeted area. Moreover, although up to 38% of Area X could be targeted by injection of the virus, the distribution of the virus in the brain is restricted and the optimal distribution of virus in the brain has to be determined experimentally. Nevertheless, knockdown of FoxP2 in an average Area X volume of ~20%, caused learning deficits. Since Area X expands considerably in both size and cell number between injection at PHD23 and analysis at PHD90, the fraction of Area X infected during the song learning period was likely larger than that measured at PHD90 (Nordeen and Nordeen, 1988). These results are in line with a previous study on virally injected rats, in which blocking neural plasticity in 10-20% of lateral amygdala neurons was sufficient to impair memory formation (Rumpel et al., 2005).

Gene-specific knockdown by RNAi requires careful experimental control. The induction of RNAi by expression of shRNA can have non-specific side effects. including the activation of the interferon system and off-target effects (Jackson and Linsley, 2004). To avoid a confounding influence of unspecific RNAi effects we used two different shRNA with independent targets in the FoxP2 mRNA. Both hairpins had a similar knockdown efficiency and resulted in similar song deficits, indicating specific targeting of FoxP2. Expression of shRNA also seems to inhibit miRNA expression, suggesting that shRNAs compete with miRNAs. This might lead to an oversaturation of cellular RNAi pathways,

which can be fatal to the cell (Grimm et al., 2006). Using the TUNEL method, we ruled out that the knockdown induced apoptosis either by oversaturation of miRNA pathways or in consequence of the reduction of FoxP2. Since the RNA-induced silencing complex (RISC) which is essential for knockdown of gene expression is involved in the formation of long-term memory in *Drosophila* (Ashraf et al., 2006), we also used the shGFP virus to exclude a possible influence of lasting RISC activation on song learning. There were no significant differences in song learning behavior between shGFP injected and shControl injected animals. Taken together, these data confirm that the observed behavioral effects can be specifically attributed to knockdown of FoxP2.

The most striking behavioral consequence of FoxP2 knockdown was an incomplete and inaccurate imitation of the tutor song. The reduced accuracy of FoxP2 knockdown animals in copying tutor syllables raises the question whether knockdown animals were limited in generating particular sounds. However, this seems unlikely, because syllables with similar spectral features could be learned or omitted by the same animal (e.g. tutor syllables E and G and pupil syllable E in Figure 3.21 C). Also, omitted syllables did not differ in their spectral feature composition from those that were learned by knockdown animals (data not shown). Consistent with this, the distribution of mean syllable feature values (data not shown) and mean duration (Figure 3.31) across the syllable repertoire was indistinguishable between knockdown and control birds. Therefore, it is improbable that knockdown animals had problems with producing particular syllable types. Moreover, syntax was unaffected by knockdown of FoxP2. The mean syntax consistency was similar in shControl and shFoxP2 animals. Since stereotypy of motif delivery is a hallmark of ‘crystallized’ adult song, this suggests that both knockdown animals and controls had reached the end of the sensory-motor learning period (Williams, 2004). Therefore, knockdown of FoxP2 was unlikely to have caused a general developmental delay, but rather interfered specifically with the vocal imitation process.

The limited learning success of FoxP2 knockdown birds could result from an imprecise neural representation of the tutor model. Although there is evidence for an involvement of Area X in sensory learning (Singh et al., 2005), the fact that the upregulation of FoxP2 in Area X coincides with the sensory-motor phase makes an involvement of FoxP2 in sensor-motor integration more likely (Haesler et al., 2004). In light of the function of cortico-basal-ganglia loops in reinforcement-based motor learning (Graybiel, 2005) we propose

that FoxP2 knockdown animals failed to reconcile their own vocalization with the memorized tutor model. This hypothesis is supported by the phenotypic overlap of song deficits observed in FoxP2 knockdown birds and birds that were prevented from matching vocal output with memorized tutor song. For instance, perturbed auditory feedback provokes syllable repetitions (Leonardo and Konishi, 1999) and juvenile Area X lesions increase the variability of syllable duration (Scharff and Nottebohm, 1991). Given that electrolytic lesions and specific gene knockdown in a particular cell type are different experimental approaches, it is not surprising that song deficits of FoxP2 knockdown birds were not identical to those of birds with early Area X lesions. Different from FoxP2 knockdown, juvenile Area X lesions result in reduced sequence consistency. Moreover the repertoire of birds with juvenile Area X lesions contains unusually long syllables (Scharff and Nottebohm, 1991) which was not observed in FoxP2 knockdown finches (Figure 3.31).

How does FoxP2 affect song learning and vocal variability? In Area X, pallial auditory and song motor efference information converges with nigral dopaminergic reinforcement signals in the medium spiny neurons, which express FoxP2 (Reiner et al., 2004). Therefore, the integration of these signals provides a candidate mechanism for tuning the motor output to the tutor model during learning. The increase of FoxP2 expression in Area X of zebra finches during times of vocal plasticity could be functionally related to this process. FoxP2 might mediate adaptive structural and functional changes of the medium spiny neurons while the song is learned. In canaries, increased FoxP2 expression in the fall months might similarly be involved in seasonal song modifications. Since FoxP2 is a transcription factor, it could act by positively or negatively regulating plasticity-related genes. Neuronal plasticity can indeed be associated with large-scale changes in gene expression (Tropea et al., 2006) and these gene expression changes are likely mediated through key transcription factors that integrate neural activity on the cellular level. An example of a transcription factor acting positively in this process is the cyclic AMP response element binding protein (CREB) in the striatum which was shown to be necessary for synaptic plasticity and procedural memory formation (Pittenger et al., 2006). Since overall, neural plasticity has to be balanced, it is not surprising, that there are also examples of molecules that stabilize neural circuits. The PirB protein restricts ocular-dominance plasticity in the visual cortex, resulting in reduced expression of the immediate early gene Arc (Syken et al., 2006). How does FoxP2 act in the medium spiny neurons in

Area X? If FoxP2 functions as a plasticity-promoting factor, knockdown animals should have been less plastic during learning, resulting in impoverished imitation and abnormally invariant song. Syllable omissions of FoxP2 knockdowns are consistent with this notion, but more variable syllable production is clearly not. Alternatively, if FoxP2 restricts neuronal plasticity, knockdown birds should sing more variable. In fact this is the case, but syllable omissions are not easily explained then. In light of the fact that FoxP2 is down-regulated when adult zebra finches sing slightly variable, undirected song, but not when they sing highly stereotyped female-directed song (Teramitsu and White, 2006), it seems however most plausible that FoxP2 negatively regulates plasticity. The vocal variability during undirected singing likely result from some form of underlying neural plasticity, as suggested by strong induction of the immediate early gene ZENK (Jarvis et al., 1998). If FoxP2 promoted plasticity it should therefore be upregulated, but not down-regulated by undirected singing. Given the complementary expression patterns of FoxP2 and ZENK, we speculate that the transcriptional repressor FoxP2 restricts neural plasticity by repressing genes induced by recurrent neuronal activity. The identification of the downstream target genes of FoxP2 and the electrophysiological characterization of medium spiny neurons with reduced FoxP2 levels will shed further light on the function of FoxP2 in vocal learning.

The vocal behavior of FoxP2 knockdown zebra finches offers an interesting interpretation of the speech abnormality in individuals with genetic aberrations of FoxP2 (Watkins et al., 2002), possibly extending to apraxia of speech in general (Ogar et al., 2005). The human core deficit affects the production of rapid sequential mouth movements which are required for speech articulation (Vargha-Khadem et al., 2005). Perhaps this deficit results from a problem with adjusting vocal output to articulation rules during speech learning. Finally, the fact that FoxP2 is essential for both song learning and the development of speech and language, provides further evidence for the hypothesis (Fisher and Marcus, 2006; Scharff and Haesler, 2005), that during evolution, ancestral neural systems were adapted in the human brain to assemble the unique framework capable of language.

4.3 Molecular Evolution of FoxP2 in Avians

Genes containing a Forkhead Box DNA binding domain are among the oldest genes in the history of life. Forkhead box transcription factors were found in the yeast *Saccharomyces*

cerevisiae and other fungi (Mazet et al., 2003), the demosponge *Reniera* (Larroux et al., 2006) and the sea anemonae *Nematostella vectensis* (Magie et al., 2005). Whereas yeast has 4, the latter species already has 15 different Fox genes. No Fox gene has yet been identified in plants, suggesting an evolutionary origin of the Fox gene family in a clade of unicellular organisms that gave rise to both the fungal and animal lineage. Orthologs of the FoxP subfamily were identified in *Drosophila melanogaster*, *Aopheles gambiae* and *Caenorhabditis elegans* (Mazet et al., 2003). However no FoxP gene was found in other invertebrates. Homologs of the FoxP2 gene exist in vertebrates species ranging from the zebra fish *Danio rerio* (Bonkowsky and Chien, 2005) to humans. Analysis of the molecular evolution of FoxP2 suggests that the gene has been the target of positive selection during recent primate evolution, which ultimately led to the fixation of a human-specific amino acid in the human population (Enard et al., 2002; Zhang et al., 2002). Given that FoxP2 is indispensible for developing proper and speech and language, at least in modern humans (Lai et al., 2001), it seems possible that FoxP2 was pivotal for the evolution of speech and language. By analogy, if this human-specific amino acid change was pivotal to the evolution of speech in hominids, similar selection pressure could have acted during the evolution of vocal learning in birds. Following the avian phylogeny proposed by (Sibley and Ahlquist, 1990) vocal learning has either been gained independently in 3 orders or lost in 4 orders of birds from the last common ancestor [Wada et al., 2004 (Figure 4.1)].

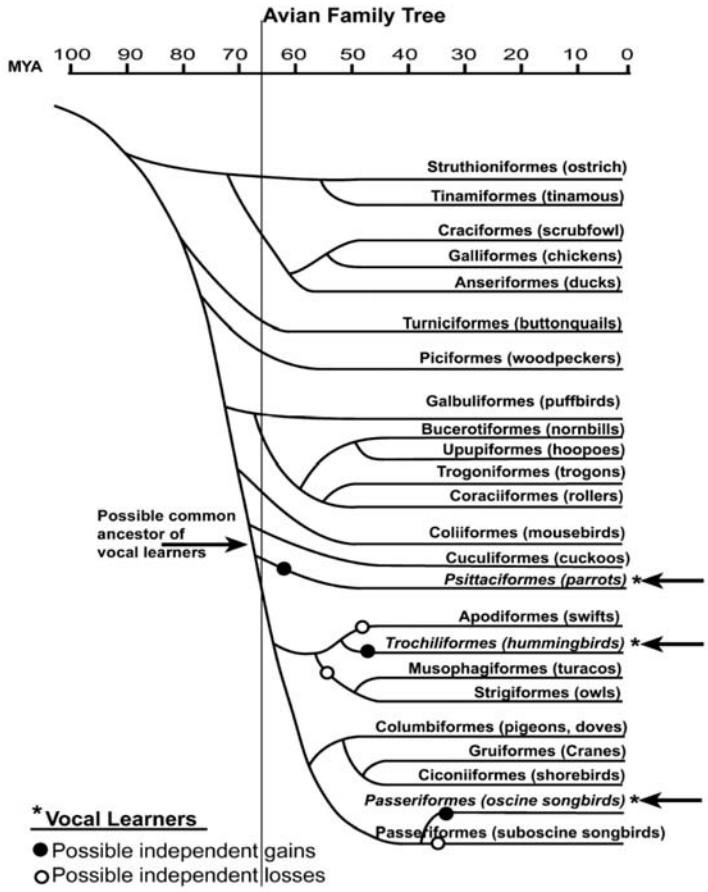


Figure 4.1 Evolution of vocal learning in the family tree of living avians. The Latin name of each order is given along with examples of common species. Passeriformes are divided into its two suborders: suboscine and oscine songbirds. The vertical line down each tree indicates the cretaceous-tertiary boundary, the time of the dinosaur extinction; MYA millions of years ago. Open and closed circles show the minimal ancestral nodes where vocal learning could have either evolved independently or been lost independently (Figure from (Jarvis, 2006)

Counting potential trait gains and losses however critically depends on the position of the branching points in the phylogenetic tree. In turn, tree-building relies on the experimental approach used to obtain discrete distance measures between species as well as the algorithm used to calculate the tree from the experimental data. The DNA hybridization technique used by (Sibley and Ahlquist, 1990) has a limited resolution, and most trees were constructed using the "Unweighted Pair Group Method with Arithmatic Mean" (UPGMA) which assumes uniform rates of DNA evolution across lineages. This assumption does not necessarily need to be correct, since genetic distance likely "accumulates" more rapidly in taxa with short generation times as opposed to taxa with long generation times (Johns and Avise, 1998). Due to these experimental and theoretical

constraints, phylogenetic trees are often ambiguous and frequently more than one solution is obtained for grouping similar species. It is therefore not surprising that many aspects of the avian phylogeny proposed by Sibley and Ahlquist, 1990 are still a matter of debate (O´Hara, 1991) and alternative trees based on DNA sequence analysis have been put forward (see e.g. Fain and Houde, 2004) for a tree of non-passerine birds, i.e. all birds except the oscinces and suboscines). Although it seems thus difficult to determine the exact number of potential gains or losses of vocal learning during the evolution from the last common ancestor, a common feature of all avian phylogenetic trees is that vocal learning is absent on the majority of branches suggesting it was rather gained independently than lost frequently. The most likely scenario is therefore, that evolutionary constraints favored the development of vocal learning where it offered a selective advantage.

To test the possibility that gains or losses of vocal learning were associated with specific amino acid substitutions in the FoxP2 protein, we analyzed the FoxP2 sequence from 11 species covering 3 orders in which vocal learning did not evolve and the 3 orders of vocally learning birds. For further comparison, we analyzed the FoxP2 sequence form the crocodile, the closest non-avian relative. None of the 12 species studied harbored the human-specific amino acid. Moreover, there was no correlation between a species' capacity for vocal learning and a particular version of their FoxP2 coding region. These findings are consistent with (Webb and Zhang, 2005) who analyzed the FoxP2 coding sequence corresponding to human exon 7 in a similar set of birds. In conclusion, FoxP2 was either not directly involved in the evolution of vocal-learning in birds and non-human mammals or selection acted on the large non-coding regions of FoxP2, which were not examined in this and other studies. The latter possibility is supported by theoretical and experimental evidence that point out the importance of regulatory sequences in the evolution of complex traits (Carroll, 2005). The fact that the most abundant FoxP2 transcript in zebra finches has the same size as the most abundant transcript in humans (Haesler et al., 2004), whereas mice lack a transcript of that size further supports this hypothesis. Possibly similar isoforms with specific untranslated regions (UTR´s) are transcribed exclusively in vocally learning species to subserve a specific function in the context of vocal learning. This hypothesis is consistent with the specific gene expression pattern of FoxP2 in the song system of vocal learners (see above).

The apparent discrepancy between the requirement of FoxP2 for human speech and birdsong (Lai et al., 2001) and its overall conservation among all vertebrates, most of which are not capable of auditory-guided vocal imitation leads to the question why the FoxP2 protein changed so little during vertebrate evolution. Under the assumption of a neutral model of evolution (Kimura, 1968) in which random genetic drift provides a large source for genetic variation this strongly suggests that the FoxP2 gene was under negative selection unrelated to vocal learning. Consistent with this, homozygous knockout of FoxP2 in mice causes perinatal death (Shu et al., 2005), although it remains open why lack of FoxP2 is lethal. Most likely, multiple selective constraints act on the FoxP2 gene, indicating that FoxP2 function is important in several biological processes. A more detailed analysis of the different functional domains and the specific amino acids of the FoxP2 protein in both vocally learning and non-learning species is needed to disentangle the different evolutionary constraints from a functional perspective.

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6 Appendix

6.1 Abbreviations

18S and 28S	Ribosomal RNA in Svedberg unit
^{32}P / ^{33}P	Radioactive phosphor isotopes
^{35}S	Radioactive sulphur isotopes
AFP	Anterior forebrain pathway
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeleton-associated protein
Area X	Used as proper name
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumine
C2H2 type zinc finger	Classical zinc finger domain containing two conserved cysteines and histidines which coordinate a zinc ion
cDNA	Copy DNA, complementary DNA
c-fms promoter	Colony stimulating factor 1 receptor, formerly McDonough feline
c-Fos	Cellular oncogene fos
ChAT	Choline acetyltransferase
CREB	Cyclic-AMP response element-binding protein
CtBP1	C -terminal binding protein 1
DARPP-32	Dopamine- and cyclic AMP-regulated phosphoprotein with molecular weight 32kDa
DLM	Medial nucleus of the dorsolateral thalamus
DM	Dorsomedial motor nucleus of the intercollicular region
DT	Dorsal thalamus
dUTP	2'-Deoxyuridine 5'-Triphosphate
DVD	Developmental verbal dyspraxia
EST	Expressed sequence tags
EtOH	Ethanol
FITC	Fluorescein-isothiocyanate
FM	Frequency modulation
Fox	Forkhead box
FoxP1	Forkhead box transcription factore, subfamily P, member 1
FoxP2	Forkhead box transcription factore, subfamily P, member 2
FoxP4	Forkhead box transcription factore, subfamily P, member 4
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase gene
GFP	Green fluorescent protein
h	Hour

HEK293-T	Human embryonic kidney cell line 293 transformed with the SV40 large T antigen
HIV	Human immuno-deficiency virus
Hmbs	Hydroxymethylbilane synthase
Hprt	Hypoxanthine phosphoribosyltransferase
Hu	ELAV (embryonic lethal, abnormal vision, Drosophila)-like gene product
HVC	Used as proper name
IGFII	Insulin-like growth factor 2 (somatomedin A)
kb	Kilobase
kDA	Kilo-Dalton
KE-family	Pseudonym for family in which FoxP2 mutations were first described
KO	Knockdout
LANT6	neurotensin-related hexapeptide
LGE	Lateral ganglionic eminence
lMAN	Lateral magnocellular nucleus of the anterior nidopallium
LSD	Least Significant Difference
mGluR2	Metabotropic glutamate receptor 2
min	Minute
miRNA	Micro-RNA
MMSt	Magnocellular nucleus of the medial striatum previously called LPOm
MO	Mesopallial song nucleus
mRNA	Message RNA
MYA	Million years ago
Nif	Nucleus interfacialis
nNOS	Nitric oxide synthase 1 (neuronal)
NPY	Neuropeptide Y
NR2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B
nXIIts	hypoglossal nucleus, pars tracheosyringealis
Oligo	Oligonucleotide
ORF	Open-reading-frame
PB	Phophate buffer
PBS	Phophate buffered saline
PCR	Polymerase chain reaction
Pfkp	6- phosphofructokinase, platelet type
PG	Pitch goodness
Pgk1	Phosphoglycerate kinase 1
PHD	Post hatch day
polyQ	Poly-glutamine stretch
PSA-NCAM	Polysialylated form of neural cell adhesion molecule 1
PSL	Pallial-subpallial lamina

R553H	Mutation from arginine to histidine at amino acid # 553
RA	Robust nucleus of the archistriatum
RACE	Rapid Amplification of CDNA Ends
RISC	RNA-induced silencing complex
RNAi	RNA interference
rpm	Rounds per minute
RT	Room temperature
SAP	Sound Analysis Pro software
SEM	Standard error of the mean
shControl	Short hairpin RNA with non-targeting control sequence
shFoxP2	Short hairpin RNA targeting FoxP2
shGFP	Short hairpin RNA targeting GFP
Shh	Sonic hedgehog (Shh) pathway
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
ssDNA	Single stranded DNA
STDEV	Standard deviation
TH	TyrosineHydroxylase
TOPRO3	DNA-binding dye from Molecular Probes (Eugene, USA)
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
UTR	Untranslated region
VAS	vocal nucleus of the anterior striatum
VSVg	Vesicular stomatitis virus glycoprotein
ZENK	Avian homolog of the mammalian zif268/EGR-1/ NGFI-A/krox24 gene
Zn-finger	Zink-finger domain

6.2 Abstract

The FoxP2 gene, which encodes a forkhead box transcription factor is essential for developing the full articulatory power of human language. Mutations of FoxP2 cause a speech and language disorder which compromises the fluent production of words and affects the correct use and comprehension of grammar. FoxP2 patients have structural and functional abnormalities in the striatum of the basal ganglia, which also expresses high levels of FoxP2. But how FoxP2 affects brain function remains unknown. The first part of my thesis addresses this question in songbirds, since learning to speak bears behavioral and neural parallels to how songbirds learn to sing. In zebra finches, FoxP2 expression increases in Area X, a basal ganglia structure necessary for song acquisition, during the time when song learning occurs. In canaries, FoxP2 expression levels in Area X are similarly associated with vocal plasticity. Using lentivirus-mediated RNAi in zebra finches we shown that FoxP2 knockdown in Area X impairs song learning. This suggests that auditory-guided vocal learning in the basal ganglia requires FoxP2. These findings provide the first example of a functional gene analysis in songbirds, a widely studied neuroethological model system. Finally, the fact that FoxP2 is involved in both birdsong and speech suggests that the molecular substrate from which the uniquely human capacity of language evolved might not be exclusive to the hominid lineage.

Consistent with this, the FoxP2 protein sequence shows a high degree of conservation among vertebrates. However, human FoxP2 contains changes in amino-acid coding and a pattern of nucleotide polymorphisms which suggests that it has been the target of selection during recent human evolution. This indicates that FoxP2 might have been pivotal for the development of human language. Although language is a uniquely human trait, learned vocalizations are also found in a few other species, among them whales, bats, and most prominently in three orders of birds. Thus, in the second part of my thesis I compared the FoxP2 sequence from 11 bird species covering the 3 orders of vocally learning birds as well as 3 orders in which vocal learning did not evolve and the crocodile, the closest non-avian relative. There was no evidence for an association between specific amino acid substitutions and the capacity for vocal learning. In conclusion, FoxP2 was either not directly involved in the evolution of vocal-learning in birds or selection acted on the large non-coding regions of FoxP2, which were not examined in this study.

6.3 Zusammenfassung

Mutationen im FoxP2-Gen führen zu einer Sprech- und Sprachstörung (developmental verbal dyspraxia, DVD), welche vor allem durch gestörte Artikulationsfähigkeit und Probleme mit dem Verständnis und Gebrauch von Grammatik gekennzeichnet ist. Patienten mit DVD zeigen funktionelle und strukturelle Auffälligkeiten im Striatum der Basalganglien. Im Striatum ist FoxP2 auch stark exprimiert, aber wie genau sich die FoxP2 Mutationen auf die Sprachfähigkeit auswirken ist unbekannt. Im ersten Teil meiner Dissertation nähere ich mich dieser Fragestellung durch Experimente in Singvögeln, da zwischen Sprachlernen und Gesangslernen viele neurobiologische und ethologische Parallelen bestehen. Das Expressionsmuster von FoxP2 in Gehirn der Singvögel stimmt mit den bereits beschriebenen Mustern aus der Maus und dem Mensch überein. Darüber hinaus ist in Area X von Zebrafinken, einer für das Gesangslernen essentiellen Struktur, die Expression von FoxP2 während der Gesangslernphase höher als davor und danach. Das Expressionsniveau von FoxP2 korreliert also zeitlich mit der Lernphase. In Kanarienvögeln ist FoxP2 ebenfalls dann besonders stark experimentiert, wenn sich die Vögel in einer plastischen Phase ihres Gesangs befinden. Dies weist auf eine mögliche Funktion von FoxP2 bei der Gesangsplastizität hin. Durch die Verwendung eines lentiviralen Expressionssystems zur Induktion von RNAi im Zebrafinken wird gezeigt, daß die experimentelle Reduktion von FoxP2 das Gesangslernen tatsächlich beeinträchtigt. Das bedeutet, daß FoxP2 notwendig für auditorisch geleitetes, vokales Lernen ist. Diese Ergebnisse liefern das erste Beispiel einer funktionellen Genanalyse in einem Singvogel. Darüber hinaus deutet die Tatsache, daß FoxP2 sowohl notwendig für die Sprache des Menschen als auch den Gesang von Singvögeln ist darauf hin, daß die Ähnlichkeiten zwischen Gesangslernen und Spracherwerb bis auf die molekulare Ebene hinabreichen. Das molekulare Substrat für die Evolution der menschlichen Sprachfähigkeit ist demnach nicht ausschließlich bei den Hominiden zu finden.

Im Einklang damit steht, daß die Proteinsequenz von FoxP2 in allen Vertebraten extrem stark konserviert ist. Allerdings wurden beim Menschen Aminosäuresubstitutionen und ein Muster an Sequenzvariationen gefunden, welche darauf schließen lassen, daß FoxP2 in der jüngeren Vergangenheit der menschlichen Evolution unter Selektionsdruck gestanden hat. FoxP2 könnte demnach entscheidend zur Evolution von Sprache beigetragen haben. Zwar ist Sprache dem Menschen vorbehalten, aber einige wenige Arten, darunter Wale,

Fledermäuse und drei Ordnungen von Vögeln, sind in der Lage ihre Vokalisation durch Imitation zu erlernen. Im zweiten Teil meiner Dissertation, untersuche ich daher, ob ein Zusammenhang zwischen der Fähigkeit zur erlernten Vokalisation und dem Muster an Aminosäuresubstitutionen im FoxP2-Gen besteht. Hierzu werden die FoxP2-Sequenzen von 11 Vogelspezies, darunter Vertreter aus den drei Ordnungen von Gesangslernern und aus drei nicht-lernenden Vogelordnungen verglichen. Zur besseren phylogenetischen Einordnung wird zusätzlich die FoxP2-Sequenz des Krokodils, dem nächsten Verwandten der Vögel, analysiert. Es zeigen sich jedoch keine Hinweise darauf, daß die Fähigkeit zum Gesangslernen mit einer bestimmten FoxP2-Sequenz einhergeht. Demnach war FoxP2 entweder nicht direkt an der Evolution des Gesangslernens beteiligt, oder aber die Selektion hat auf die nicht-kodierenden Regionen von FoxP2 gewirkt, welche hier nicht untersucht wurden.

6.4 *curriculum vitae*

Education

01/02-12/06 **Max-Planck-Institute for Molecular Genetics, Berlin, Germany**
PhD thesis with Prof. C. Scharff: "Studies on the evolution and function of *FoxP2*, a gene implicated in human speech and language, using songbirds as a model"

12/01 **University of Hohenheim, Stuttgart, Germany**
Master of science, grade A- (very good), Major in Molecular Plant Genetics

07/95 **Goethe-Gymnasium, Frankfurt, Germany**
Baccalaureate (grade A-)

Professional Experience

03/01 - 10/01 **Max-Planck-Institute for Plant Breeding Research, Cologne, Germany**
Master Thesis with Prof. J. Schell in the field of homologous recombination and gene targeting plants

10/00 - 03/01 **Institute for Biochemistry at the University of Stuttgart, Germany**

Bioinformatic classes, project on 3D-homlogy-modelling of a monoxygenase

07/00 - 10/00 **Weickmann & Weickmann, patent attorneys, Munich, Germany**
Practical training, introduction to German and European patent law

06/99 – 10/99 **Nunhems Zaden B.V., seed company, Haelen, Netherlands**
Practical training within the Molecular Research Department

10/95 – 10/96 **School for mentally handicapped children, Mainz, Germany**
Community service, taught classes and one-to-one tutoring

06/92 – 08/92 **Wellesley College Boston, USA**
"Exploration" Summer School

Languages

German, French, English

6.5 Publications

1. Kalscheuer VM, Freude K, Musante L, Jensen LR, Yntema HG, Gecz J, Sefiani A, Hoffmann K, Moser B, Haas S, Gurok U, **Haesler S**, Aranda B, Nshedjan A, Tzschach A, Hartmann N, Roloff TC, Shoichet S, Hagens O, Tao J, Van Bokhoven H, Turner G, Chelly J, Moraine C, Fryns JP, Nuber U, Hoeltzenbein M, Scharff C, Scherthan H, Lenzner S, Hamel BC, Schweiger S, Ropers HH; Mutations in the polyglutamine binding protein 1 gene cause X-linked mental retardation. *Nature Genetics*, 2003; **35**:313-5.

2. **Haesler S**, Wada K, Nshdejan A, Morrisey EE, Lints T, Jarvis ED, Scharff C; *FoxP2* expression in avian vocal learners and non-learners. *Journal of Neuroscience*, 2004; 24:3164-75.

3. Scharff C, **Haesler S**; An evolutionary perspective on FoxP2: strictly for the birds? *Current Opinion in Neurobiology*, 2005; 15: 694-703.

4. Wada K, Howard JT, McConnell P, Lints T, Rivas MV, Whitney O, Horita H, Patterson MA, White SA, Scharff C, **Haesler S**, Zhao S, Sakaguchi H, Hagiwara M, Shirakih T, Hirozane-Kishikawah T, Skenea P, Hayashizakih Y, Carninci P and Jarvis ED; A molecular neuroethological approach for identifying and characterizing a melody of behaviorally regulated genes. *Proceedings of the National Academy of Science USA*, 2006;103:5212-7.

5. **Haesler S**; Also sprach der Zebrafink. *Gehirn&Geist*, Heft 12, 2006

6. **Haesler S**, Licznerski P, Georgi B, Osten P, Scharff C; Knockdown of FoxP2 in songbird basal ganglia impairs song learning. *submitted*

7. **Haesler S**, Schneider R, Schweiger S, Scharff C; The mammalian TAP42 homologue is a negative regulator of TOR signaling. *manuscript in preparation*

Poster-presentations

- 1th Symposium of the "National Genome Research Network", 2002, Berlin, Germany
- Berlin Neuroscience Forum, 2002, Liebenwalde, Germany
- Brain Research Symposium 2003, "Neurogenomics of mice and men", New Orleans, USA
- 33th Annual Meeting of the Society for Neuroscience, 2003, New Orleans, USA
- Berlin Neuroscience Forum, 2004, Liebenwalde, Germany
- 1st European Meeting of the Molecular and Cellular Cognition Society, 2004, Lissabon, Portugal
- 4th Forum of European Neuroscience, 2004, Lissabon, Portugal
- 34th Annual Meeting of the Society for Neuroscience, 2004, San Diego, USA
- Hertie/FENS Winter School 2004 on "Research strategies for the study of animal models of cognition and its pathologies", Kitzbühel, Austria,
- Gordon Research Conference on "Neural circuits and plasticity", 2005, Newport, USA
Berlin Neuroscience Forum, 2006, Liebenwalde, Germany
- 36th Annual Meeting of the Society for Neuroscience, 2006, Atlanta, USA

Oral presentations

- 6th Schloessmann Symposium of the Max-Planck society on "Cognitive Neuroscience of Human Ontogeny", 2005, Doellnsee, Germany
- 10th Conference of the International Association for the Study of Child Language (IASCL), 2005, Berlin, Germany
- "Day of Science 2005"; Seminar of the MPI for Molecular Genetics, Berlin, Germany
- "Birdsong in Behavioral and Neurobiological Research", International Symposium at Freie Universität Berlin, 2006, Germany

Awards and Fellowships

- Fellow at the Hertie/Federation of European Neuroscience Societies (FENS) Winter School on "Research strategies for the study of animal models of cognition and its pathologies", Kitzbühel, Austria,
- 1st prize for research proposal presented at the 6th Schloesmann Symposium of the Max-Planck society on "Cognitive Neuroscience of Human Ontogeny" and postdoc stipend, 2005, Doellnsee, Germany
- Poster prize at Berlin Neuroscience Forum, 2006, Liebenwalde, Germany

Patent

International patent (WO/2003/096964) *Means for use in treating dieseases correlated with or caused by non-physiological levels of microtubule-associated PP2AC.*

Activities

- music (violin, piano)
- member of "Student Association" at MPI for Molecular Genetics
- Organisation of the seminar series "Branching Out - Professional possibilities after your PhD"
- "McKinsey educates", 2005, initiative for the promotion of early scientific education of children by McKinsey&Company

6.6 Erklärung

Ich versichere hiermit, diese Arbeit selbstständig verfaßt und nur die angegebenen Hilfsmittel und Hilfen in Anspruch genommen zu haben. Alle Experiemente und Analysen wurden von mir eigenständig durchgeführt, mit Ausnahme der *in situ* Hybridisierungen (mit Kazuhiro Wada, Duke University) und der Analyse der Phylogenetischen Verwandtschaft der FoxP2 Gene (mit Wolfgang Enard, MPI für Evolutionäre Anthropology).

Sebastian Haesler Berlin, Dezember 2006

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Berlin im Dezember 2006