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.

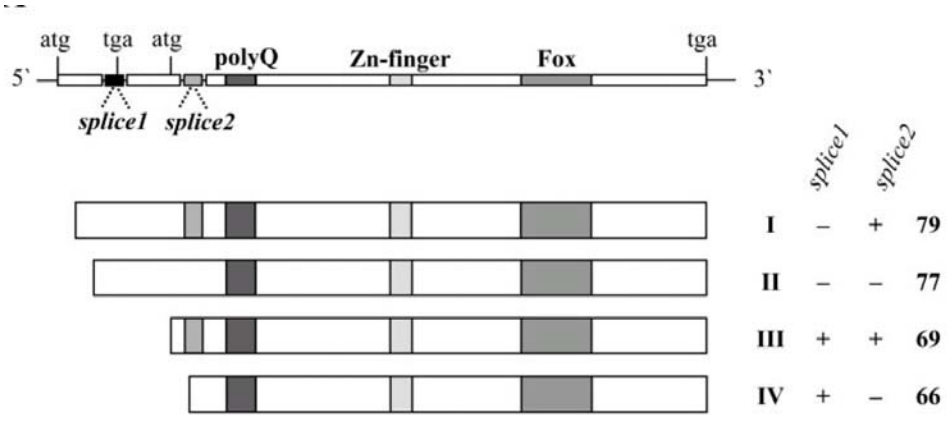

Figure 3.1 FoxP2 isoforms from the zebra finch. Identification of the zebra finch FoxP2 mRNA. Schematic representation of the zebra finch FoxP2 mRNA structure and its four predicted protein isoforms (I-IV). Positions of start (atg) and stop (tga) codons, the polyglutamine tract (polyQ), zinc finger (Zn-finger), 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

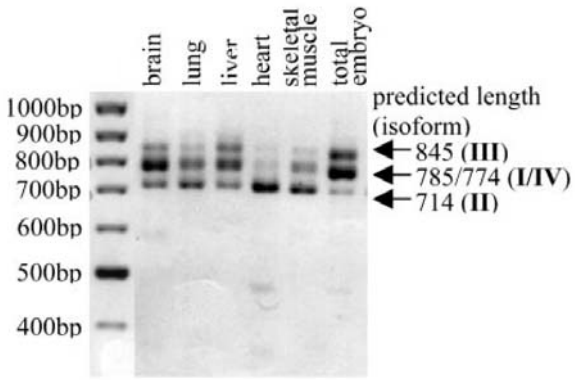

Figure 3.2. Length [in base pairs (bp) and amino acid (AA)] **of the zebra finch FoxP2 isoforms (I-IV) and the length of the RT-PCR products spanning the alternatively spliced region.** RT-PCR on RNA of different zebra finch tissues spanning the alternatively spliced region, but not the entire ORF, yields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

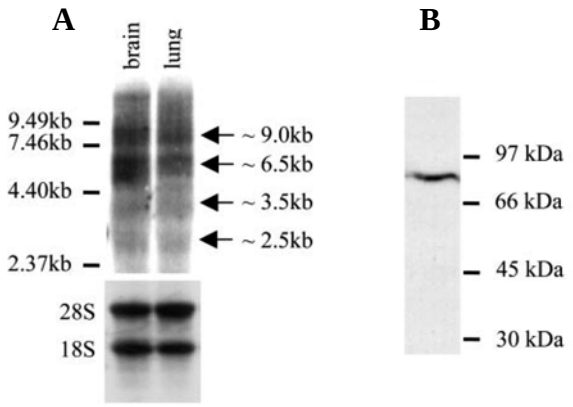

Figure 3.3 Transcript and protein analysis of zebra finch FoxP2. (A) Northern blot analysis of 20 µg of total RNA from adult zebra finch brain and lung was performed with a ^{32}P-labeled DNA fragment spanning bp 114 -959 (relative to the first start codon of isoform III). Ethidium bromide staining of 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)].

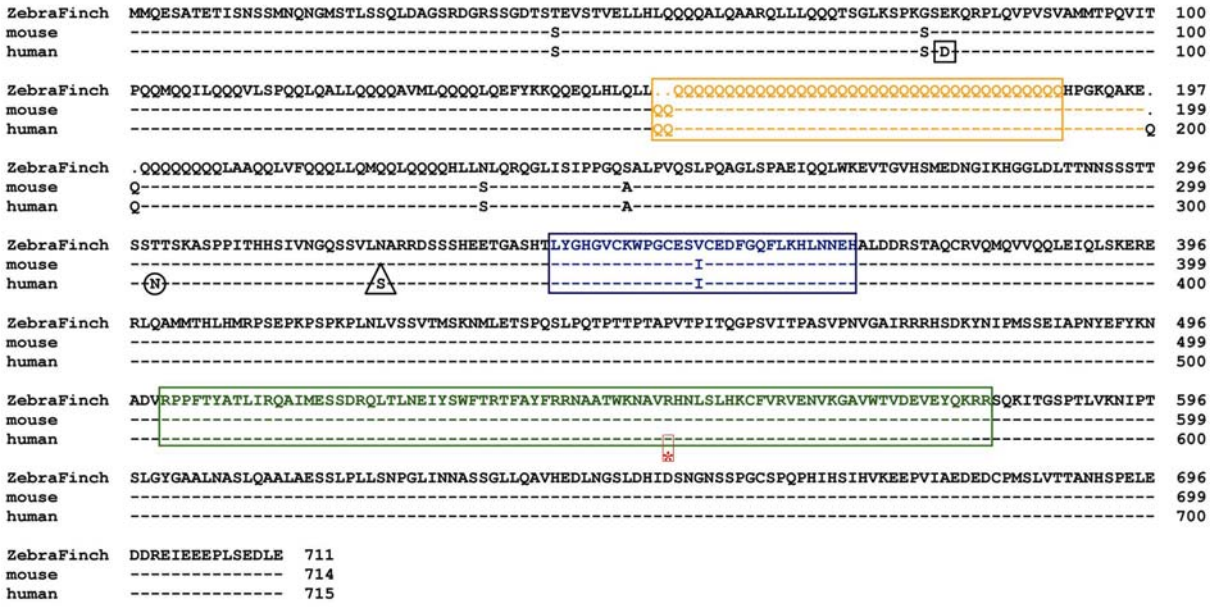

Figure 3.4 Comparison of zebra finch FoxP2 with human and mouse FoxP2. In the human sequence the R553H mutation that is associated with developmental verbal dyspraxia is marked with an asterisk (*), a primate-specific amino acid is boxed, a carnivore-specific amino acid is circled and the unique human-specific 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.

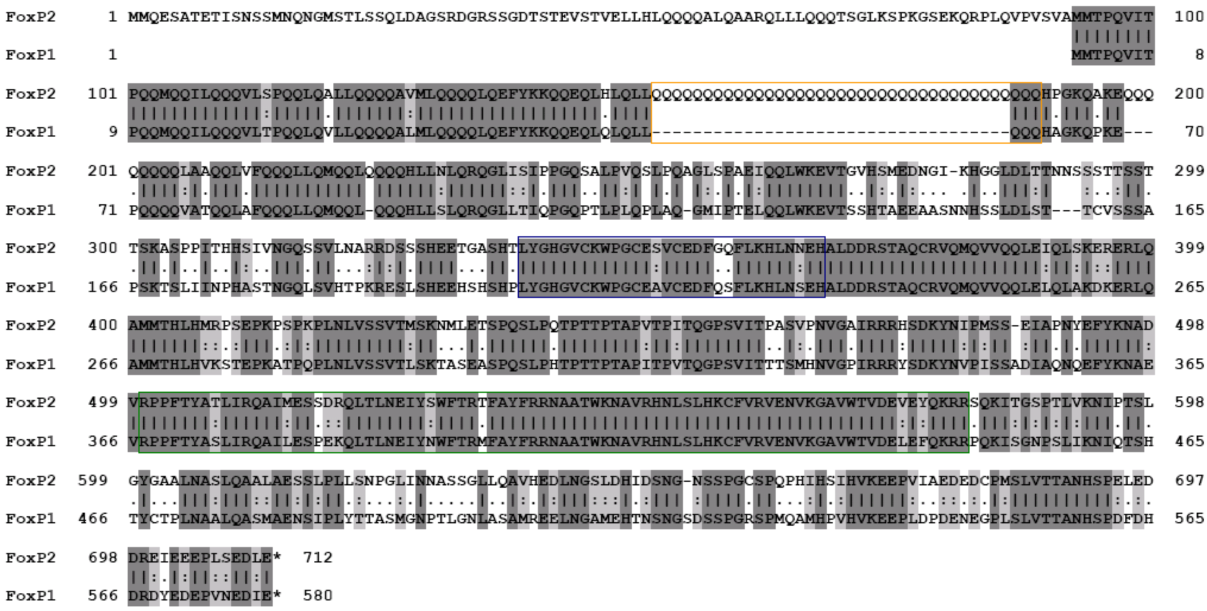

Figure 3.5. Comparison between FoxP2 and FoxP1 from the zebra finch. Alignment of zebra finch FoxP1 and FoxP2 protein sequences. Identical amino acid are shaded in dark grey, similar amino acids are shaded in light grey and non-similar comparisons remain white.

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.

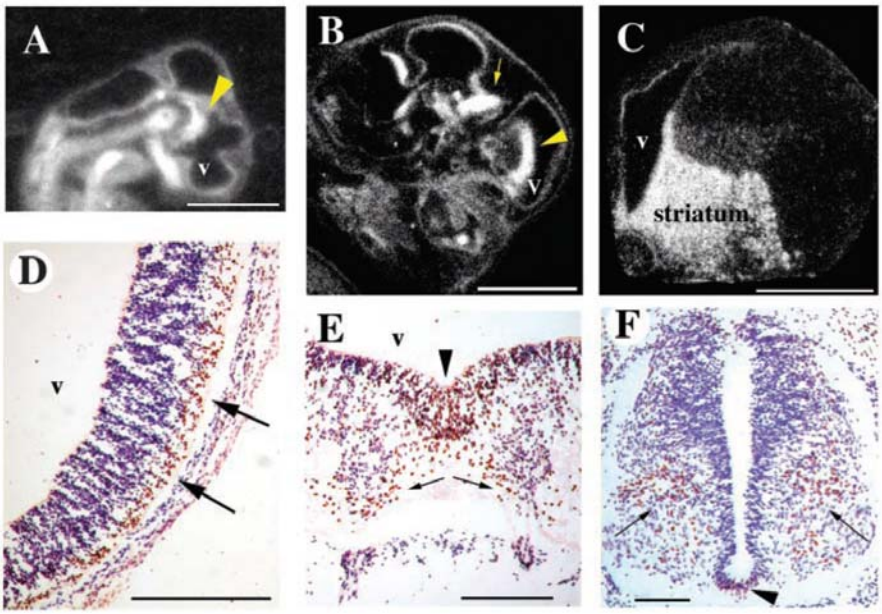

Figure 3.6 FoxP2 expression in the developing embryo. Embryonic FoxP2mRNA **(A-C)** and protein **(D-F)** expression. Sagittal sections through stage 26 **(A)** and 34 **(B)** zebra finch embryos show expression in presumptive striatum (arrowheads) and presumptive dorsal thalamus (arrow). The heads face 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.

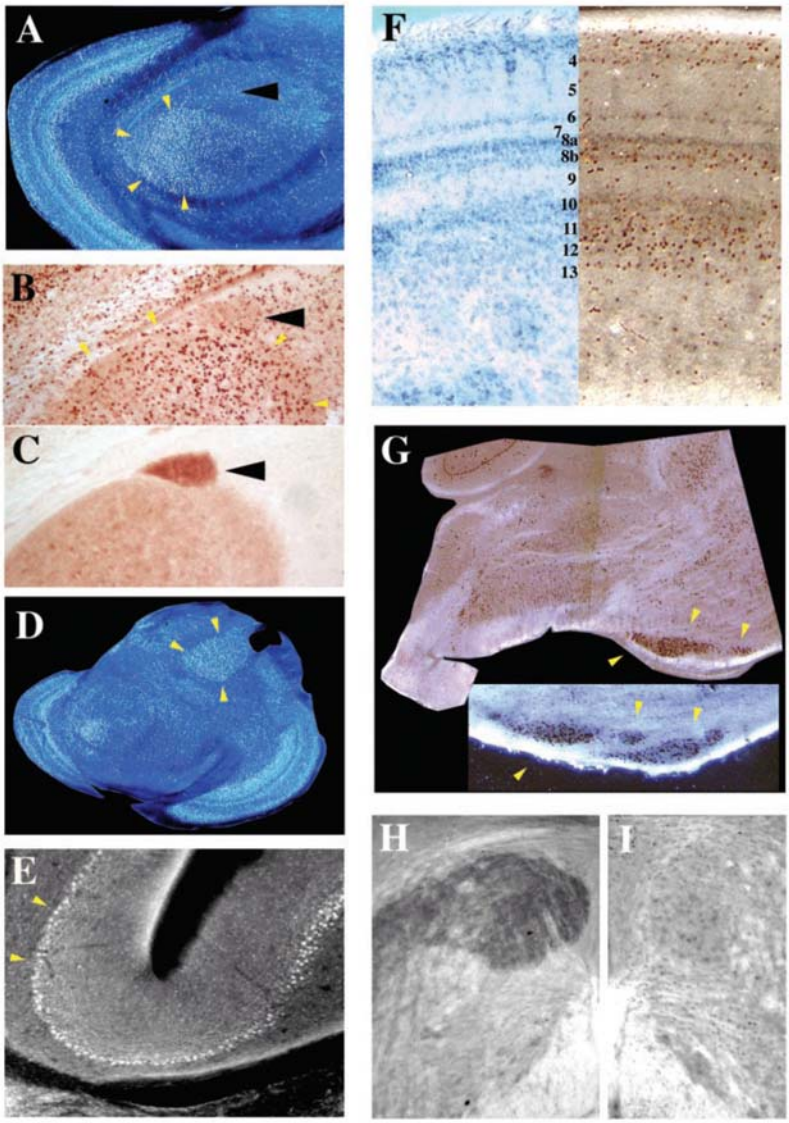

Figure 3.7 Subtelencephalic FoxP2 epxression in the adult zebra finch. (A-H) FoxP2 expression in subtelencephalic regions was associated more with afferent sensory or multimodal areas rather than with pure motor areas. Auditory nucleus MLd (dorsal part of the lateral mesencephalic nucleus) expressed 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.

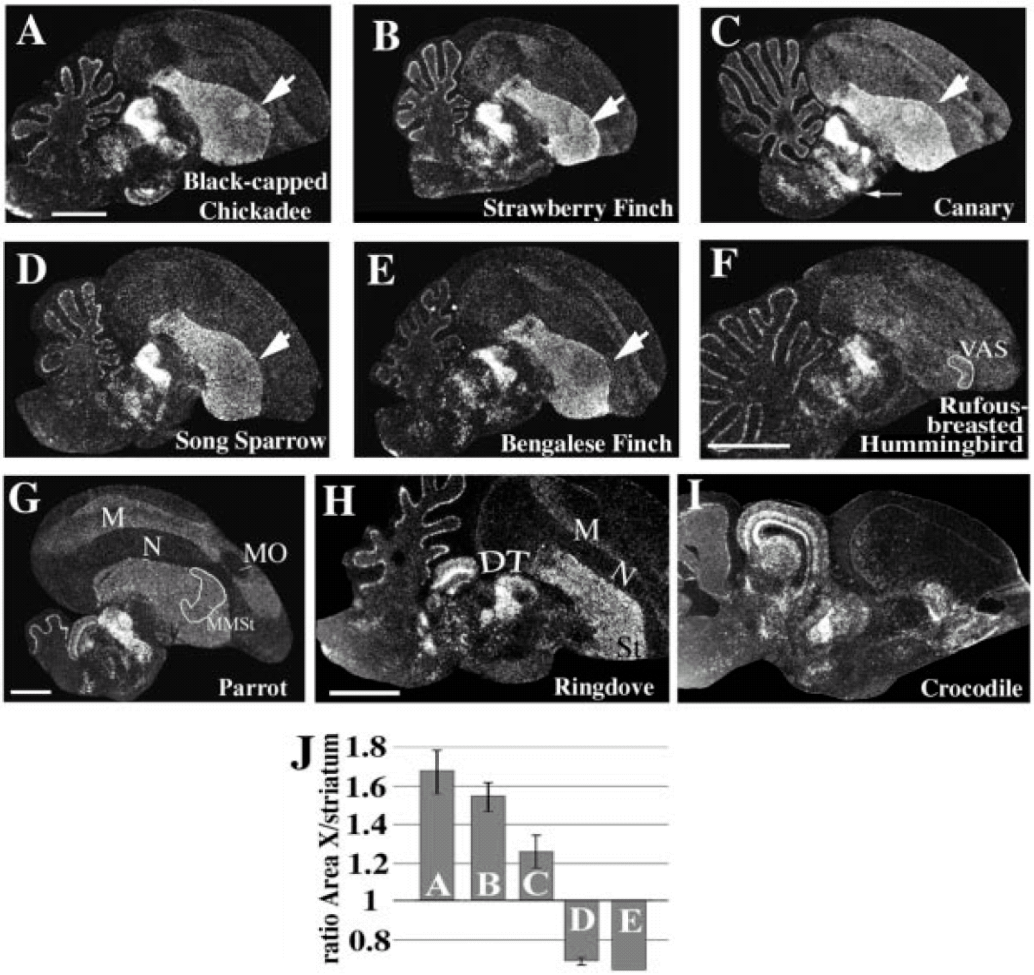

Figure 3.8 FoxP2 expression in adult vocal learners, vocal non-learners and the crocodile. FoxP2 expression in the striatum and dorsal thalamus was conserved among vocal learners, non-learners, and crocodile species. It was exclusive however to the striatal vocal control nucleus of vocal learners (Area 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.

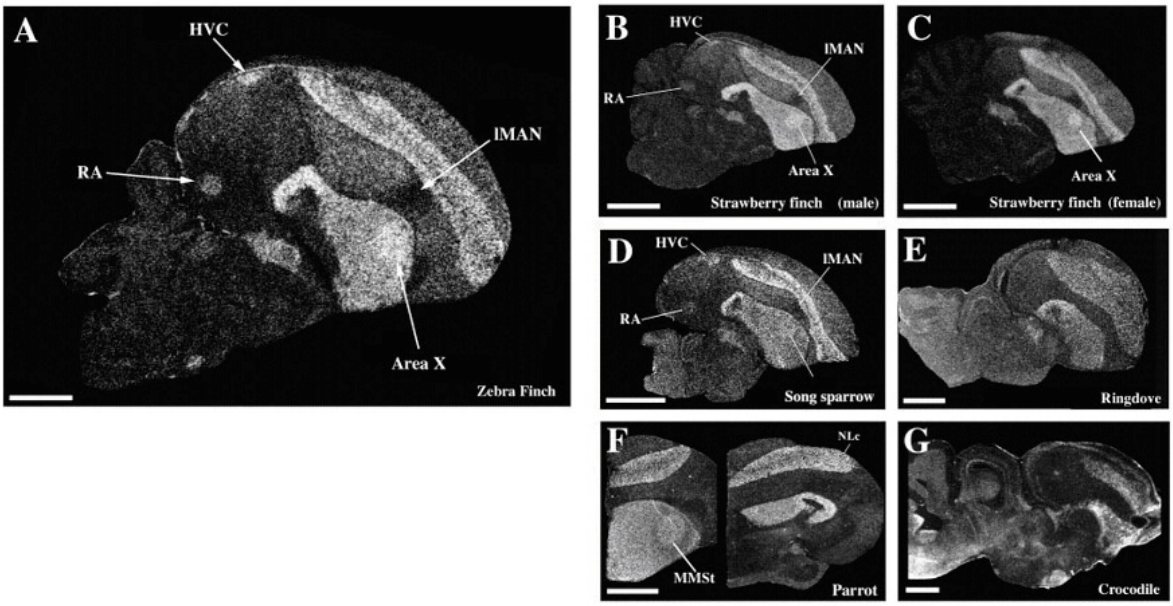

Figure 3.9 FoxP1 expression in the adult brain. (A) Expression pattern of FoxP1 was distinct from but partially overlapping with that of FoxP2. FoxP1, like FoxP2, was expressed in the dorsal thalamus and striatum in adult zebra finches. In addition, it was expressed in vocal nuclei HVC, RA, and Area 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.

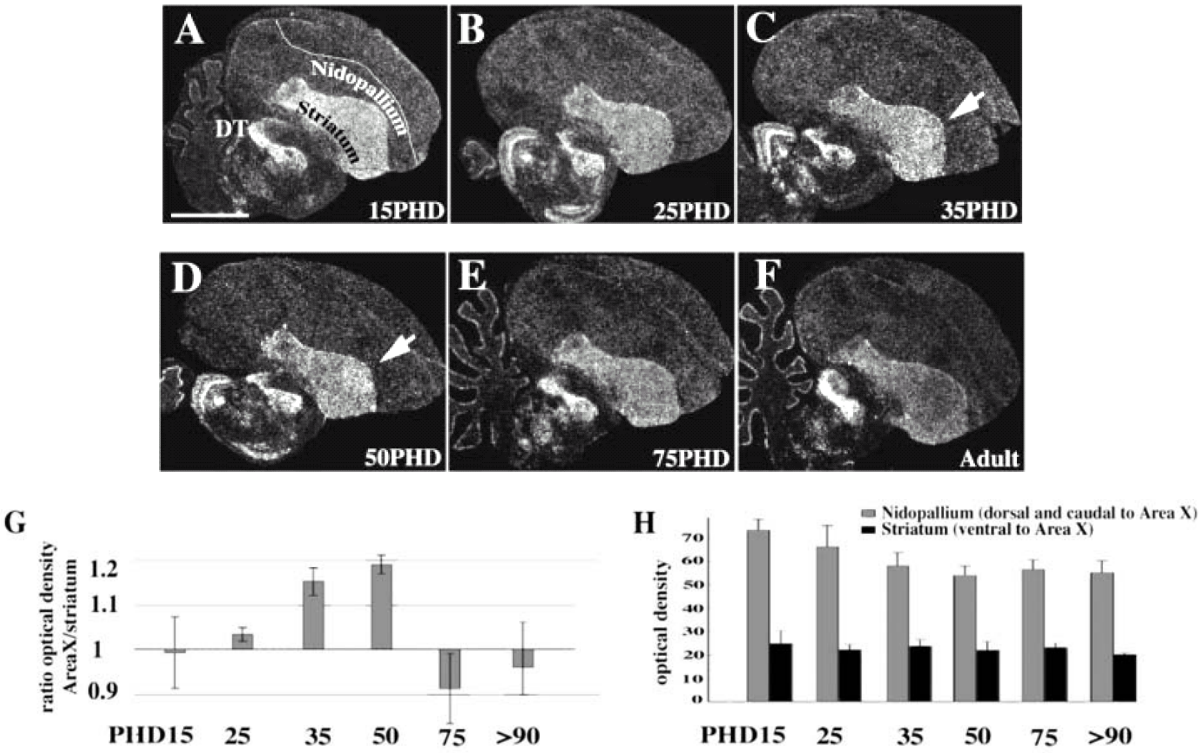

Figure 3.10 Differential FoxP2 expression in Area X during post-hatch zebra finch development. (A-F) Area X expressed more FoxP2 than the surrounding striatum only at PHD35 and 50 **(C, D, arrowheads),** which is the time when zebra finches learn to imitate song. **(G and H)** show the results of autoradiographic 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).

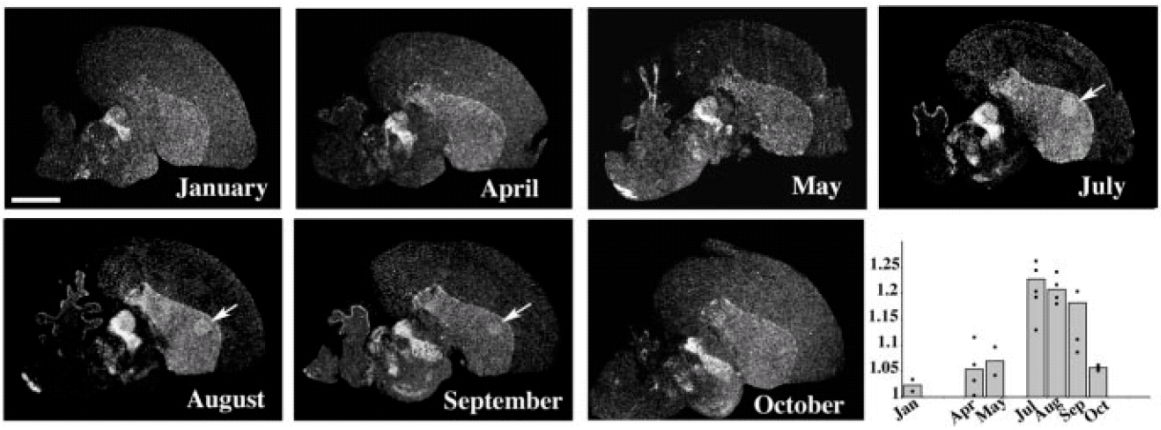

Figure 3.11 FoxP2 expression in canaries varies seasonally. FoxP2 expression in Area X of adult canaries varied seasonally. Area X expressed noticeably more FoxP2 than the surrounding striatum only during the months of July, August, and September, resulting in higher ratios of Area X to striatum expression (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).

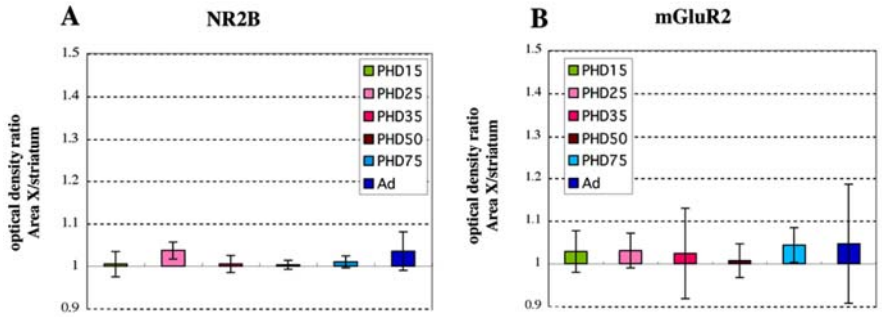

Figure 3.12 Gene expression in Area X is not globally increased during song learning. The expression of the zebra finch glutamate receptor subunit NR2B and subtype mGluR2 was measured in adjacent sections to those in **Figure 3.10**. In contrast to developmental differences in the ratio of FoxP2 mRNA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

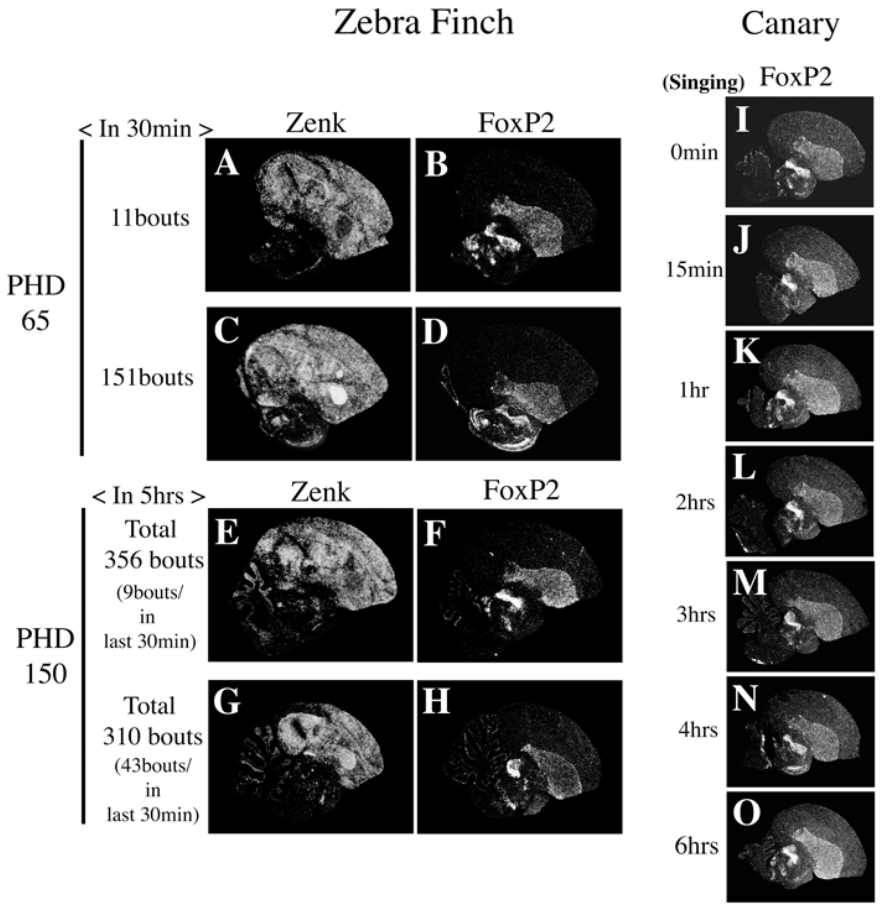

Figure 3.13 FoxP2 expression in Area X is not induced by singing. **(A-H)** In both young (PHD 65, B, D) and adult (PHD 150, F, H) zebra finches, singing undirected song does not induce FoxP2 expression in Area X, whereas in adjacent sections of the same animals, the amount of ZENK expressed reflects 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).

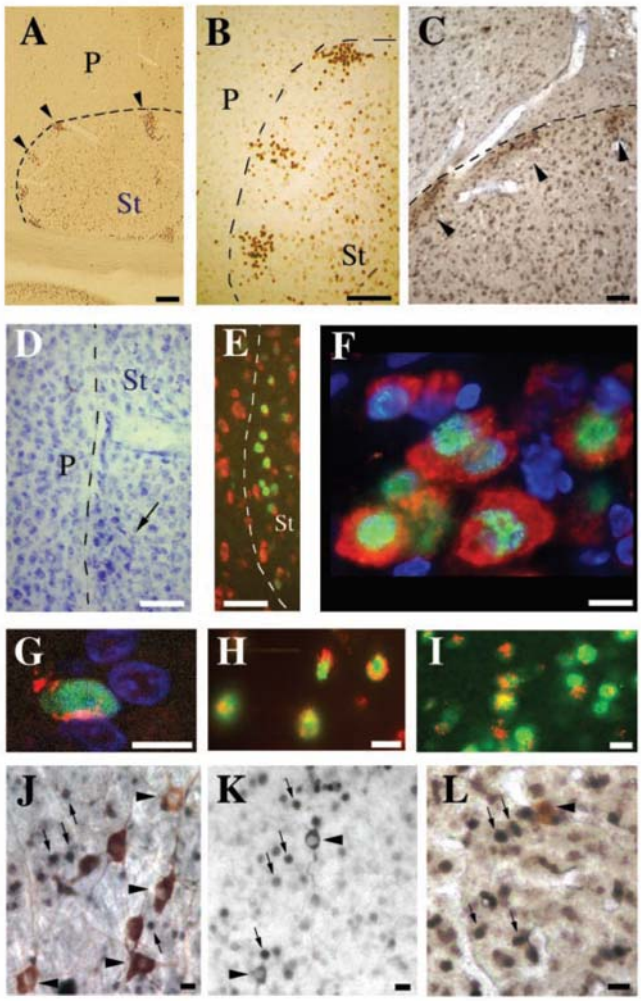

Figure 3.14 FoxP2 expression in distinct populations of neurons in adult zebra finches. Low **(A)** and high **(B)** magnification of a sagittal section showing the dorsolateral extent of the subpallial-pallial (P) border with the striatum (St; black dashed line), where clusters of cells in the dorsal 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).

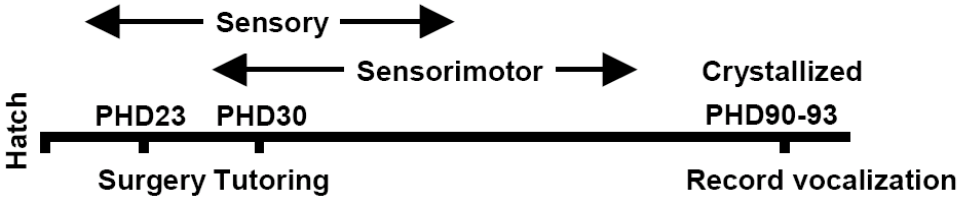

Figure 3.15 Timeline of experiments. By PHD20, fathers and older male siblings were removed from family cages to prevent experimental zebra finches from instructive auditory experience prior to the onset of tutoring. At the beginning of the sensory learning period on PHD23, virus was injected into Area 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)].

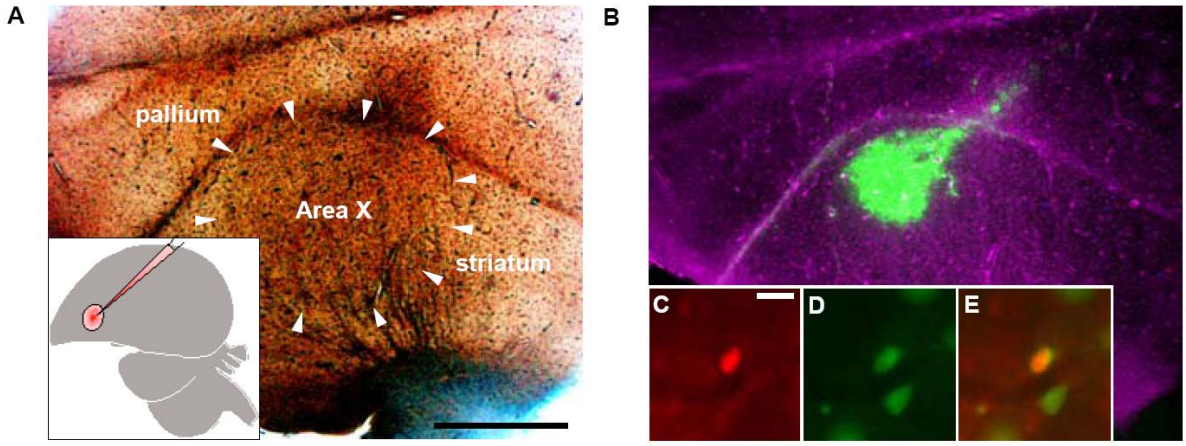

Figure 3.16 Targeting of virus into Area X. (A) Phase contrast image of a male zebra finch 50µm thick sagittal brain slice. Area X is outlined by white arrows (►; scale bar 1mm). The position of Area X within the brain is represented in the inset. **(B)** Fluorescent microscopy image of **(A)**. Virus 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).

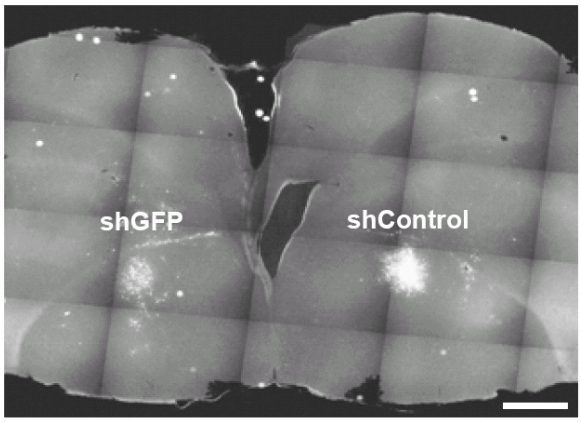

Figure 3.17 Lentivirus-mediated gene knockdown can persist long term. RNAi-mediated knockdown persisted for at least 3 month. Frontal 50µm thick brain slice of zebra finch injected with the indicated virus 105 days prior to perfusion. The intensity of GFP expression, visible as white signal, was reduced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

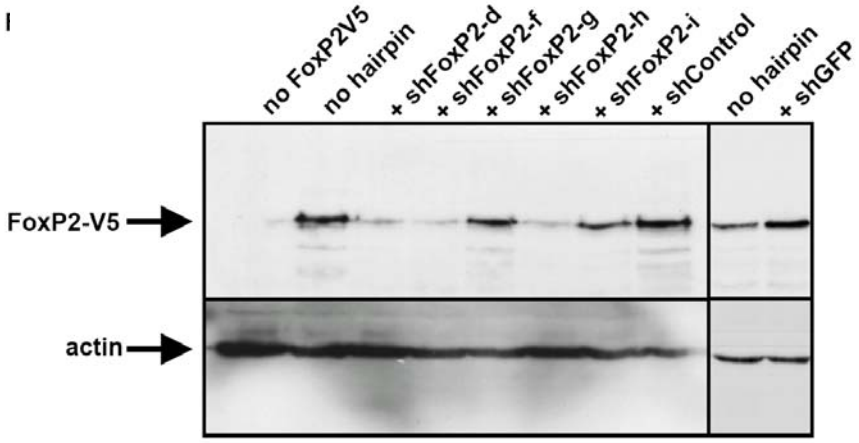

Figure 3.18 Identification of functional shRNA targeting FoxP2 *in vitro*. Hairpin expression constructs were tested for their knockdown efficiency in HEK293-T cells by simultaneous overexpression of zebra finch FoxP2, tagged with the V5 epitope and one of different hairpin constructs (shFoxP2-d - shFoxP2-i). 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.

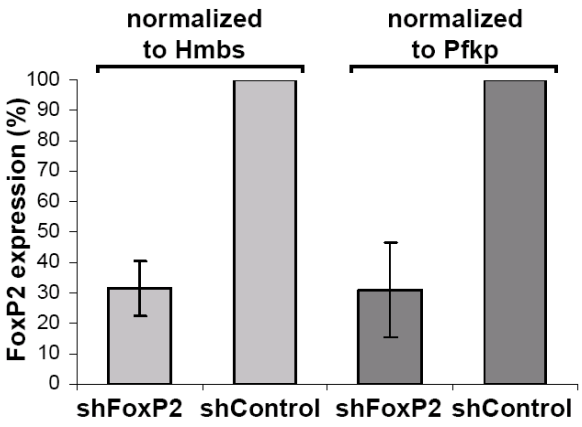

Figure 3.19 Quantification of *in vivo* knockdown efficiency. Real-time PCR quantification of FoxP2 expression in Area X on PHD50. Animals were injected with shControl in one hemisphere and shFoxP2 virus in the contralateral hemisphere, on PHD23. Bars represent relative gene expression between shControl 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.

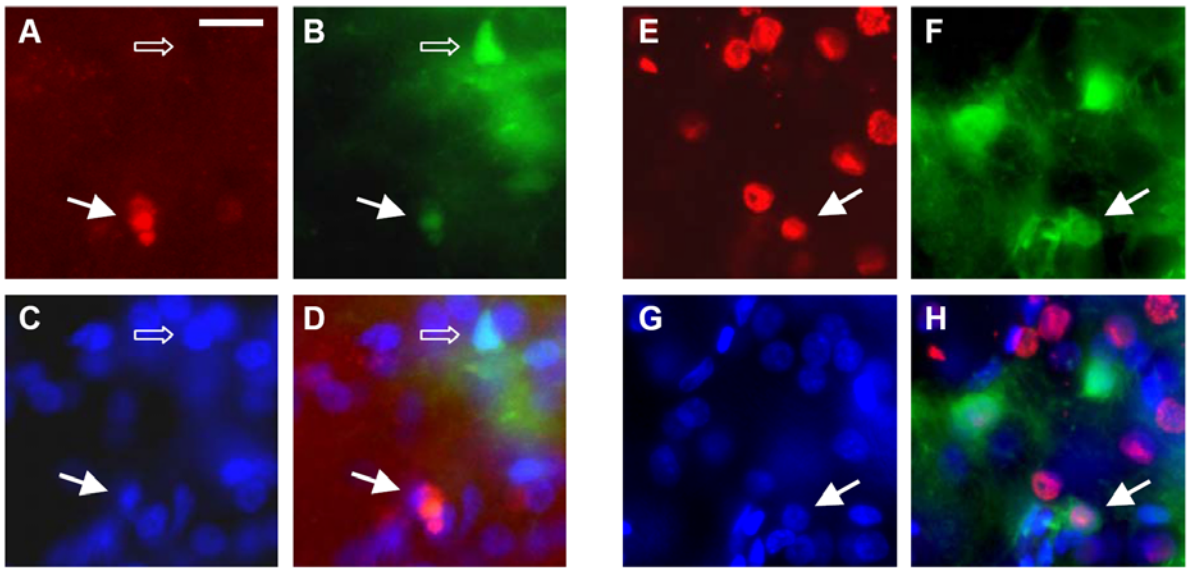

Figure 3.20 Infection with shFoxP2-virus does not induce apoptosis. (A) We labeled apoptotic cells in 50µmsagittalsections from PHD29 male zebra finch brains, injected with shFoxP2 virus on PHD23. DNA double strand breaks characteristic of apoptotic cells were detected using the TUNEL method withFluorescein(FITC)-marked 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.

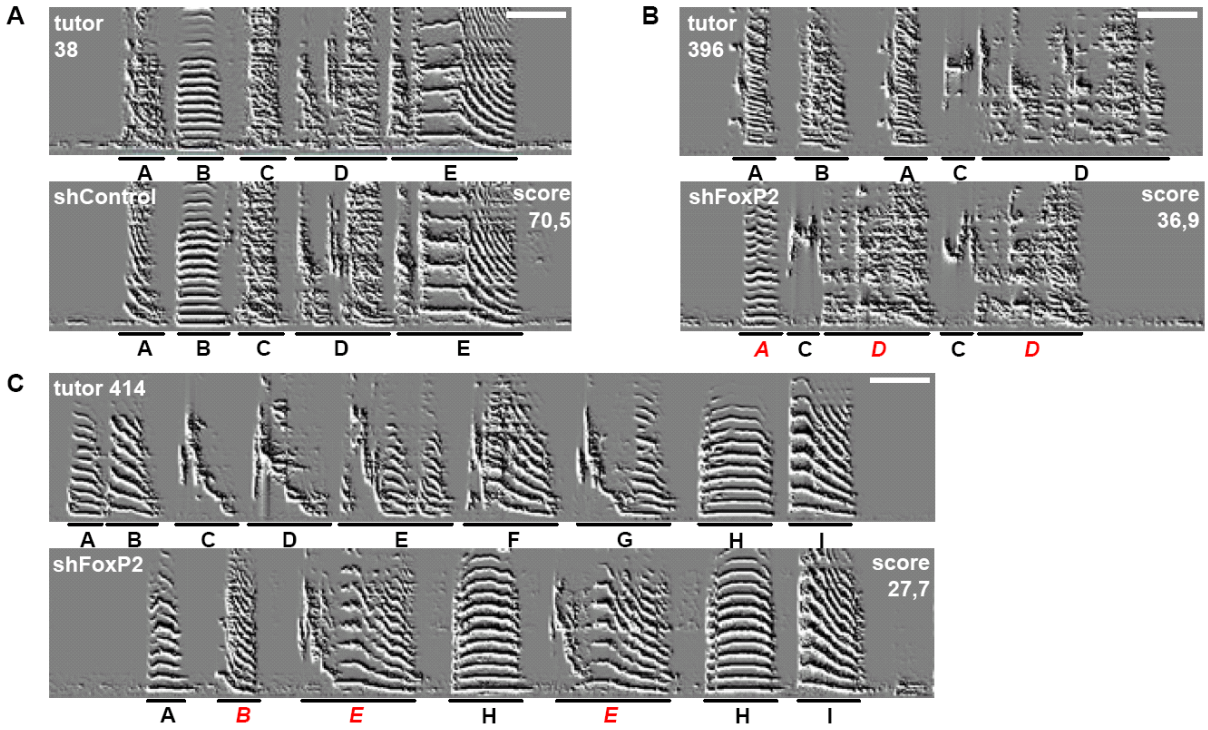

Figure 3.21 Song learning in FoxP2 knockdown birds. Sonograms from FoxP2 knockdown and control birds. Each sonogram depicts a representative motif of one animal (scale bars 100ms, frequency range 0-8600Hz). Tutor syllables are underlined with black bars and identified by letters. The identity of pupil 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).

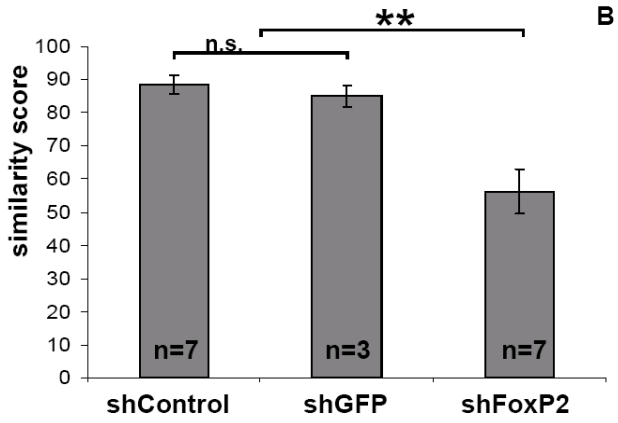

Figure 3.22 Knockdown of FoxP2 reduces motif similarity. The mean similarity between pupil and tutor motifs was significantly lower in shFoxP2 injected animals than in shControl and shGFP-injected birds, indicating that knockdown animals copied less acoustic material from their tutors [± standard error 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.), $P>0.5$].

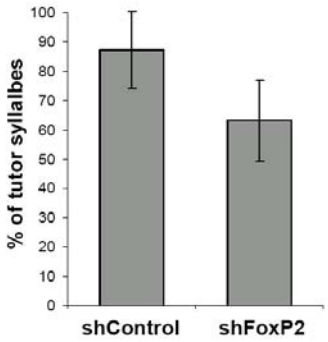

Figure 3.23 Manual counting of syllables copied by knockdown and control animals. All syllables that matched a tutor syllable by visual inspection on a sonogram, were counted for shFoxP2 and shControl-injected animals. Bars represent the mean percentage of tutor syllables copied by the pupils (±STDEV,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

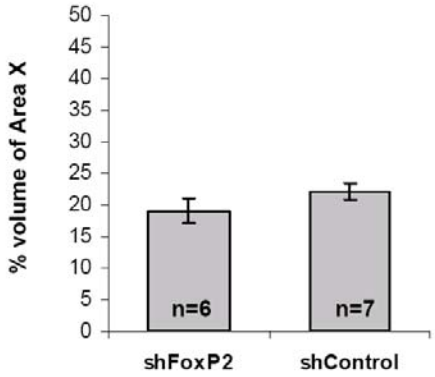

Figure 3.24 The volume of Area X targeted by virus injection was not significantly different in FoxP2 knockdown (shFoxP2) and control animals (shControl; two-tailed t-test $P>0.5$). Bars represent the percentage of total Area X volume, averaged across hemispheres, expressing the viral reporter GFP (±SEM).

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).

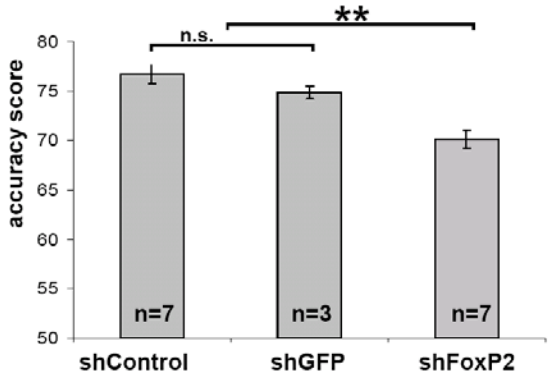

Figure 3.25 Knockdown of FoxP2 reduces motif accuracy. Average motif accuracy was significantly reduced in shFoxP2 knockdown animals compared to control animals, indicating that they imitated their tutors less exactly (± SEM; two-tailed t-test, $^{**}P<0.001$, Bonferroni-corrected α-level). shControl 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.

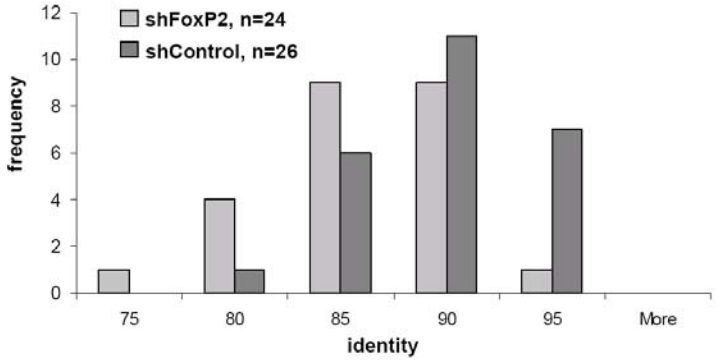

Figure 3.26 Knockdown zebra finches copied all syllables less precisely. Histogram of syllable identity scores obtained from pairwise comparison of visually identified pupil/tutor syllable pairs. In FoxP2 knockdown animals the distribution of the scores was shifted towards lower values indicating that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

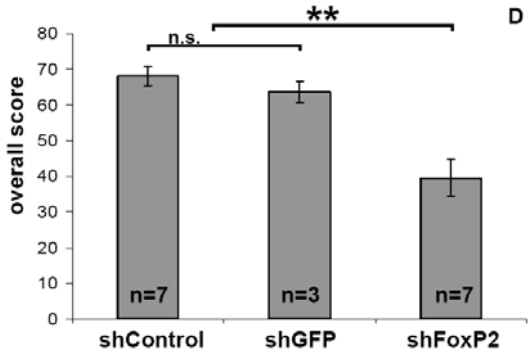

Figure 3.27 FoxP2 knockdown birds have reduced overall imitation scores. The overall imitation score was significantly lower in shFoxP2-injected birds, than in the control group (± SEM; two-tailed t-test, $^{**}P<0.001$, Bonferroni-corrected α-level). Birds injected with shControl or shGFP virus respectively 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).

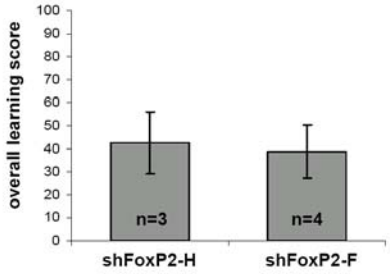

Figure 3.28 Both hairpin constructs targeting FoxP2 impaired song-learning to a similar degree. Bars indicate the overall imitation score of zebra finches injected with either shFoxP2-F or shFoxP2-H (±STDEV).

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).

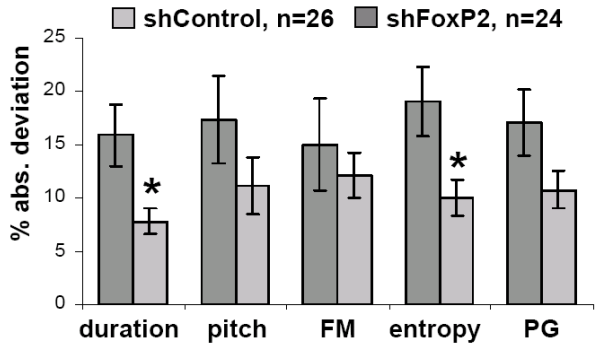

Figure 3.29 Analysis of individual syllable features. Comparison of mean acoustic feature values between pupil syllables and their respective tutor syllables revealed that the absolute deviation in % from the tutor mean values tended to be higher for shFoxP2-injected zebra finches than for shControl-injected 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.

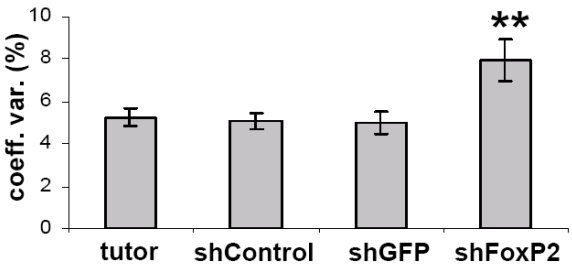

Figure 3.30 Syllable length varied more from rendition to rendition in knockdown birds (shFoxP2) than in controls (shControl and shGFP) and tutors, as indicated by a higher mean coefficient of variation of syllable duration (± STDEV, ANOVA, LSD post hoc test; shFoxP2 versus control: $P<0.002$; shFoxP2 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).

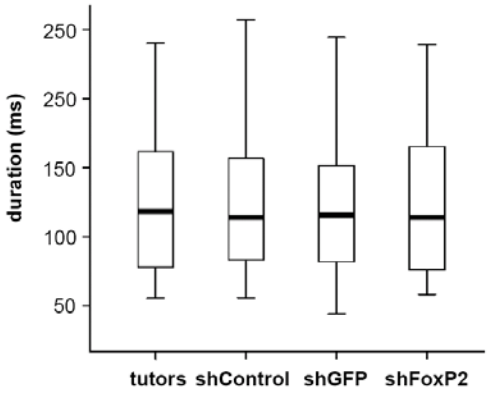

Figure 3.31 Syllables from knockdowns and control zebra finches were similar in their duration. Box plots represent the mean duration of all syllables from tutors and each experimental group (shControl, shGFP and shFoxP2-injected zebra finches). Boxes indicate the interquartile range of the distribution.

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).

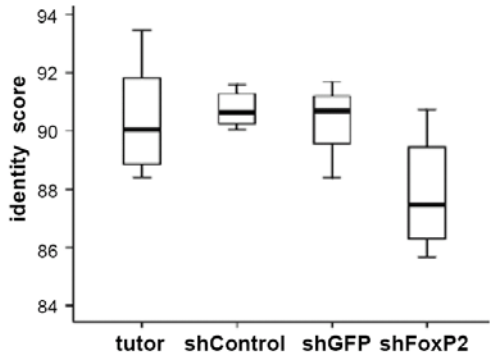

Figure 3.32 Variable song performance in FoxP2 knockdown zebra finches. Acoustic variability of syllables from rendition to rendition was higher in shFoxP2-injected than in control animals (shGFP and shControl injections), as indicated by significantly lower syllable identity scores (ANOVA, LSD post 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).

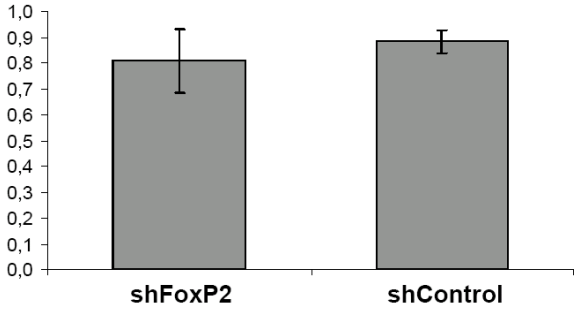

Figure 3.33 The consistency of motif performance was similar in shFoxP2 and shControl-injected birds (two tailed t-test, $P>0.4$). The syntax consistency score was calculated based on the entropy of 300 successive syllables (1-entropy).

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).

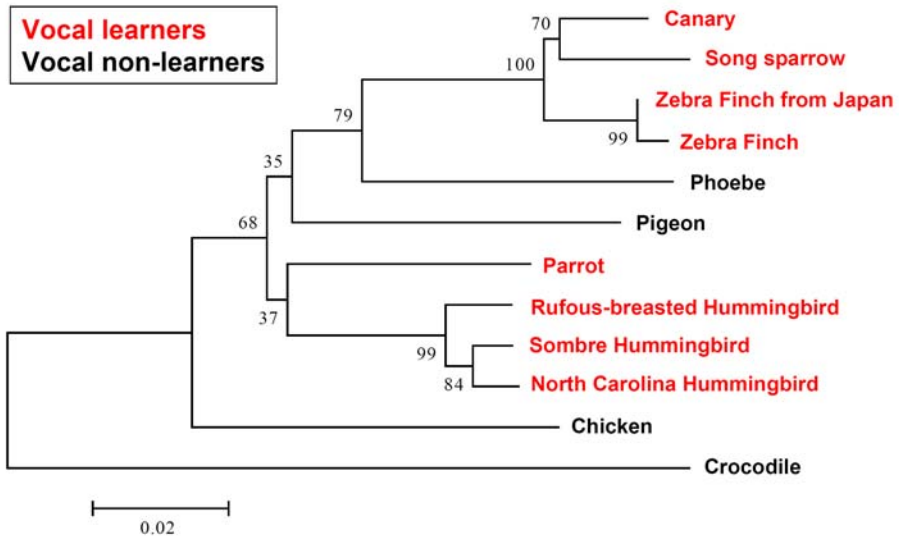

Figure 3.34 Phylogenetic relationship between FoxP2 genes from birds and the crocodile. Species capable of vocal learning are marked in red, non-vocal learners are marked in black. The phylogenetic tree was calculated according to the method of (Li et al., 1985). Bootstrap values on each branching point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

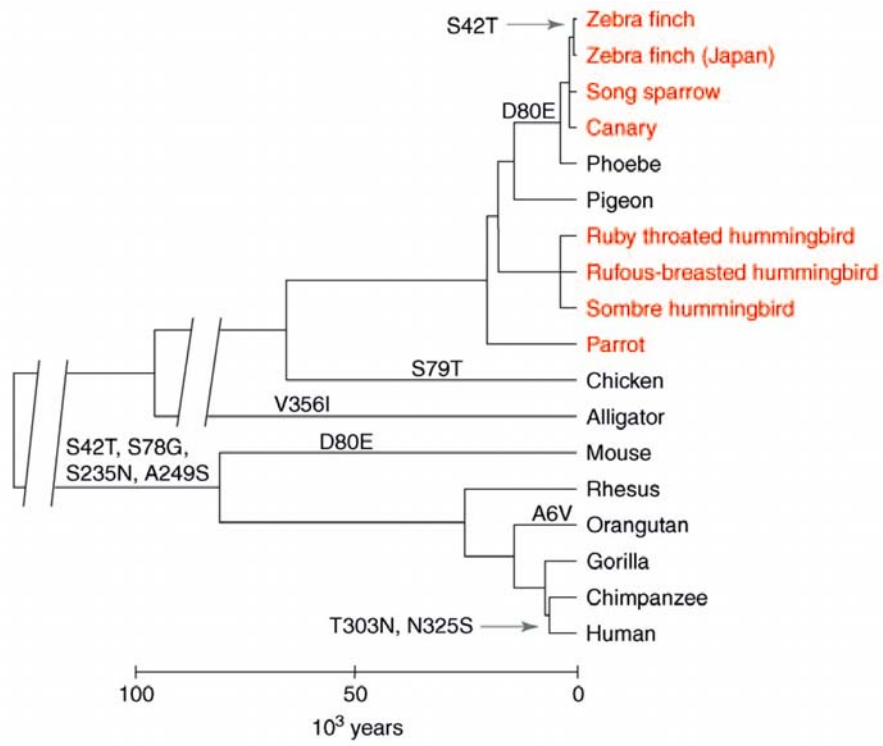

Figure 3.36 FoxP2 amino acid changes mapped on the phylogenetic tree of the species indicated. The seven song-learning avian species are marked in red, all other species, including the three non-song-learning birds, appear in black. Amino acid changes were inferred by parsimony and the phylogenetic tree 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.