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)].

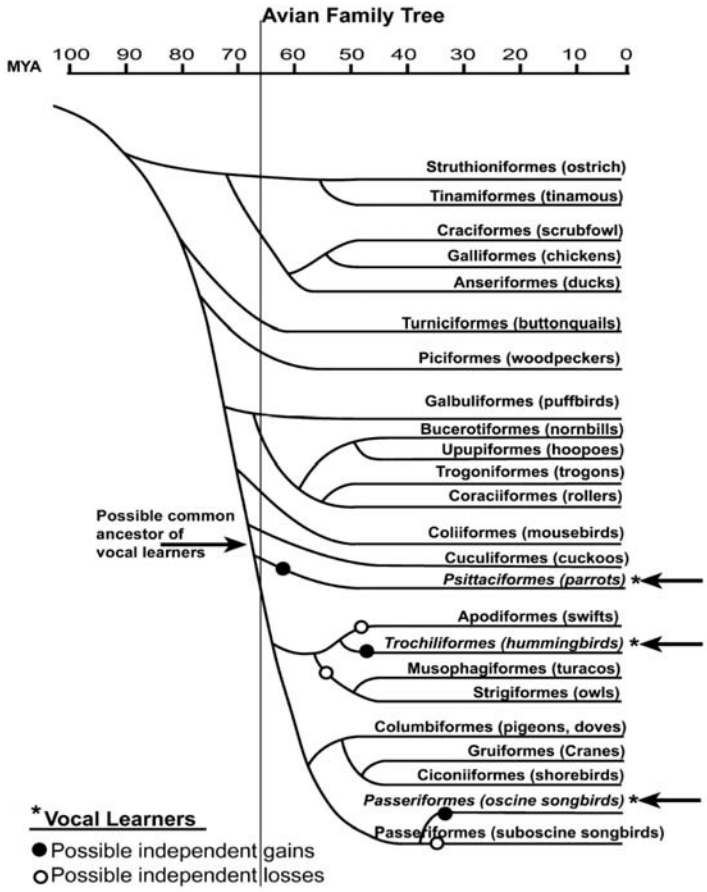

Figure 4.1 Evolution of vocal learning in the family tree of living avians. The Latin name of each order is given along with examples of common species. Passeriformes are divided into its two suborders: suboscine and oscine songbirds. The vertical line down each tree indicates the cretaceous-tertiary 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.