# FoxP1, FoxP2 and FoxP4 in the song control system of zebra finches: molecular interactions and relevance for vocal learning

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LE MAÁX MA´TU P´ILIK U YICH
BAJLA´E´, MIS BIK´IN U P´ILIK
WHO DID NOT OPEN HIS EYES TODAY
WILL NEVER DO IT.
MAYAN PROVERB
ICH WEIß DASS ICH NICHTS WEIß.
SOKRATES
LO DOCTOR NO QUITA LO PENDEJO.
PROVERBIO POPULAR BASADO EN SOKRATES
'GLAUBE'
HEIßT
NICHT-WISSEN-
WOLLEN, WAS
WAHR IST.
FRIEDRICH NIETZSCHE
DER VOGEL KÄMPFT SICH AUS DEM EI.
DAS EI IST DIE WELT. WER GEBOREN WERDEN WILL,
MUSS EINE WELT ZERSTÖREN.
HERMANN HESSE
"EL UNIVERSO NO ES PREDECIBLE,
NO IMPORTA LO QUE LOS CIENTÍFICOS TE DIGAN"
CARLOS CASTANEDA

# Table of Contents


**Introduction ..... 7**

1.1 Human speech and bird song: Parallels and Differences ..... 7
1.2 Genes for Vocal learning? ..... 13
1.3 FoxP2 mutations and human speech ..... 17
1.4 FoxP2 and vocal learning in birds ..... 22
1.5 FoxP2 expression in the brains of various species ..... 23
1.6 Need for dimerization of FoxP subfamily members ..... 24
1.7 FoxP1 mutations and speech ..... 25
1.8 FoxP1 expression in the brain of various species ..... 26
1.9 FoxP4 ..... 27
1.10 FoxP4 expression in the brain of various species ..... 28
1.11 Molecular function of FoxP subfamily members ..... 29
1.12 Analysis of gene function by genetic manipulation in songbirds ..... 35
1.13 Aims of this study ..... 41

**1. Methods ..... 42**

2.1 Solutions and buffers ..... 42
2.2 Enzymes ..... 44
2.3 Nomenclature ..... 45
2.4 Molecular Biology ..... 45
2.4.1 Sex determination of young zebra finches ..... 45
2.4.2 RNA extraction from zebra finch tissue ..... 45
2.4.3 Cloning of FoxP4 from zebra finch ..... 46
2.4.4 Cloning of FoxP1, FoxP2 and FoxP4 *in situ* probes ..... 47
2.4.5 Cloning of FoxP1, FoxP2 and FoxP4 FLAG and V5 tagged expression constructs ..... 48
2.4.6 Cloning of R553H and ΔFox Box FLAG and V5 tagged expression constructs ..... 49
2.4.7 Cloning constructs encoding short hairpin RNA for FoxP1 and FoxP4 ..... 49
2.4.8 Preparation of plasmid DNA ..... 50
2.4.9 Sequencing ..... 50
2.4.10 Sequence analysis ..... 51
2.4.11 *In situ* hybridization ..... 51
2.4.12 Double *in situ* hybridization ..... 52
2.4.13 PCR ..... 53
2.4.13.1 Normal PCR ..... 53
2.4.14 Agarose gel electrophoresis: analytical and preparative ..... 56
2.4.15 DNA isolation and purification from agarose gels ..... 56
2.4.16 DNA restriction digest ..... 56
2.4.17 Ligation of DNA inserts into linearized vectors ..... 56
2.4.18 Transformation of chemically competent E. coli cells ..... 57
2.4.19 Colony PCR ..... 57
2.4.20 Spectrophotometric DNA and mRNA concentration determination ..... 58
2.4.21 Luciferase assays ..... 58
2.5 Protein Biochemistry ..... 59
2.5.1 Protein extraction from culture cells ..... 59
2.5.2 Protein extraction from zebra finch tissue ..... 59
2.5.3 Western blot ..... 60

2.5.4 Co- immunoprecipitation ..... 60
2.6 Knock down efficiency of hairpin constructs in vitro ..... 61
2.7 Generation of Lentivirus ..... 62
2.8 Surgery and stereotactic injection of virus and retrograde tracers ..... 63
2.9 Behavioural paradigm and song analysis ..... 65
2.10 Histology ..... 65
2.11 Immunohistochemistry ..... 65
2.12 Microscopy ..... 66
2.13 Antibodies ..... 66
2.14 Bacterial strains ..... 67
2.15 Plasmids ..... 68
2.16 Constructs ..... 69
2.17 Primers ..... 70
2.18 Short hairpin target sequences for FoxP1 ..... 72
2.19 Short hairpin target sequences for FoxP4 ..... 73
2.20 ssDNA sequences encoding shRNA for FoxP1 ..... 74
2.21 ssDNA sequences encoding shRNA for FoxP4 ..... 75
2.22 Others ..... 76
2.22.1 Mammalian cell lines ..... 76
2.22.2 Software and internet resources ..... 76
***3. Results*** ..... **78**
3.1 Expression pattern of FoxP1, FoxP2 and FoxP4 in the zebra finch ..... 78
*3.1.1 Cloning of FoxP1, FoxP2 and FoxP4 genes* ..... 78
*3.1.2 Comparisons of FoxP1, FoxP2 and FoxP4 sequences* ..... 78
*3.1.3 Expression of FoxP1, FoxP2 and FoxP4 genes in the adult zebra finch brain* ..... 81
*3.1.4 Expression of Foxp1, FoxP2 and FoxP4 at different ages in the zebra finch brain* 85
*3.1.5* Characterization of *FoxP1+* and *FoxP4+* cells in HVC ..... 86
*3.1.6 Double in situ* hybridization; are *FoxP* subfamily members in the same cells? ..... 87
3.1.7 Specificity of FoxP1, FoxP2 and FoxP4 antibodies ..... 90
3.1.8 Neurons in Area X express different combinations of FoxP1, FoxP2 and FoxP4 .. 91
3.2 FoxP1, FoxP2 and FoxP4 interaction ..... 95
3.2.1 Hetero- and homo- dimerization of zebra finch FoxP1, FoxP2 and FoxP4 *in vitro* 96
3.2.2 Hetero- dimerization of zebra finch FoxP1, FoxP2 and FoxP4 *in vivo* ..... 98
3.3. Function of the interaction of FoxP1, Foxp2 and FoxP4 ..... 102
3.4 Interaction consequences of FoxP2 mutant variant with FoxP1, FoxP2 and FoxP4 proteins ..... 104
3.4.1 Interaction of mutant FoxP2 proteins ..... 105
3.4.2 Interaction of mutant proteins with FoxP1, FoxP2 and FoxP4 proteins ..... 106
3.4.3 Mutant FoxP2 variants change their cellular localization if expressed with wild type FoxP proteins ..... 108
3.4.4 Co-expression of mutant FoxP2 variants affect the regulation of wild type FoxP proteins *in vitro* ..... 109
3.5 Knock down of FoxP1 and FoxP4 in vivo ..... 110
*3.5.1 In vitro* selection of specific short hairpins to downregulate zebra finch FoxP1 and FoxP4 ..... 110
3.5.2 FoxP1 and FoxP4 knockdown affects song learning in zebra finches ..... 117
**4. Discussion** ..... **129**
4.1 FoxP1, FoxP2 and FoxP4 expression in the zebra finch ..... 129
4.2 Analysis of FoxP1, FoxP2 and FoxP4 interaction ..... 133

4.3 Analysis of the interaction of mutant FoxP2 and wild type FoxP proteins ..... 134
4.4 Analysis of FoxP1 and FoxP4 function in vivo ..... 136
**5. References ..... 142**
**6. Appendix ..... 151**
6.1 Abbreviations ..... 151
6.2 MIQE Q-PCR ..... 156
6.3 Abstract ..... 159
6.5 Zusammenfassung ..... 161
6.6 Curriculum vitae ..... 164
6.7 Affidavit ..... 166
6.8 Acknowledgments ..... 167

# Introduction

## *1.1 Human speech and bird song: Parallels and Differences*

What is vocal learning? White (2010) defines it as "the experience-dependent modification of one's vocal motor output with the goal of mimicking other members of one's species (conspecifics) or of creating new sounds". Although many animals produce complex communication sounds, few of them learn these vocal signals via imitation (Doupe and Kuhl, 1999). Most vertebrates that vocalize appear to have inherited patterns of vocalizations that do not depend upon auditory feedback. On the other hand, some groups of birds (songbirds, parrots, hummingbirds) and mammals (whales, bats, possibly elephants and humans) have been shown to learn vocalizations (Kikusui *et al.,* 2011).

Besides human language, birdsong is arguably the acoustically most complex and diverse communication signal known (Knudsen and Gentner, 2010). Human speech and birdsong have numerous parallels. First, both birdsong and human speech are complex acoustic signals whose production has to be learned. Second, vocal learning requires both perception of sound and the capacity to produce sound (Doupe and Kuhl, 1999). The architecture and connectivity of avian and mammalian brains is analogous and may depend on partly homologous circuits and molecular networks (Bolhuis *et al.,* 2011). Both songbirds and humans demonstrate that learning is not solely dependent on innate predispositions and acoustic cues. Social factors can also dramatically alter learning since both learn better from a live tutor than from playbacks. And finally, a critical (sensitive) period is evident in both species (Doupe and Kuhl, 1999). Last, and a point of debate, is that birds can behave in ways that imply a sensitivity to syntactic rules, a characteristic that was long though to be unique for human language (Gentner *et al.,* 2006; Abe and Watanabe, 2011).

Evidence of vocal learning in human and birds comes from the fact that in both cases the initial vocalizations are immature. Young human babies babble, and young songbirds produced what is called subsong (Doupe and Kuhl, 1999). Initially, both humans and songbirds produce vocalizations that are different from those of adult conspecifics. During development, their vocalizations gradually come to resemble their adult form (Bolihuis *et al.,* 2011). Additionally, specific aspects of song depend on the specific auditory experience young birds have. Many cross fostering experiments show that birds can also learn the song, or aspects thereof, of the fostering species (Doupe and Kuhl, 1999). Another line of evidence supporting vocal learning is the development of abnormal vocalizations when humans or birds

with normal hearing are socially isolated and therefore not exposed to the vocalizations of others (Doupe and Kuhl, 1999). However, a predisposition is also evident, because isolate song retains certain species-specific features (Bolhuis *et al.,* 2011).

Humans and birds share also the fact that both have characteristic speech and song learning phases. Human infants first babble, then build one word, eventually string two words together before building simple sentences. Likewise, in many songbird species, such as the zebra finch, song learning has distinct phases: a memorization phase, during which the tutor song is stored in long-term memory, and a sensorimotor phase during which the bird's own vocal output is 'matched' with the memorized information (Figure 1.1) (Bolhuis *et al.,* 2011). During the memorization phase, young birds acquire an acoustic memory of song by listening to adult sing. Some time later, in the sensorimotor phase, juvenile birds begin to sing subsong, analogous to babbling in infants.. With further practice the song increasingly resembles the tutor song. This 'plastic song' is also louder and better structured, but still variable in form. Finally, song becomes crystallized in structure as the bird's produce a stereotyped version of the tutor song to which they were exposed earlier (Brenowitz *et al.,* 1997). In both humans and songbirds, the strong dependence of vocal behaviour on hearing early in life lessens in adulthood (Doupe and Kuhl, 1999). Experiments in white crowned sparrow show that when a bird has developed crystallized song, removing auditory input by deafening had no or little effect on the production of stable song (Brenowitz *et al.,* 1997).

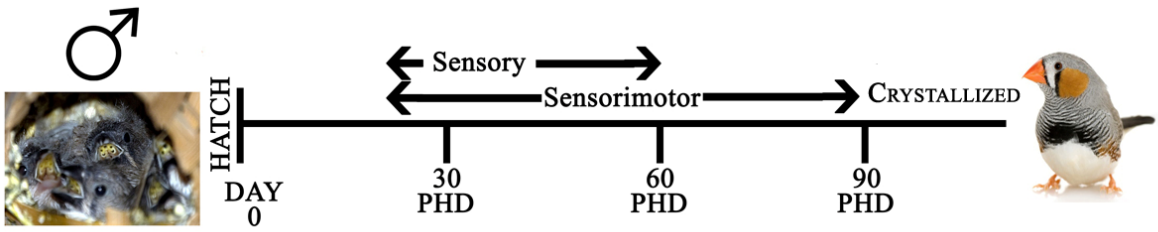



**Figure 1. 1 Timeline of song learning in zebra finches. The sensory and sensorimotor phases both start around 30 post hatching days (PHD) and overlap most of the time. Around 60 PHD the sensory phase ends. During this first phase the bird listens to and learns the tutor song. The learned song, called template, cannot be simply translated into the correct motor pattern. The second phase, sensorimotor, is where this vocal practice takes place; the bird must actively compare the template memorized song and match its own vocalizations, using auditory feedback.**

Parallels between human speech and birdsong not only exist on the behavioural level, but also on the level of the neural circuits mediating these behaviours. The architecture and connectivity of avian and mammalian brains are much more similar than had been recognized previously (Figure 1.2). Edinger, the father of comparative neuroanatomy, formulated a unified theory of brain evolution that formed the basis of a nomenclature that has been used to

define the cerebral subdivisions of all vertebrates. According to this theory, the avian cerebrum is almost entirely composed of basal ganglia. The basal ganglia were thought to be involved only in instinctive behaviour, and the malleable behaviour that was thought to typify mammals exclusively required the so-called neocortex. However, towards the end of the twentieth century, a wealth of evidence accumulated that these viewpoints were incorrect. The avian cerebrum has a large pallial territory that performs functions similar to those of the mammalian cortex (Jarvis *et al.,* 2005). For instance, avian pallial 'song' regions bear functional similarities with human auditory and motor cortices and the importance of the basal ganglia for both speech and birdsong is starting to be understood mechanistically (Bolhuis *et al.,* 2011).

Song behaviour in birds is regulated by a discrete network of interconnected nuclei that undergo dramatic changes in anatomical, neurochemical, and molecular organization during periods of learning (Brenowitz *et al.,* 1997). These nuclei are involved in song recognition, production and learning (Bolhuis *et al.,* 2011) and are collectively called the "song system". The song system is divided into two connected main neural pathways: Motor pathway and anterior forebrain pathway (AFP), in addition the auditory pathway is also important for vocal learning.

First I will discuss the avian auditory pathway and point out differences and similarities to the human one. Auditory information enters the avian brain via sensory transduction in the inner ear, where vibrations on the basilar membrane of the cochlea are transduced into the action potentials propagated to the cochlear nuclei in the brainstem (Figure 1.2a). Afferents from the brainstem converge in the midbrain structure mesencephalicus lateralis dorsalis (MLd). MLd is anatomically analogous to the mammalian inferior colliculus (IC), and in many respects it is also functionally analogous. Like the mammalian IC, MLd neurons show a wide variety of tuning characteristics, are not particularly sensitive to level differences, and have temporally precise responses that preserve the timing information in the acoustic signal. MLd sends afferents into the avian auditory thalamus, the Nucleus Ovoidalis (Ov). Thalamic efferents project from Ov mainly into the Field L complex (shortened here to Field L), the avian analogue of the mammalian primary auditory cortex. Specifics of cytoarchitecture and neural connectivity allow further subdivision of the Field L complex into regions L1, L2a, L2b, L3 and L. From Field L, forebrain processing proceeds along a number of parallel and interconnected pathways. Field L itself projects to the caudomedial nidopallium (NCM), which surrounds Field L anatomically, and to the lateral portion of the caudal mesopallium (CLM). The medial portion of the caudal mesopallium (CMM) shares reciprocal projections

with both CLM and NCM. Work with immediate early genes (IEG) like Zenk shows that NCM and CMM are involved in processing of conspecific song. In addition to connections between regions within the auditory system, anatomical and functional connections have been observed between auditory processing areas and the song production system, providing a path for feedback during the acquisition and maintenance of a bird's own song (Knudsen and Gentner, 2010). NCM and the CMM are analogous with the mammalian auditory association cortex, in particular with belt and parabelt regions (Bolhuis *et al.*, 2010). The avian forebrain lacks the gross anatomical structure of mammalian cortex. Nonetheless, birds and mammals use their auditory systems to solve many similar behavioural problems. Structural and functional similarities present in the auditory regions of both classes of animals at the cellular and systems levels, and across the whole of the forebrain may reflect these common behavioural goals (Knudsen and Gentner, 2010).

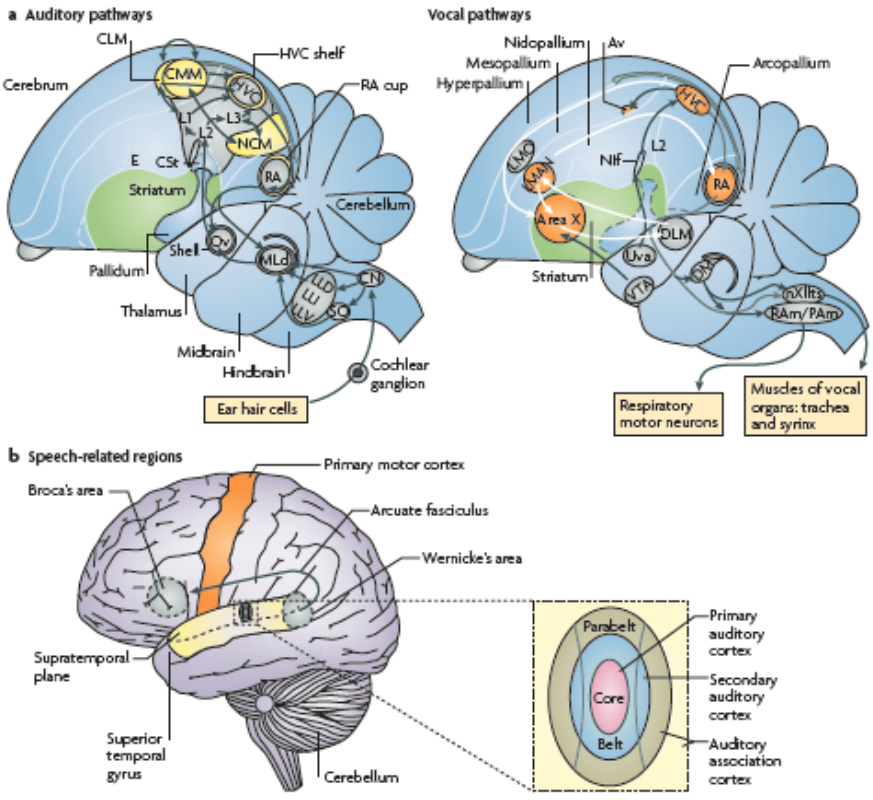



**Figure 1. 2 The bird brain and the human brain. (Taken from Bolhuis *et al.*, 2010). "a" Schematic diagram of a composite view of parasagittal sections of a songbird brain, giving approximate positions of nuclei and brain regions. Auditory pathways, with the known connections between the Field L complex, a primary auditory processing region and some other forebrain regions are shown (left panel). Brain regions that show increased neuronal activation when the bird hears song are represented in yellow. The caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) regions are thought to contain the neural substrate for tutor song memory. Vocal motor pathways are also shown (right panel). Lesion studies in adult and young songbirds led to the distinction between a posterior pathway, known as the song motor pathway (SMP) (shown by grey arrows), considered to be involved in the production of song, and a rostral pathway (shown by white arrows), known as the anterior forebrain pathway (AFP), that is**

**thought to play a role in song acquisition and auditory–vocal feedback processing. The two networks together are called the song system. The orange nuclei in the song system show substantially enhanced neuronal activation when the bird is singing. "b" Schematic view of the left side of the human brain, showing regions that are involved in speech and language. Broca's area is particularly involved in (but not limited to) speech production, whereas Wernicke's area is involved in speech perception and recognition, as well as other language-related tasks. The two regions are connected by the arcuate fasciculus. Area X, Area X of the striatum; Av, avalanche; CLM, caudal lateral mesopallium; CN, cochlear nucleus; CSt, caudal striatum; DLM, dorsal lateral nucleus of the medial thalamus; DM, dorsal medial nucleus of the thalamus; E, entopallium; L1, L2, L3, subdivisions of Field L; LLD, lateral lemniscus, dorsal nucleus; LLI, lateral lemniscus, intermediate nucleus; LLV, lateral lemniscus, ventral nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; LMO, lateral oval nucleus of the mesopallium; MLd, dorsal lateral nucleus of the mesencephalon; NIf, interfacial nucleus of the nidopallium; nXIIts, tracheosyringeal portion of the nucleus hypoglossus (nucleus XII); Ov, ovoidalis; PAm, para-ambiguus; RA, robust nucleus of the arcopallium; RAm, retroambiguus; SO, superior olive; Uva, nucleus uvaeformis; VTA, ventral tegmental area.**

The second pathway I will discuss is the Motor Pathway. The motor pathway controls the production of sound, and some part of it might also be important in song learning. This circuit consists of projections from the thalamic nucleus UVA and the nucleus NIf to HVC (Figure 1.2 Vocal pathway, grey arrows). HVC projects to the nucleus of the archistriatum or RA in the forebrain, and RA projects both to the dorsomedial part of the intercolicular nucleus in the midbrain (not sown in the Figure 1.2) and to the trachiosyringeal part of the hypoglossal motor nucleus in the brain stem (nXIIts). Motor neurons in nXIIts send their axons to the muscles of the syrinx. Since the motor pathway is important in song production any disruption to this pathway eliminates the ability to sing (Brenowitz *et al.,* 1997). This vocal motor pathway is similar to mammalian motor corticobulbar pathways (Jarvis *et al.,* 2005).

The third pathway is the anterior forebrain pathway (AFP) (Figure 1.2 Vocal pathway, white arrows). The AFP originates in HVC, which in addition of its projection to RA also projects to Area X, then Area X projects to DLM in the thalamus, from DLM to the lateral portion of the magnocellular nucleus of the anterior neostriatum (lMAN), and finally to the motor pathway nucleus RA. In addition lMAN neurons that project to RA send collaterals to Area X, thus providing the potential for feedback within this pathway (Brenowitz *et al.,* 1997). The vocal nuclei that are involved in the imitation of vocalizations form a pallial–basal ganglia–thalamic–pallial loop. This vocal learning pathway is similar to mammalian cortical–basal ganglia–thalamic–cortical loops, which are involved in motor learning, sensorimotor integration and addictive behaviours (Jarvis *et al.,* 2005). In mammals, afferents from frontal cortex densely innervate the striatum of the basal ganglia, which also receives inputs from several other areas of the cortex. The striatum controls behavioural sequencing in many species. Spiny neurons, the principal cells of the striatum, have properties that make them ideal for recognizing patterned sequences across time. In human adults, the neural substrate of motor representations of speech is traditionally thought to involve regions in the inferior

frontal cortex (including Broca's area), whereas perception and memory of speech is considered to involve regions in the superior temporal cortex (Wernicke's area and surrounding regions, Figure 1.2 b). Finally, the AFP loop in the song system bears strong similarities in connectivity, neurochemistry and neuron types to the mammalian basal ganglia, and both the lMAN and the HVC have been tentatively suggested to correspond functionally to Broca's area. Of the AFP, I want to point out Area X, because this is a nucleus that expresses FoxP2 (Haesler *et al.,* 2004), which I will discuss latter. Area X is also important in developing birds to learn the species-specific and individual-specific song (Scharff and Nottebohm, 1991; Sohrabji *et al.,* 1990). Once song is stably learned, Area X continues to be relevant for online monitoring of song. Area X lesions in juvenile zebra finches result in unusually variable song that persists into adulthood (Scharff and Nottebohm, 1991; Sohrabji *et al.,* 1990; Kao *et al.,* 2005). Adult song production deteriorates without Area X post lesion (Kobayashi *et al.,* 2001). Area X lesions in adult also show that recognition of the birds own song (BOS) against song of other zebra finches is impaired (Bolhuis *et al.,* 2010). Last, Area X is one of the song nuclei that show incorporation of new neurons through the whole life of the bird (Rochefort *et al.,* 2007). Area X possesses all 4 types of cells present in the mammalian striatum and also receives a dense dopaminergic innervation from the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) (Gale and Perkel, 2010). These types of cells include spiny neurons, fast spiking interneurons, low spiking threshold interneurons, and cholinergic interneurons. In addition, Area X presents a fifth class of neurons not present in the mammalian striatum, these are the pallidal- like neurons, called like that because they have pallidal properties although they are in the striatum (Farries and Perkel, 2002). A difference between mammalian spiny neurons and the avian spiny neurons in Area X is that the last ones do not send projections to the pallidum or substantia nigra pars rediculata; instead the pallidal-like neurons project to the thalamus (Person *et al.,* 2008). Another difference of Area X spiny neurons is that it expresses both dopamine receptors (Kubikova *et al.,* 2009; Ding and Perkel, 2002), in mammals there are two separated populations, one that expresses D1-dopamine receptors and co-localizes with substance P and another one that has D2-dopamine receptors and co-localizes with enkephalin (Smith *et al.,* 1998). The lack of thalamic input into Area X is another important difference to mammalian striatum. A functional parallel between the mammalian basal ganglia and Area X is that both are involved in rewarded behaviours (Gale and Perkel, 2010).

Lastly, a comparison of vocalization pathways among terrestrial mammal species has revealed that only humans exhibit a direct pathway from the motor cortex to the motorneurons

controlling the larynx muscles. This could me the most important derived feature in the human lineage regarding the ontogeny of speech (Fischer *et al.*, 2011)

In summary, there are various parallels between vocal learning of humans and birds, parallels that involve not only the learning phases but also neural and molecular networks, (discussed below). To date, birds have been proven to be a good model for studying vocal learning. There is no evidence that the genetically more amenable animal model, the mouse, learns to produce their vocalizations. A recent study has shown that the courtship ultrasonic vocalizations that they emit are not learned, because cross fostered animals emit the vocalization of their genetic parents (Kikusui *et al.,* 2011).In addition, studies on male song vocalizations in mouse did not show a sustained response of female mice, since they only responded to those songs the first time they where exposed to them (Fischer *et al.*, 2011) Other models that might be interesting for vocal learning could be the bats in which new interest is merging in the last years (Scharff and Petri, 2011).

## *1.2 Genes for Vocal learning?*

What characteristics might genes have that are involved in vocal learning? They should probably be expressed in vocal learning regions, either during their development or during the acquisition of song. They might be regulated by vocalizations or audition. What about genes involved in human language disorders, or other brain disorders, might they also play a role?
Many studies have tried to find vocal learning genes, using criteria mentioned above, and some interesting genes were found. Let us start with genes enriched in song control nuclei. To date there is no gene found that is exclusively expressed in song control nuclei; rather genes are enriched or present in those regions, but not exclusively so.

Below I will summarize the studies that have addressed molecular correlates for song learning and song behaviour. The first review on this subject was published by Clayton (1997). He summarized molecules that are expressed in song nuclei and change their expression according to different learning phases. Among those are MEK-1, a signal transduction kinase, is differentially expressed in HVC. *n*-Chimaerin (aka HAT-2), which is a cytosolic signal transduction protein, as well as NFm (neurofilament) and MBP (myelin basic protein), are all differentially expressed in HVC and RA. α- synuclein, a presynaptic lipid binding protein with differential expression in HVC, lMAN and RA,(Clayton and George, 1999). These nuclei also express GAP-43 or NEUM (neuromodulin), an axonal growth factor

protein. HAT-14 (aka NRGR, neurogranin), which is a dendritic signal transduction protein is differentially expressed in HVC, RA and Area X. NEUM and NRGR expression in Area X and RA genes declines at around 30 PHD and stay low into adulthood. NRGR and NEUM are also in Area X and HVC expressed at higher levels before 35 PHD than later. Co-occurrence on pre- and post-synaptic sites early in song development could indicate a concerted program of regulation based on these signalling systems in the HVC-Area X ("anterior forebrain loop") pathway early in song development (Clayton, 2009).

Zenk (egr-1) and C-jun, are transcription factors that belong to the class of immediate early genes (IEG), so named because their mRNA are rapidly transcribed after stimulation. IEGs have gained a lot of attention as a marker of differential activation of the song system by social context song behaviour (Mello *et al.,* 1994; Mello *et al.,* 1995 and 1998; Kubikova *et al.,* 2007 and 2009; Zapka *et al.,* 2010) ALDH-1, which is involved in the production of retinoic acid, showed also a differential expression in HVC, RA and lMAN (Clayton, 1997) and is important for song learning in HVC (Denisenko-Nehrbass *et al.,* 2000). Receptors of steroid hormones, like androgen receptors (AR) and estrogen receptors (ER), are differentially expressed in HVC, RA and lMAN (AR), and NCM (ER) (Clayton, 1997).

Dopamine receptors were also studied in the song system because of their role in the reward system and thus a potential influence on learning. Dopamine is released in Area X (Gale and Perkel; Ding and Perkel 2002, 2005; Heimovics *et al.,* 2009), a region particularly relevant for the present thesis, and D1A, D1B and D2 receptors are expressed in this region. Half of the cells in Area X express D1 and D2 receptors. This is different from mammalian basal ganglia, where only a small percentage of cells express both receptors. The expression of these receptors changes during development in Area X, and singing in different social contexts activates neurons that express D1 and D2 receptors differentially (Kubikova *et al.,* 2009). Area X medium spiny neurons have different intrinsic properties after stimulation with dopamine (Ding and Perkel, 2002). Dopamine is important for LTP to occur in these neurons of birds from 47 PHD on (Ding and Perkel, 2004).

The expression of all glutamate receptors was studied in zebra finches and non vocal learners. From the 26 glutamate receptor units known in mammals, 21 exist in birds and their sequence is highly similar to those of their mammal counterparts (82-90%), with the exception of mGluR2.. 19 were cloned and their pattern of expression was studied. These 19 subunits of glutamate receptors cover all 4 subfamily of glutamate receptors known: 1) α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA), 2) kainate (KA), and 3) N-methyl-D-aspartate (NMDA); and 4) the metabotropic subfamily. All of the receptors have a

unique pattern of expression in the zebra finch brain, The family and presence of glutamate receptors was highly conserved between the brain of birds and mammals and it is thought that the physiological function of most receptors is also similar in birds and mammals. All 19 of the 21 subunits/subtypes are differentially expressed in one or more of these larger vocal nuclei (8 subunits/subtypes in HVC, 9 subunits/subtypes in RA, 11 subunits/subtypes in lMAN, and 9 subunits/subtypes in Area X). HVC and RA potentially could be dominated by GluR2/GluR3 heteromers resulting in faster desensitization times than the surrounding brain areas, with higher GluR2/GluR1 and GluR2/GluR4 heteromers. Area X would be expected to have a lower number of GluR2/GluR4 receptors relative to the surrounding striatum that is compensated for by a higher number of GluR2/GluR1 receptors (Wada *et al.,* 2004). NMDA receptors are critical for many forms of learning and synaptic plasticity. It is thought that experience is stored in the brain as a consequence of an activity-dependent process of synaptic strengthening (related to LTP) and weakening (related to LTD), governed in turn by the relationship between presynaptic activity and postsynaptic firing. Most of the synapses that express LTP also express LTD, and NMDA receptors have been linked to both of these forms of synaptic plasticity (Lisman 2003). It has been proposed that a change in NMDA receptors subunits expression could be the leading force that changes plasticity in neurons when the sensitive period closes. Furthermore, systemic blockade of NMDA receptors impairs song learning when it overlaps with restricted periods of tutoring, but not if it occurs in non tutoring days. These data suggest that NMDA receptors in the AFP are necessary for normal song learning (Nordeen and Nordeen, 2004).

FnTm2 is a novel fibronectin type III protein found in a screening looking for differences between HVC and RA. It is expressed in HVC that project to Area X projecting neurons, Area X and lMAN. It is not regulated by singing but hearing can change its expression in lMAN, since deafened birds have less expression in this nucleus. FnTm2 is expressed in mice in the "limbic system" and might be involved in affective and/or attentional states. Its precise role is still unknown (Agate *et al.,* 2007).

In a study using micro dissection and microarray techniques 33 genes were found which were activated by singing in the different song nuclei. The functions of all 33 singing-regulated genes (including egr-1 and c-fos) spanned a range of categories: signal transduction proteins (egr-1, c-fos, c-jun, sim junB, Atf4, Hspb1, UbE2v1, HnrpH3, Shfdg1, and Madh2), chromosome scaffold proteins (H3f3B and H2AfX), actin-interacting cytoskeletal proteins (Arc, sim Fmnl, Tagln2, ARHGEF9, and β-actin), a $Ca^{2+}$-regulating protein (Cacyb), cytoplasmic proteins with enzymatic (Prkar1a, Atp6v1b2, and Ndufa5), protein kinase

(Gadd45β), folding (Hsp70-8), binding, and transporting functions (Hsp40, Hsp90α, and Hsp25), and membrane (Stard7, Syt4, and Ebag9) and synaptically released proteins (JSC, BDNF, and Penk). All these genes can be grouped into four anatomical expression categories: those regulated in (i) all major pallial vocal nuclei (HVC, RA, and lMAN) and the striatal vocal nucleus (Area X); (ii) a combination of 1 or 2 pallial and the striatal vocal nucleus; (iii) pallial (P) vocal nuclei only; and (iv) the striatal (S) vocal nucleus only. Area X had the highest percentage (94%) of genes regulated by singing. These results further suggest that each vocal nucleus has unique but overlapping signal-transduction pathways that are activated during singing behaviour. Area X is the only nucleus found where genes are down-regulated by singing; one of these (ARHGEF9) is a GTPase that acts as a molecular switch to regulate actin cytoskeleton formation during cell signalling (Wada *et al.,* 2006).

The above mentioned genes are not linked to human language or its disorders. In contrast, FOXP2 is a gene which is relevant for language and vocal learning in birds: mutations lead to a language disorder (Lai *et al.*, 2001; MacDermont *et al.*, 2005), it is expressed in regions important for vocal learning (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004), it is regulated by singing in birds (Teramitsu *et al.*, 2006; Miller *et al*., 2008), and many other characteristics which will be discuss further on. Deletions and mutations of FOXP1, the closest homolog of FOXP2, also lead to language disorders (Pariani *et al.*, 2009; Carr *et al.*, 2010; Horn *et al.*, 2010; Hamdan *et al.*, 2010), which I will discuss also later.

Interestingly, a gene whose transcription is regulated by FoxP2 (Vernes *et al.,* 2008) also plays a role in language. In 2006 a group of Old Order Amish children who harboured a mutation in the gene known as contactin- associated protein-like 2 CNTNAP2 were reported to exhibit several deficits including intractable seizures, autism, and, relevant here, language regression (Strauss *et al.,* 2006). This gene was identified by three separated groups as an autism susceptible gene (White 2010; Alarcon *et al.,* 2008). In children with a Specific Language Impairment (SLI) it was discovered that genetic polymorphisms correlate with their ability to perform a non-word repetition task. There are striking differences in the pattern of expression of CNTNAP2 between vocal learners and non-vocal learners in mammals. CNTNAP2 has a strong expression in regions of the cortico-basal ganglia thalamic circuitry in foetal human brain, in contrast to a broad expression pattern in mice and rats, all of which is consistent with the language phenotype (Abrahams *et al.,* 2007). The pattern of expression in the zebra finch was also assessed using *in situ* hybridization. Differences were found between males and females in the pattern of expression of CNTNAP2, which supports an involvement of the gene in the formation and function of the neural circuits necessary for vocal learning in

birds. Differential expression between ages where found in lMAN, were there was an up-regulation of the gene at 50 PHD, not found in females. In Area X the expression did not change at any age. RA had and up-regulation starting at 6 PHD in males, again this change in expression was not evident in females (Panaitof *et al.,* 2010).

In summary, there are many genes that are expressed in regions of vocal learning in birds but their importance to human vocal learning is not known, except for FOXP1, FOXP2 and CNTNAP2.

## *1.3 FoxP2 mutations and human speech*

FoxP2 is a transcription factor that belongs to the Forkhead Box (Fox) protein family (discussed in 1.11).

The first FOXP2 mutations that were found in humans were the R553H mutation and the R328X. Both mutations lead to a substantial impairment of expressive and receptive language, called developmental verbal dyspraxia (DVD) or childhood apraxia of speech (CAS) (Lai *et al.,* 2001). DVD is a motor speech disorder that affects the quality of speech. Mostly the *planning and programming* of speech is affected, and execution of individual speech sounds is preserved but co-articulation and sequencing seems to be impaired (Liegeois and Morgan, 2011). After these mutations, other mutations of the FOXP2 gene were found, the Q17L mutation and Q40-Q44 mutations, all leading to similar phenotypes (MacDermont *et al.,* 2005). Data of deletions of FOXP2 or the regions in which FOXP2 is (7q31.1) adds to the body of evidence which supports the role of FOXP2 in language impairment. To date there are 15 reports of cases that have deletions in the region were FOXP2 is. Seven of these reported cases had no information on speech development provided (four died in infancy); three of them had either absent speech, or speech problems with unusual crying. Lastly, five of the patients with deletions in 7q31 had speech and language impairments with oromotor dyspraxia (Lennon *et al.,* 2007).

The best studied FOXP2 mutation is the R553H mutation, which was found in a large family called the KE family (Figure 1.3). In the 1990s, scientists became aware of a British family with severe communication problems. Indeed, half the KE family suffers from a rare form of SLI in which the most prominent deficits lie in sequencing of orofacial movements, especially those required for speech. Meanwhile, non-learned orofacial control involved in chewing, swallowing or smiling is unimpaired. In addition to core deficits in orofacial control

and spoken language, affected individuals are also impaired on tests of verbal fluency and language comprehension (Vergha-Khadem *et al.*, 2005; White, 2010). Astonishingly, the pedigree of the KE family suggested a simple autosomal dominant inheritance. The responsible gene locus was mapped to the region 7q31 of chromosome 7, and eventually the gene FOXP2 was identified to be the cause for this disease (Lai *et al.,* 2001). From this day on, the press has claimed FOXP2 to be the "language gene".

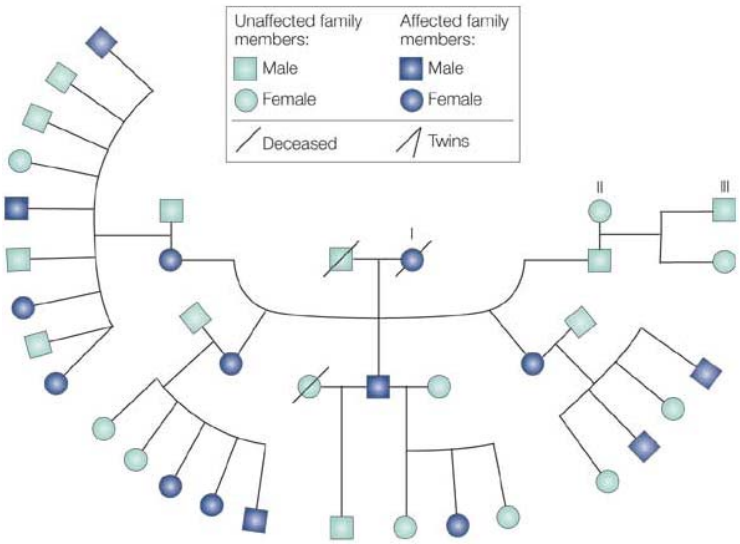



**Figure 1. 3 Inheritance pattern of the FOXP2 mutation in the KE family. This family consists of three generations, half (15) of whose members are affected by DVD, 16 are unaffected members and 6 spouses who married into the family are also displayed. Squares show males and circles females. Dark blue colour shows affected members and light blue colour unaffected. Vergha-Kadehm *et al.* 2005.**

The R553H mutation affects an amino acid, which is in the Forkhead box. It is a substitution of an arginine to histidine amino acid at position 553 of the FOXP2 protein, due to a guanine to adenine base transition in the open reading frame of the nucleotide sequence (Lai *et al.,* 2001). In cell culture, the R553H mutation affects cellular localization, since the R553H mutant is predominantly found in the cytoplasm, in contrast to normal FOXP2, which is mainly found in the nucleus. The binding to DNA is also affected by this mutation and the regulatory properties of the SV40 promoter were also affected (Vernes *et al.*, 2006). In addition another study shows that R553H can interact with importins α and β, and that is why some of the R553H mutant can enter the nucleus. Furthermore, the cellular localization of the R553H co-expressed with the normal FOXP2 protein was also assessed. In this case the mutant-normal dimer enters the nucleus, indicating that the R553H protein switched its cellular localization to the nucleus. It is interesting to see if the pathogenesis of the R553H mutation is due the cytoplasmic presence of R553H, or the nuclear localization of the mutant-

normal dimer (Mizutani *et al.,* 2007). A recent article shows that the R553H mutation also affects neurite outgrowth in primary neurons as well as in cellular models (Vernes *et al.,* 2011).

The R328X mutation was found at the same time as the R553H mutation. Both mutations served to localize the gene affected in these two unrelated families with DVD. The R328X mutation was found in a patient called CS, who had a more severe DVD syndrome. This mutation leads to a C to T transition in exon 7, yielding a premature stop codon at amino acid 328 and disrupting all functional domains of the FOXP2 protein (Lai *et al.,* 2001). This mutation leads to a more markedly cytoplasmic localization than the R553H mutation. Because the mutated protein contains no Forkhead box, it also affects the DNA binding and the repression of the SV40 promoter (Vernes *et al.,* 2006).

The Q17L and Q40-44 mutations were both found in a screening of 49 probands that had DVD. The Q17L mutation is a transvertion of an A to T in exon 2 that leads to a glutamine to leucine change in amino acid 17 of the FOXP2 protein. This mutation lies in the N terminal part of the protein and the particular aminoacid is evolutionarily conserved in all vertebrates studied, but a specific function for this region within the FOXP2 gene has not been asserted (MacDermot *et al.,* 2005). Although there was an attempt to investigate the possible function of this mutation, there was no difference in cellular localization, as well as binding to DNA and regulation of the SV40 promoter of this mutant, and to date there is no evidence that would suggest why this mutation affects speech (Vernes *et al.,* 2006).

The Q40-44 mutation is an extension of the poly Q due to a CAGCAGCAACAA insertion in exon 5 (MacDermot *et al.,* 2005). Although the function of the poly Q is not completely understood, studies have shown that the Poly Q might be involved in the strength of repression (Wang *et al.,* 2003).

In addition to the verbal and orofacial dyspraxia, KE family patients perform significantly worse than their unaffected relatives on tests that assess receptive and grammatical language. The deficit includes an impaired ability to correctly inflect words (i.e. change tense or number) consistently or to match a relative clause describing subtle relationships between objects with the corresponding pictures. Nevertheless, affected individuals score on average only slightly, but significantly lower on a non-verbal IQ-test than non-affected individuals and there is considerable overlap between the groups (Alcock *et al.*, 2000; Vargha-Khadem *et al.*, 1998; Watkins *et al.*, 2002).

First insights into the neural basis of the behavioural abnormalities shown by DVD patients came from the examination of affected and unaffected KE family members with

structural and functional brain imaging techniques. Affected KE family members displayed bilateral grey matter morphological abnormalities in the perisylvian and rolandic cortices, basal ganglia (Vargha-Khadem *et al.*, 1998; Watkins *et al.*, 2002) and the cerebellum (Belton *et al.*, 2003) and Broca´s area. Abnormally high grey matter density was found in the putamen and Wernicke´s area. Interestingly, the volume of the caudate correlated well with the performance in the test of oral praxis (Watkins *et al.*, 2002), indicating its involvement in the pathology. Given the well-established role of the basal ganglia in motor planning and sequencing, the structural abnormalities in the striatal regions of the basal ganglia (caudate and putamen) are generally consistent with an impaired central nervous system control of orofacial motor function. However, it is less clear how they specifically compromise orofacial movements, without affecting other motor functions.

Functional imaging during the performance of silent and spoken tasks revealed lateralized disturbances in language-impaired subjects. In contrast to the typical left-dominant activation pattern involving Broca´s Area that is elicited by a verb generation test in unaffected KE family members, the signal distribution in affected individuals is more bilateral. Extensive bilateralization in the activation pattern was also observed for DVD subjects in the word repetition tasks described above. Consistent with the morphological findings, an under-activation of Broca´s area and the putamen occurred in the affected family KE members (Liegeois *et al.,* 2003; Liegois *et al.,* 2011). The observed over-activation of areas normally not involved in language has been interpreted to result from compensatory recruitment of additional brain areas, increased attention or a higher cognitive effort to solve the task. All these data indicate that the frontostriatal and frontocerebellar networks could be the key circuitry affected in impaired KE family members.

Different deletions of chromosome 7 that affect FoxP2, add to the body of evidence which supports the role of FOXP2 in language impairment (Sarda *et al.*, 1988; Tyson *et al.,* 2004; Shriberg *et al.*, 2006; Lennon *et al.*, 2007). One of them, a report of a 7;13 chromosomal translocation that disrupted the transcription of FOXP2 in a mother and her daughter was also reported. Both were named the "TB family" and were subject of a study trying to reveal the core speech and language deficits of FOXP2 affection. It is still debated if the name of the phenotype of FOXP2 affection should be developmental verbal dyspraxia or not, because affected members (at least these two studied here) have deficits that would be a mixed of apraxia of speech (AOS) and spastic-flaccid dysarthria (S_DYS) disorders (Shriberg *et al.,* 2006). It is also important to point out that in almost all cases of deletion or mutation of

FOXP2 the affected allele is from paternal origin, suggesting differential parent-of-origin expression of FOXP2 (Marshall *et al.,* 2008).

FOXP2 is among the most conserved genes in humans. It has been shown that FOXP2 was under a strong selective pressure during human evolution given that it is the gene with the highest score of acceleration index, which suggests an important role of FOXP2 in the origin of human speech (Zhang *et al.,* 2002). The chimpanzee, gorilla and rhesus macaque FOXP2 proteins are all identical except for one difference from the mouse and two differences from the human protein, whereas the orang-utan carries two differences from the mouse and three from humans. These two amino-acid differences are both found in exon 7 of the FOXP2 gene and are a threonine-to-asparagine and an asparagine-to-serine change at positions 303 and 325, respectively (Enard *et al.,* 2002). The only amino acid that would be "human-unique" seems to be the one at position 303, because the one in position 325 is also found in carnivores (Zhang *et al.,* 2002). Anyway, the combination build by both of these amino acids is specific to humans. It has been speculated that these changes in FOXP2 were important in the evolution of language. Interestingly, sequencing exon 7 of the Neanderthals showed that they shared the FOXP2 version that humans have, so that it was speculated that they may have had speech (Krause *et al.,* 2007). Comparison of the potentially different cellular functions of the human FOXP2 and the ones in other primates used a number of experimental approaches. Comparison of different cell lines with over-expression of two different FOXP2 constructs used micro array analysis to show changes in the genes that were regulated by the human and the chimp FoxP2 versions. It was shown that both FOXP2 constructs differ in the targets they regulate. A micro array comparison of tissue from adult human brain and chimpanzee was used to validate their data. One criticism of this work was that the authors used site directed mutagenesis to the human FOXP2 to mimic the chimpanzee version, but did not mutate the additional Q in the poly Q. The expression of both constructs was not similar FOXP2 human version had a higher expression than the chimpanzee version (Konopka *et al.,* 2009).

In summary, human FOXP2 is one of the most conserved genes known and mutations and deletions clearly implicate this protein in language and speech disorders which involve the basal ganglia. FOXP2 has undergone positive selection, suggesting an important function in man.

## *1.4 FoxP2 and vocal learning in birds*

The pattern of expression of FoxP2 in zebra finches was assessed with *in situ* hybridization. It shows a strong expression of FoxP2 in Purkinje cells in the cerebellum. Other regions of expression are optic tectum, thalamus regions, inferior olive, and more important for vocal learning the basal ganglia including Area X (Haesler *et al.,* 2004; Teramitsu *et al.,* 2004). Expression in Area X was also found in other birds species (canaries, Black cape chickadee, strawberry finch, Bengalese finch, song sparrow, Rofous-brestead Hummingbird), with varied FoxP2 expression levels if compared to surrounding striatum. FoxP2 expression was also seen in striatum in non-vocal learning birds like the ringdove. This demonstrated that FoxP2 pattern of expression in birds vocal learners differ from vocal- non learners, especially in Area X, a part of the specialized basal ganglia forebrain network required for vocal learning that vocal non-learners do not possess. Striatal expression of FoxP2 in zebra finches started at embryonic stage 26 (Haesler *et al.,* 2004).

It is in Area X were FoxP2 levels vary, if compared to the surrounding striatum, at different ages, showing an increase of expression during 35-50 PHD, which is the time when zebra finches learn their song. Also, in canaries, expression of FoxP2 varied seasonally according to the time when they remodel their songs. In adult birds, FoxP2 expression decreases in Area X (Haesler *et al.,* 2004). At the mRNA level using *in situ* hybridization, FoxP2 is also on-line regulated in a social context, shown to be down regulated in Area X when zebra finches practice their songs singing alone, called undirected song. FoxP2 expression levels in Area X are higher when birds address song to a female, called directed song (Teramitsu and White, 2006). At the protein level changes in FoxP2 do not seem to be regulated in a same form, since in both singing conditions there was less FoxP2 protein than in non-singing conditions (Miller *et al.,* 2008). A problem of this study is that it used GAPDH to normalize the expression of FoxP2, and GAPDH was shown to be a poor housekeeping gene (De Boever *et al.,* 2008) and changes its expression with nutrition and age status (Mozdziak *et al.,* 2003) in birds, were shown.

FoxP2 in Area X is expressed in medium spiny neurons that also express DARPP-32. It is not expressed by ChAT, nNOS or parvalbumin containing interneurons (Haesler *et al.,* 2004). It was also shown that about 85% of the new neurons incorporated into Area X at postembryonic stages were FoxP2 positive (Rochefort *et al.,* 2007).

Lastly, specifically down-regulating FoxP2 in Area X using lentiviral mediated RNAi affects song learning in zebra finches. Birds injected with a short hairpin against FoxP2 only imitated

some syllables of the tutor song but omitted others, copied syllables with less fidelity and sang song more variable than control-virus-injected birds (Haesler *et al.,* 2007). This effect might be related to the fact that FoxP2 down-regulation also reduced spine density in Area X and thus likely affects the neural plasticity of neurons in this area (Schulz *et al.,* 2010).
Together these data suggest that FoxP2 function in zebra finches and humans might be homologous, since down-regulation in birds had a similar effect as having just one functional copy of FoxP2 in humans.

## *1.5 FoxP2 expression in the brains of various species*

The pattern of expression of FoxP2 in many species is known and is conserved. All species studied show expression in striatum that starts in embryonic stages, however there are species that show a decrease in adulthood. Humans and monkeys strongly express FoxP2 in the striatum in foetal brain of both species (Teramitsu *et al.,* 2004; Takahashi *et al.,* 2008). It is not known whether this striatal expression of FOXP2 persists into adulthood in humans. In monkeys the expression in striatum at the age of 6 years was very low or absent (Takahashi *et al.,* 2008). In contrast to this results in rats (Takahashi *et al.,* 2003), mouse (Ferland *et al.,* 2003) and 4 different muroid species (Campbell *et al.,* 2009), Foxp2 is expressed in striatum through development and persists into adult stages. In zebra finches the striatal expression is also persistent into adulthood stages, although there is a decrease of expression in Area X (Haesler *et al.,* 2004; Teramitsu *et al.,* 2004).

In the cerebellum FoxP2 is strongly expressed in Purkinje cells in all species studied so far (Teramitsu *et al.,* 2004; Ferland *et al.,* 2003; Haesler *et al.,* 2004; Ferland *et al.,* 2003; Campbell *et al.,* 2009).

Other regions with strong and conserved FoxP2 expression are thalamus, optic tectum and inferior olive, being positive in mouse (Ferland *et al.,* 2003; Campbell *et al.,* 2009), rat (Takahashi *et al.,* 2003), monkeys (Takahashi *et al.,* 2008) and zebra finches (Haesler *et al.,* 2004; Teramitsu *et al.,* 2004).

In summary, the conserved pattern of expression of FoxP2 supports the idea that FoxP2 is important for frontostriatal and frontocerebellar networks in all vertebrates including humans.

## *1.6 Need for dimerization of FoxP subfamily members*

Work of Shanru *et al.,* 2004 showed that the FoxP subfamily members can homo- and hetero-dimerize using the leucine zipper (Figure 1.5) present in all FOXP proteins. Mouse Foxp1, Foxp2 and Foxp4 can interact either with themselves or with the other FoxP members mentioned before. This ability is confined only to the FoxP subfamily members. It is not known what the role of dimerization is, and if this can occur *in vivo*, since there is to date no study that had shown that FoxP members can be expressed in the same cells in the brain or if they also dimerize. In the lung and oesophagus it was shown that there might be cooperation in the development of both organs (Shu *et al.,* 2007).

In addition to the homo-dimerization using the leucine zipper it was shown that FOXP2 and FOXP1 Forkhead box domain can interact in a dimerized version with DNA. In this type of dimerization there is a "sharing" of helix H3 and strands S2 and S3 between two FoxP molecules. This type of conformation was called domain swapping. This region is highly conserved in all Fox genes with the exception of the Alanin 539, which is only present in the FoxP subfamily members. This change is responsible for the domain swapping conformation, since a mutation in this alanine disrupted the domain swapping conformation in both FoxPs. This suggests that domain swapping is an adaptive structural feature of the P branch of Fox proteins (Stroud *et al.,* 2006; Chu *et al.,* 2011). If domain swapping is present in a complete protein with all domains is a question that remains to be solved.

In FOXP3, a protein not expressed in the brain but playing an important role in immune cells (Bennet *et al.*, 2001), the importance of dimerization is exemplified by the fact that a point deletion of a single glutamic acid (ΔE250) in the leucine zipper of the protein leads to the IPEX syndrome in humans. This point mutation in the leucine zipper affects DNA binding, homo-dimerization and repression of different known targets of FOXP3, leading to disease (Chae *et al.*, 2006). In the case of FOXP3 there are no heterodimers, since FOXP3 is restricted to cells of the immune system were FOXP1, FOXP2 and FOXP4 are not expressed. But the same deletion of this conserved glutamic acid in all FoxP subfamily members abolishes also dimerization, DNA-binding and repression activity (Shanru Li *et al.*, 2004).

It is not known what the function of homo- and hetero- dimerization of the FoxP subfamily is, but it is the only Forkhead family that has this ability, therefore it is likely to be important for the function of this Fox subfamily.

## *1.7 FoxP1 mutations and speech*

FOXP1 was not known to be involved in speech disorders until recently. Previous work with respect to FOXP1 mainly focused on the development of B-cell lymphomas (Banham *et al.,* 2001, 2005 and 2007). The mutant Foxp1 homozygous deficient mice show defective cardiac development which leads to embryonic death at E14.5 (Wang *et al.,* 2004).

In humans, the first insight into FOXP1 function comes from deletions of the 3p chromosome, which are in the region of the FOXP1 gene, but depending on the size of the deletion, other genes were also affected so that no clear correlation could be established (Sichong *et al.,* 1981; Crispino *et al.,* 1995; Petek *et al.,* 2003; Pariani *et al.,* 2009). Most of them report speech deficits among the phenotype described. Vernes *et al.,* reported the finding of a coding mutation and four intronic mutations in the probands of DVD that were used to identify the FOXP2 mutation. The coding mutation was a P215A mutation that was found in a single proband. Here a clear correlation of FOXP1 and DVD could not be assessed, because the same mutation was found in 20 (out of 146) control probands, for which speech deficits were not investigated. In addition, the P215A mutation of FOXP1 lies in a region of unknown function, although the Proline in 215 is conserved among all species.

Carr *et al.,* 2010 reported a 41 month old boy that had a deletion affecting the FOXP1 gene exclusively. At 41 months, the patient was found to be imitating sounds and attempting words. He had difficulty articulating entire words and was only able to verbalize one or two syllables of multisyllabic words. Most often, he could verbalize vowels but not consonants. The patient exhibited no deficits of oromotor coordination, had no feeding difficulty, was able to suck through a straw, and did not dribble excessively. Because of the effective use of sign language, the patient's speech deficit appears to be a problem of verbal expression and not a failure of language development. All of these characteristics resemble the phenotype of FOXP2 mutations. Furthermore, the patient reported clearly correlated a FOXP1 deficiency with motor and speech deficits. Two unrelated studies further show the involvement of FOXP1 in speech deficits. A karyotyping analysis of 1532 patients with mental retardation with or without speech and language deficits found three heterozygous overlapping deletion of chromosome 3p14.1 affecting solely FOXP1 in three unrelated patients with moderate mental retardation and significant language and speech deficits. All were *de novo* deletions of paternal origin in all three cases (Horn *et al.,* 2010). Finally, a male patient bears a FOXP1 point mutation (R525X) in the Forkhead box.. Luciferase reporter assays with this mutation show that its transcriptional function is affected. The behavioural phenotype is more severe

than the FOXP2 phenotype. Patients with FOXP1 mutations not only have severe language impairment, but in addition mood liability with physical aggressiveness, and specific obsessions and compulsions. The difference between the phenotypic consequence of FOXP1 and FOXP2 haploinsufficiency might be explained at least in part by some difference in their expression patterns. Alternatively, FOXP heterodimers and homodimers may have different biochemical properties and regulatory consequences (Hamdam *et al.,* 2010).

In summary, the recent emerging data on FOXP1 deletion and mutations clearly correlates the gene with language impairment in humans, but the phenotype is more severe than that of FOXP2. Other similarities are that the majority of affected chromosomes are of paternal origin (Horn *et al.*, 2010; Marshall *et al*., 2008). Last, it remains the question if the differences in the pattern of expression are responsible of the different phenotype, and what the role of dimerization between FOXP2 and FOXP1 could be.

## *1.8 FoxP1 expression in the brain of various species*

The expression pattern of FoxP1 has been studied less than that of FoxP2. However since it is the closest FoxP2 homolog, some studies have compared the expression of both genes, e.g. in mice (Ferland *et al.,* 2003), rats (Takahashi *et al.,* 2003), monkeys (Takahashi *et al.,* 2008), different birds (Haesler *et al.,* 2004, Teramitsu *et al.,* 2004) and human foetal brain (Teramitsu *et al.,* 2004). In all species investigated, both genes' expression overlap in the basal ganglia, starting at embryonic ages. In most of the species, the expression of FoxP1 and FoxP2 persists into adulthood with the exception of the monkey, where a decrease of FoxP1 and FoxP2 was reported by an age of 6 years (Takahashi *et al.,* 2008). In the basal ganglia of birds, in the song nucleus Area X, FoxP1 is usually expressed at higher levels than the surrounding striatum. Unlike FoxP2, FoxP1 does not seem to be regulated in Area X seasonally (Haesler *et al.,* 2004). About 70% of Foxp1 positive cells in the mouse striatum co-localize with DARPP-32, and are NPY, PV and ChAT negative (Tamura *et al.*, 2004). Interestingly FoxP2 positive neurons in zebra finch striatum are also DARPP-32 positive and PV, NPY and ChAT negative (Haesler *et al.*, 2004). This could be an indirect hint that FoxP1 and FoxP2 might be expressed in the same neurons, although the analyses were done in different species. Overlapping expression of FoxP1 and FoxP2 also occurs in all species studied except humans in thalamus and inferior olive (Ferland *et al.*, 2003; Takahashi *et al.*, 2003; Takahashi *et al.*, 2008; Haesler *et al*., 2004; Teramitsu *et al.*, 2004).

In contrast, in the cerebral cortex of mice Foxp1 and FoxP2 cells mark different populations and does not co-localize (Hisaoka *et al.* 2009). FoxP2 is expressed in layer 6, whereas FoxP1 is expressed in layers 3-5 (Ferland *et al.,* 2003, Tamura *et al.*, 2003); in monkeys FoxP2 was moderately expressed in layer VI, whereas FoxP1 was highly expressed in layers IV and V, and moderately in layer VI (Takahashi *et al.,* 2008). Differential expression of both genes also occurs in the cerebellum. FoxP2 is highly expressed in Purkinje cells, and FoxP1 is not expressed in those cells in birds (Haesler *et al.,* 2004), in mice (Ferland *et al.,* 2003) and rats (Takahashi *et al.,* 2003). In birds there are also differences found in pallial regions, FoxP1 was found to be expressed in mesopallium and two nuclei of the song system (HVC and RA), that do not express FoxP2. So FoxP1 is expressed in all three important nuclei of the song system, whereas FoxP2 is only expressed in Area X (Haesler *et al.,* 2004, Teramitsu *et al.,* 2004).

In summary, some regions, like the cortex, express FoxP1 and FoxP2 in different populations, and in other regions they are expressed in the same region, e.g. basal ganglia and thalamus.

## *1.9 FoxP4*

FoxP4 is the last FoxP subfamily member to be discovered, and is the least studied also far. The first report isolated FoxP4 from mouse pulmonary tissue. Northern blots revealed that FoxP4 was expressed in the brain, as well as heart, spleen, liver, lung, testes and kidney (Lu *et al.,* 2002). In addition, using northern blots, a weak expression of FoxP4 was reported at embryonic stage E7, peaking at E11 (Teufel *et al.,* 2003). Heterozygous knock down mouse mutants exhibit no obvious defects. The majority of homozygous FoxP4 mutants died at E12.5. Histological analysis of FoxP4 homozygous mutants between days E8.5 and E12.5 revealed the development of two complete hearts. Foxp4 mutants exhibited grossly normal ventral morphogenesis and embryonic turning, suggesting that cardia bifida was not due to secondary defects in these processes (Li *et al.,* 2004). FoxP4 has stronger repressing action on the SV40 and CC10 promoters that FoxP1 and FoP2 (Shanru *et al.,* 2004; Vernes *et al.,* 2006). A difference to Foxp1 and Foxp2 mouse version is that Foxp4 has no functional CtBP-1 binding domain and because of this, it has no change of repression in the presence of this co-factor, as FoxP1 and FoxP2 have (Shanru *et al.,* 2004).

## *1.10 FoxP4 expression in the brain of various species*

Northern blots and *in situ* pattern in the Allen Brain atlas also indicate that FoxP4 is expressed in the mouse brain (Teufel *et al.,* 2003 and Lu *et al.,* 2002 (See also2.22.2)). In pulmonary and gut tissue immunohistochemistry against Foxp1, Foxp2 and Foxp4 showed differential expression of all three members and no co-localization (Lu *et al.,* 2002). The expression pattern of *Foxp4* was described by Takahashi *et al., (*2008). Expression in rat brain was at different embryonic stages up to adulthood and compared to the expression pattern of *Foxp1* and *Foxp2*. There are regions of overlapping expression of all Foxp members studied in striatum, cerebral cortex and thalamus during development. In the forebrain *Foxp4* and *Foxp2* were detected as early as E13, whereas *Foxp1* was detected as early as E14.

Between E14 – E15 *Foxp4* is expressed in the ventricular zone (VZ), subventricular zone (SVZ) and intermediate ventricular zone (IVZ) with a decrease in expression from medial to lateral telencephalon. In these regions there was no expression of *Foxp2* or *Foxp1*, since this last one was not expressed at all at this time point. *Foxp4* was also expressed in the cortical plate (CP) were it had an overlapping expression with *Foxp2* only in the CP of the lateral telencephalon. *Foxp4* expression was observed in the medial telencephalon, including the hippocampal anlage, and both regions had no expression of *Foxp1* and *Foxp2* at this age. In the subpallium, there was an overlapping expression of *Foxp2* and *Foxp4* in the mantel zone of the lateral ganglionic eminence (LGE), and *Foxp1* was expressed at the caudal part of the LGE. No Foxp subfamily member was expressed in medial ganglionic eminence (MGE). In the developing brain, *Foxp4* was expressed in proliferating cells and differentiating cells of the epithalamus (ET), the dorsal and ventral thalamus, and the hypothalamus. Similarly to *Foxp4* expression, *Foxp2* mRNA was also highly expressed in the dorsal thalamus (DT), but, interestingly, its expression pattern was complementary to that of Foxp4.

Between E20-P7 in the developing cerebral cortex, *Foxp4* was detected in the SVZ, CP, and subplate (SP), both in the developing ventral cortex and in the dorsal cortex, *Foxp2* was abundant in the lower part of layer 6 of the developing lateral cortex, and *Foxp1* was detected in layer 5 and the upper part of layer 6 of the developing ventral and medial cortex. During this period he expression patterns of *Foxp2* and *Foxp4* changed dramatically in the developing striatum. By contrast, *Foxp1* was highly and homogeneously expressed in the striatum during this period. From P0 to P4 *Foxp2* expression becomes patchy, presumably expressed in developing striosomes, which are striatal compartments (Takahashi *et al.,* 2003; 2008). *Foxp4* expression started to show some heterogeneity at E20 being at P0 expressed in a medial to

lateral gradient in a heterogeneous pattern in the lateral part. At P4 *Foxp4* was expressed in striatum in a pattern similar to calbindin and was devoid of MOR (mu-opoid receptor, a marker of striosomal cells) positive patches, it had a matrix pattern in the lateral striatum. By P7 *Foxp4* was barely detectable in striatum. In the amygdala *Foxp4* was expressed differentially from *Foxp2*, and *Foxp1* was not expressed in any amygdalar nucleus. At P1 in the developing cortex there was expression of *Foxp4* in layers 2-6, in contrast to *Foxp2* which was only expressed in layer 6 of the lateral cortex and *Foxp1* was expressed in layer 5 and in the upper part of layer 6 of the ventral and medial cortex. In the periform cortex only expression of *Foxp4* was detected. Cortical expression of *Foxp4* declined between P4 and P7 (Takahashi *et al.,* 2008).

Expression of *Foxp1*, *Foxp2* and *Foxp4* of the Allen Brain atlas in mouse show a contradictory result regarding the striatal expression of *Foxp4* in adult stages. In mouse the expression of *Foxp4* in striatum is persistent in adulthood stages. This contradiction of striatal expression can be also found in *Foxp2* if one compares monkeys and all other species. *FoxP2* striatal expression in monkeys was shown to be down regulated after 6 years (Takahashi *et al.,* 2008), whereas in all other species reported *Foxp2* expression persists into adulthood. So it could be that striatal *FoxP4* expression is persistent into adulthood in birds and other species. Purkinje cells in the cerebellum also express FoxP2 and *Foxp2* but not *Foxp1*. The inferior olive, parts of the thalamus and the striatum express all three FoxPs (Tamura *et al.*, 2003) raising the possibility that co-localization occurs in the same neurons.

In summary *Foxp4* has a specific pattern of expression that partly overlaps with if *Foxp2* and *Foxp1* in the rat brain in different ages. If this pattern of expression is conserved in different species is not known. However, expression patterns of *FoxP1* and *FoxP2* were conserved among various species, so that the chance that the expression pattern of *Foxp4* is conserved is high.

## *1.11 Molecular function of FoxP subfamily members*

From the molecular perspective, the FoxP subfamily belongs to a large family of winged helix transcription factors that are characterized by a conserved Forkhead box (Fox) DNA-binding domain. The Forkhead box binds to distinct sequences in promoter regions of a specific set of target genes, allowing their transcriptional regulation. Forkhead proteins are not among the largest transcription factor families but display a remarkable functional diversity

and are involved in a wide variety of biological processes (Carlsson and Mahlapuu, 2003). Fox proteins affect cell fate and differentiation in various tissues, and mutations cause developmental disorders (Lehmann *et al.,* 2003). Among the mutations of Fox proteins for which there is a human phenotype are: FoxC1 involved in various developmental defects in the anterior segment of the eye; congenital glaucoma, Axenfeld–Rieger anomaly; FoxC2 involved in Lymphedema combined with distichiasis, ptosis and/or cleft palate; FoxE1 involved in Thyroid agenesis, cleft palate, and choanal atresia; FoxE3 involved in malformations in the anterior segment of the eye including Peters' anomaly; FoxL2 involved in Blepharophimosis/ptosis/epicanthus inversus syndrome (BPES); can be associated with ovarian failure (BPES type I); FoxN1 involved in T cell immunodeficiency combined with lopecia and dystrophic nails (Carlsson and Mahlapuu, 2003); FoxP1 involved in mental retardation and speech disorders (Hamdan *et al.,* 2010; Horn *et al.,* 2010); FoxP2 severe speech and language disorder (Lai *et al.,* 2001); and FoxP3 involved in an Immune dysregulation, polyendocrinopathy, enteropathy syndrome (IPEX) (Carlsson and Mahlapuu, 2003).

*Drosophila* Forkhead (Fkh) and rat Foxa1 (also known as Hnf3a) are founding members of the Forkhead-box (FOX) family of transcription factors (Katoh and Katoh, 2004). The Fox family is characterized by a 110 amino acid DNA binding domain called Forkhead box or FOX box (Mazet *et al.,* 2003). This domain folds into a helix–turn–helix structure created by a core of three α helices capped at one end by three antiparallel β strands, and accompanied by two large loops called 'wings', from which derives the term "winged helix" (Tu *et al.,* 2006). Fox genes have been identified in the genomes of animals and fungi, but not plants. Animals appear to have more Fox genes than fungi, with four genes identified in *Saccharomyces* and *Schizosaccharomyces*, at least 15 in the cnidarian sea anemone *Nematostella vectensis*, 17 in *Drosophila melanogaster*, 29 in *Ciona intestinalis* and over 40 in the human and other vertebrate genomes. Phylogenetic analysis of the Forkhead domains has led to placement of most of these genes into 20 subclasses named *FoxA* to *FoxS*, with a small number of 'orphan' genes of unclear relationships defying classification (Wotton *et al.,* 2006; Mazet *et al.,* 2003).

The FoxP subfamily comprises in humans and vertebrates 4 members (FoxP1-FoxP4) (Katoh and Katoh 2004) and is one of the Fox subfamilies that is present, with only one member, in invertebrates (Santos *et al.,* 2010; Mazet *et al.,* 2003; Tu *et al.,* 2006; Lee *et al.,* 2004; Kiya *et al.,* 2008; ) (Figure 1.3). This led to the hypothesis that all other FoxP members evolved via gene duplication events (Santos *et al.,* 2010). It might be that the regulation of targets of FoxP in insects share some homologies with the mammalian ones. Or it might also

be that molecular cascades that regulate FoxP might be shared also across all taxa. Since it is known that some gene networks are conserved in different taxa, like the *Hox* (Shubin *et al.*, 2009) and *Pax6* genes (Fernald, 2006), it might be that the FoxP network could be also one of those exemplary gene families were deep homologies are found (Shubin *et al.*, 2009; Scharff and Petri, 2011).

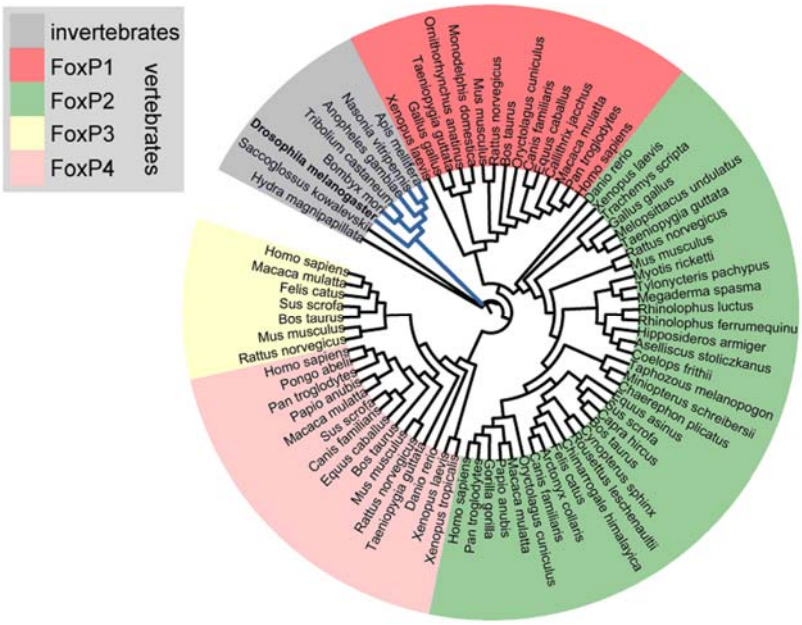



**Figure 1. 4 Phylogenetic tree of the FoxP subfamily members across different species. The single invertebrate FoxP gene probably corresponds to the ancestral form from which the four vertebrate genes have arisen by serial duplication given rise to the four subfamily members known in vertebrates (Mendoza, E. unpublished data).**

The FoxP subfamily is characterized by having the following functional domains, described from N terminal to C terminal (Figure 1.5): A Poly glutamine tract or Poly Q (purple in Fig, Figure 1.5); a zinc finger domain (in blue); leucine zipper (in yellow); and the characteristic domain of the Fox proteins, the Forkhead box (in green) (Shu *et al.* 2001).

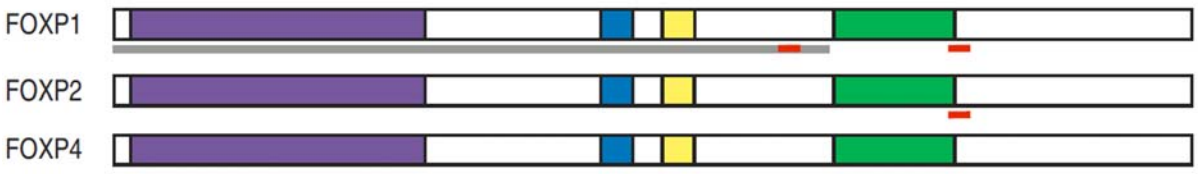



**Figure 1. 5 Functional domains of the FoxP subfamily members. Purple: glutamine rich region; Blue: zinc finger; Yellow: leucine zipper; Green: Forkhead domain; Grey Bar: region involved in repression; Red Bar: EH1 motif, sites 398–408 and 501–511 for FOXP1, sites 501–511 for FOXP2. Taken from Fetterman *et al.*, 2008.**

FOXP2 and FOXP1 have isoforms that contain the polyglutamine tract (Wang *et al.,* 2003; Banham *et al.,* 2001) whereas FoxP4 does not. Work on the different FoxP1 isoforms show that isoforms lacking the poly glutamine have stronger repression ability, than isoforms that have it. This part of the FOXPs may serve as a modulator of repression activity. It could act as a polar zipper to join other transcription factors bound to separate DNA segments (Perutz *et al.*, 1994), creating a multiprotein transcriptional unit. This hypothesis is consistent with the proximity of a binding site for FoxP1 to a number of other transcription factor binding sites in the *c-fms* promoter, a physiological target of FoxP1 (Shi *et al.*, 2004). Regulation of *c-fms* expression by FoxP1 depends on the polyglutamine-repeat. Mutations of the poly Q tract of FOXPs are only known from FOXP2, were an addition of 4 glutamines was found in a case of DVD (MacDermont *et al.*, 2005), showing the importance of this domain.

All members of the FoxP subfamily have a zinc finger domain (in blue; Figure 1.5). It was shown that this domain contributes to the dimerization of FoxP proteins (Wang *et al.*, 2003). But C2H2 zinc finger motifs are also known for binding DNA (Wolfe *et al.*, 2000). It was also shown that C2H2 zinc fingers can bind to RNA and proteins (Iuchi, 2001). The function of the zinc finger domain in FoxP is not clear. It may be that the zinc finger cooperates with the Forkhead box to bind to the target.

The leucine zipper (in yellow; Figure 1.5) is clearly involved in homo- and hetero-dimerization of FoxP members, which is a unique characteristic of this family. Mutations in the leucine zipper domain are known from FOXP3 X-linked autoimmunity and allergic dysregulation syndrome (IPEX) patients where a deletion of a conserved glutamic acid was found to be the cause of the disease (Chatila *et al.*, 2000). Furthermore, it was shown that with this mutation dimerization is affected and that this affects also the function of the protein (Chae *et al.*, 2006). In a similar experiment the leucine zipper was deleted or the same glutamic acid was deleted for assessing the homo- and hetero- dimerization of mouse Foxp1, Foxp2 and Foxp4, showing that all members are able to homo- and hetero-dimerize and that dimerization is needed for the proper function of the proteins as well as binding to DNA (Shanru-Li *et al.*, 2004).

The co-repressor C-terminal binding Protein 1 (CtBP-1) domains, which is between the leucine zipper and Forkhead box (not shown in Figure 1.5) was found in mouse Foxp1 and Foxp2, but not FoxP4. This domain was found to be important for the regulation of Foxp1 and Foxp2, but not for Foxp4 in mice (Shanru-Li *et al.*, 2004). Upon the binding of CtBP the

activity of Foxp1 and FoxP2 was repressed, but not for Foxp4, because in mice there is a leucine to proline substitution in the core of the CtBP-1 domain.

The DNA-binding Forkhead box domain (in green; Figure 1.5) is the most evolutionary conserved part of all FoxP members, similarities range from 88% comparing FoxP1 with FoxP2, to 92% comparing FoxP1 and FoxP4. There are several human mutations known that affect this domain (Bennett *et al.*, 2001; Wildin *et al.*, 2001; Lai *et al.*, 2001; Hamdam *et al.*, 2010).; it was shown that the R553H mutation and that deletions of this domain in FOXP2 affect DNA binding (Vernes *et al.*, 2006). The crystal structure of the Forkhead box of FoxP2 bound to DNA shows that the third helix of this domain is the one that binds to DNA. It was also shown that the FoxP family can bind as a Forkhead dimer, doing domain swapping due to a conserved alanine only present in the P-family (Stroud *et al.*, 2006; Chu *et al.* 2011). The exact function of domain swapping is not known. In FOXP1 and FOXP2, in the Forkhead box an Engrailed Homology 1 EH1 was found (red underline; Figure 1.5). This motif could be used by transducin-like enhancer of split (TLE) proteins that would act as co-repressors of FoxP1 (Chen and Courey, 2000) a further way of regulating these proteins. It is also known that in this domain there are at least two nuclear localization sequences (NLS). First insights came from the fact the mutant R553H and R328X, as well as isoforms lacking the Forkhead box, showed a cytoplasmic expression that was not evident with the wild type FOXP2 (Vernes *et al.*, 2006). In another work the exact location of these NLS was mapped for FOXP2 and it was shown that importins bind to these two NLS and are the ones involved in importing the protein to the nucleus. More interesting is the fact that when co- expressing mutant R553H and normal FOXP2, both proteins are imported to the nucleus (Mizutani *et al.*, 2007).

In addition to these domains in FOXP3 it was found that there are two pro-protein cleavage sites (N and C terminal), and that cleavage of FOXP3 was needed for FOXP3 to bind chromatin. The RXXR motif characteristic of this pro-protein cleavage sites was predicted to be also in FOXP1 and FOXP2, so a regulation via cleavage could be also possible (de Zoeten *at al.*, 2009).

There are almost no data about upstream transcriptional regulators. One study showed that in zebra finches (*Taeniopygia guttata*) the administration of cannabinoid agonist increases FoxP2 expression in striatum and persisted into adulthood (Soderstrom *et al.* 2010); another study suggests that LEF1 regulates FoxP2 in zebra fish (*Danio rerio*) (Bonkowsky *et al.*, 2008). Interestingly, transcriptional profiling of cells of the developing eye of mouse and chicken showed that FOXP2 was one of the genes being enriched in the peripheral margin of

the optic cup at E12.5 and the developing ciliary body at E16.5. Furthermore, it was shown that FoxP2 was downstream of the Wnt signalling pathway, since manipulation of this pathway by over-expressing the active form of β catenin, affected the expression of Foxp2, which suggest that FoxP2 is directly downstream of Wnt signalling pathway (Trimarchi *et al.* 2009). Lef1 is known to be the Wnt signal transducer, so these last two studies suggest that FoxP2 might be regulated by the Wnt signalling pathway. Until now no one has cloned any promoter of FOXP1, FOXP2 or FOXP4 and shown which factors might regulate them or which molecular cascades are involved in their activation. Evo-devo studies in the regulation of FOXP2 would bring interesting results on how a gene important in speech is regulated and maybe differences between vocal learners and non-vocal learners are found. The only promoter of FOXP subfamily members known to date is the one of FOXP3. It was shown that NFAT and AP-1 bind to the FOXP3 promoter and *trans* activate FOXP3 expression and that cyclosporine A acts as a repressor of FOXP3 expression (Mantel *et al.*, 2006).

The FOXP subfamily members 1, 2 and 4 act primarily as transcriptional repressors (Shanru-Li *et al.*, 2004), but they can also act as activators (Vernes *et al.* 2010, 2011; Spiteri *et al.* 2010; Konopka *et al.*, 2009). The mechanism by which this shift from repressor to activator occurs is not known. Promoters that have been shown to be regulated and repressed by FOXP members are: CC10 (Shanru-Li *et al.*, 2004), SV40 (Vernes *et al.,* 2006) for all FOXP subfamily members; T1α was shown to be a direct target of Foxp1 and Foxp2 in lung tissue (Shu *et al.*, 2007); and *c-fms* (Shi *et al.* 2004), IL-2 (Wang *et al.*, 2003), IL-6 (Chokas *et al.*, 2010) for FOXP1. Since the binding site of all FOXP subfamily members is very similar it is possible that all of them are able to bind to those promoters. There is growing data about the neural targets of FOXP2. There have been various studies trying to identify FOXP2 targets (Vernes *et al.*, 2010, 2011; Spiteri *et al.*, 2010). The identified potential FOXP2 targets are involved in mediating synaptic plasticity, neurodevelopment, neurotransmission and axon guidance. The only target of FOXP2 that also affects language is CNTNAP2, discussed above (1.2.).

Transcription factors bind to co-factors that may change their activity; in the case of FOXP subfamily members it is know that they homo- and hetero-dimerize in order to bind to DNA, but if this interaction affects or alters the activity of the dimer is still not known. It may be that specific FOXP dimers bind to different co-factors and thereby have different regulatory properties or activity. An example of this would be the differential regulation of the mouse Foxp subfamily members by CtBP-1. It is known that Foxp1 and Foxp2 are regulated by CtBP-1, but Foxp4 is not (Shanru-Li *et al.* 2004). What happens if a Foxp1-Foxp4

heterodimer encounters a CtBP-1 protein is not known. Another co-factor that has been shown to interact with Foxp1, Foxp2 and Foxp4 is p66β, a component of the NuRD/MeCP1 chromatin-remodelling complex. The zinc-finger and leucine-zipper domains of the FOXP proteins are important for this interaction to occur and p66β regulates the repression of Foxp1 and Foxp4, but not of Foxp2. Further interaction was shown between Foxp1 and Foxp4 and HDAC1/2 proteins. HDAC1 and HDAC2 are core components of the NuRD complex and are required for NuRD-mediated chromatin remodelling and transcriptional repression. Only Foxp1 was shown to interact with the metastasis associated 1 protein (MTA), which is also a member of the NuRD complex (Chokas *et al.*, 2010). It is long known that Foxp1 is specifically expressed in the motor neuron region of the spinal cord in the brain of developing mice (Shu *et al.*, 2001; Tamura *et al.*, 2003). In this region Foxp1 played a critical role regulates the formation and connection of motor neurons to the target muscles, and this by acting as an accessory factor of Hox transcription factors (Dasen *et al.*, 2008; Arber, 2008; Rousso *et al.*, 2008; Surmeli *et al.*, 2011).

In summary, FOXP subfamily members have well defined protein domains for which specific functions are known. They act mostly as transcriptional repressors. Except for FOXP2 and FOXP3 (not addressed here), there is not much known about targets they might regulate and less is known about possible molecular routes upstream them. The presence of multiple protein-protein interaction motifs in the Foxp subfamily members suggests that these factors have the ability to interact with a wide variety of co-factors. Such a large repertoire of interactions would allow for a single factor to modulate gene expression in a spatial and temporal specific fashion depending on the expression of such co-factors.

## *1.12 Analysis of gene function by genetic manipulation in songbirds*

In principle, all methods that are used for making transgenic mouse should work also for birds, and since the first genetic modifications were done in the mouse in the 80´s, there has been a lot of effort into trying to apply this method in birds. Interest in making transgenic chickens not only come from the fact that it could be a good model for studying developmental processes and cell fate, but mainly because of its interest for the pharmaceutical industry and for their use as bioreactors (Sang, 2004). One of the problems that arise from trying to apply methods of mouse to the birds is that they have huge differences in their reproductive system. Most critically, birds lay eggs and mice do not. The

embryo in a newly layed egg comprises 50,000 – 60,000 cells on the surface of the yolk mass (Mozdziak and Petitte, 2004); this is the so called stage X embryo.

Methods that are used for transgenic manipulations in mice are: intracytoplasmic injection, pronucleus injection, spermatozoa as carrier, nucleus transplantation, manipulation of embryonic stem cells (ESCs) and retroviral vectors.

The first, intracytoplasmic injection, has the problem of DNA degradation in mice (Perry *et al.*, 1999), it has not been established in birds.

The pronucleus injection method that was established in mice and since then used for other mammalian species can also not be applied to birds. Problems arise again from the fact that the fertilized zygote is relative inaccessible and difficult to handle in birds, if compared to mammals. Although newly fertilized zygote can be recovered by sacrificing a laying hen and cultured methods are established, the efficiency of germ line transgenic chicken achieved is low and labour intensive (Sang, 2004).

Sperm-mediated gene transfer has made some recent advances. Briefly, a DNA construct is bound to sperm, and the semen/sperm is used to inseminate a fertile female. However, sperm-mediated gene transfer has not yet provided any convincing data to suggest that a transgene of interest can successfully be incorporated into the avian germline (Mozdziak and Petitte, 2004).

Methods for manipulating embryonic stem cells have shown also promising results. Current targets for germ line modification include the mature oocyte/spermatozoa, the newly fertilized egg, and primordial germ cells (PGCs) during their establishment, migration, and colonization of the gonad. A major difficulty for the embryonic manipulation of freshly laid eggs is achieving a sufficient hatchability to generate an acceptable number of G0 offspring to screen for the incorporation of the transgene. To date there are only two methods for manipulating the birds embryos, the windowing and the ex ovo culture (Mozdziak and Petitte, 2004). An advantageous characteristic for the method using spermatozoa as carrier is that spermatogenesis in birds and mammals is very similar. This technique uses the biological process of spermatogenesis and uses the putative spermatogonial stem cells (SSCs) as inducers of bird transgenesis. It was shown that these SSCs express germline markers similar to those present in primordial germ cells (PGCs) and can be successfully maintained *in vitro* for 5 months. Manipulations of these cells could be a useful method for transgenic bird production (Han, 2009). The isolation of pluripotent cells from the inner cell mass of mouse blastodermal cells, embryo stem (ES) cells has led to the development of more sophisticated methods for generation of transgenic mice. Efforts to isolate the chick equivalent of mouse ES

cells have focussed on manipulation of cells from the embryos from new laid eggs (stage X). Identification of cells equivalent to the inner cell mass of the mouse blastodermal embryo has not been possible as the organisation of the early chick embryo differs significantly from that of the mouse. For this reason different works have focus on the blastodermal cells (Sang, 2004). Because blastodermal cells are undifferentiated, researchers have attempted to use this cell type as the progenitor of chicken ESCs; however, this approach has had only limited success. Blastodermal cells were subcultured to promote stem cell establishment, but the reactivity of the cultured cells with several stem cell-specific markers and their pluripotency were only partially confirmed. A number of studies have focused on PGCs, instead of blastodermal cells, as a way to manipulate embryogenesis in transgenic research (Han, 2009). PGCs can be obtained in three different ways: first, they have been isolated from the germinal crescent of stage X embryos; second, exploiting the fact that there is migration of PGCs through the blood vessels during stages 13-17 they have been removed and isolated from the blood, with the problem that this type of cells are scarce; and last, collect PGCs from embryonic gonads (Han, 2009; Sang, 2004; Mozdziak and Petitte, 2004). In all cases the transferred PGCs have been shown to contribute to the germline (Sang, 2004). The efficiency of all these ways of isolating PGCs varies and the retrieval of PGCs from embryos is still inefficient, and the procedure itself is technically challenging (Han, 2009). Last development of this method requires optimisation of the PGCs isolation and transfer process, genetic modification of the PGCs before transfer and manipulation of the recipient to allow a greater contribution to the germ line of donor-derived PGCs (Sang, 2004).

The method that has given the best results until now, and is not as challenging as the mentioned above, is the injection of retroviral vectors into undifferentiated, stage X embryos. The first attempts of getting expression of transgenes were done using avian viruses, like the avian leukosis virus (ALV) used by Salter *et al.*, 1986, 1987, with a transmission of the provirus in the germline of 1-11%. Next replication defective vectors derived from reticuloendotheliosis virus (REV) and ALV were developed and used to produce transgenic birds. These last two studies resulted in production of chimeric transgenic birds with the viral vector present at low levels in founder (G0) birds and very low frequencies of germ line transmission (Bosselman *et al.,* 1989). Again the ALV vector was used to carry the *LacZ* gene. Unfortunately, beta-galactosidase expression was only noted in cultures of embryonic fibroblasts from G2 progeny, and expression was not reported in the entire embryo (Thoraval *et al.*, 1995). Until this point, although results were encouraging, the major problem was gene silencing of the inserted vectors and that the vectors were limited in size of the transgene they

could carry (Sang, 2004). A replication-deficient vector based also on ALV was successfully used by Harvey *et al.*, 2002, to express β – lactamase driven by the human cytomegalovirus promoter (CMV). They showed that expression of the protein was in the egg white and that homozygotes had a greater expression than the heterozygotes. This was the first report that showed the expression of a foreign protein in the egg and thereby the feasibility of the hen as a bioreactor. Afterwards, work by Lois *et al.*¸ 2002, showed the utility of using vectors based on the human immunodeficiency virus (HIV-1) pseudotyped with the vesicular stomatitis virus G protein (VSV-G) and carrying an ubiquitously express promoter driving the expression of GFP. First it was used in the production of transgenic mice with an efficiency of 80% and GFP expression was detected in G1 offspring. This study encourage further work in the transgenic chicken field, so that the same vector was used by McGrew *et al.*, 2004, this time driven by the CMV promoter, showing an efficiently to produce transgenic chicken that would also have a stable offspring transmission of GFP. Since then, numerous studies have tried different lentiviral vectors, like the Moloney murine leukemia virus (MoMLV) with which expression of human immunoglobulin G1 protein expression in serum and egg white of chicken using a β actin chicken promoter was achieved. The efficiency of germline transmission from G0 to G1 was relatively low (3.3%), and the expression level of this protein in G2 transgenic chickens was reduced in comparison to the G0 founders. This was the first time that a chicken promoter was used (Kamihira *et al.*, 2005). Using the same MoMLV based vector it was also possible to express enhanced green fluorescence protein (eGFP) in chicken (Koo *et al.*, 2006). Another publication showed that it was possible to do tissue-specific transgenic quails. In this study they used the same HIV-1 based vector used by McGrew and expression of GFP driven by the human synpasin 1 promoter. Expression of GFP was specific to neurons and consistent across multiple generations (Scott and Lois, 2005). Last, using the same approach it was possible to make the first songbird transgenic expressing GFP driven by the human ubiquitin C promoter (Agate *et al.*, 2009). The use of lentiviral vectors have brought transgenic songbirds into close reach. Nevertheless, with these approaches it would still not be possible to target specific genes by homologous recombination, such that a gene can be "knocked out" or replaced with an expression reporter ("knock-in"). Another problem is the fact that the mouse genome is known and promoters are also characterized. For the chicken the first map of the genome was published a few years ago (Wallis *et al.*, 2004) and recently the genome of the zebra finch was also published (Warren *et al.*, 2010). Nevertheless, it still lacks specific information about promoters and their

characterization. A last problem that is always mentioned is the low efficiency of hatching in all methods tried so far.

One method to circumvent these problems is to inject a lentiviral vector that induces RNA interference (RNAi) into defined brains areas at a defined time. RNAi is a mechanism of posttranscriptional gene silencing through sequence specific degradation of mRNA (Figure 1.6). RNAi mediated by long double stranded RNA (dsRNA) has been successfully used in various organisms including plants, planaria, Hydras, *Trypanosomes*, *Drosophila*, mosquitoes, mouse oocytes (Dykxhoorn *et al.*, 2003) and chicken (Pekarik *et al.*, 2003), where it induced cleavage of their target mRNA.

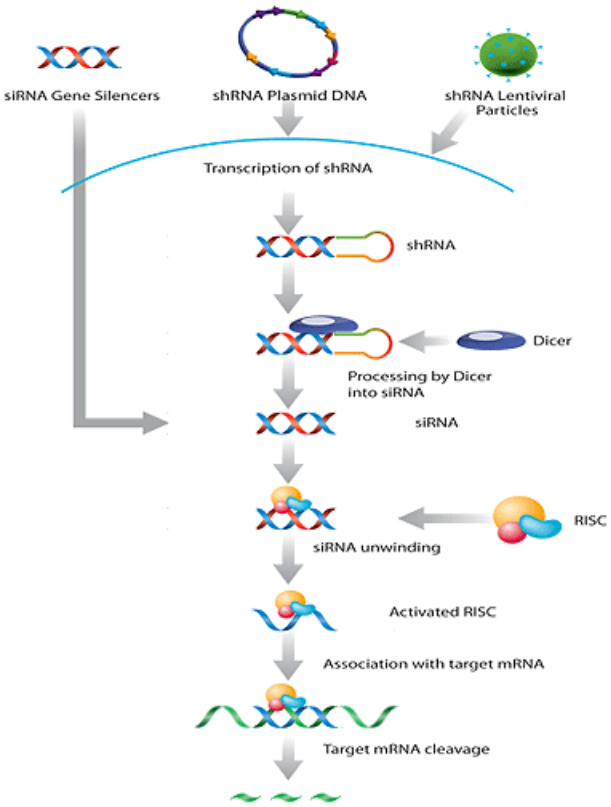



**Figure 1. 6 The RNA interference pathway. RNAi is a method that uses the micro RNA (miRNA) pathway to cleave a specific mRNA. First a carrier is inserted into the cell. It can be a short interference RNA (siRNA), a plasmid carrying a short hairpin RNA (shRNA) DNA vector or a virus carrying the information for expressing a shRNA. Then the shRNA is processed by proteins of the miRNA pathway. Dicer cleaves the double stranded RNA into a short interfering RNA (siRNA) in an ATP-dependent reaction. The siRNA is then incorporated into the RNA-inducing silencing complex (RISC). Unwinding of the siRNA duplex is again an ATP-dependent reaction. Once unwound, the single-stranded antisense strand guides RISC to the mRNA that has the complementary sequence, which results in endonucleolytic cleavage of the target mRNA. Taken from Santa Cruz Biotechnology, Inc. www.scbio.de/.../rnai-directed_mrna_cleavage.png**

The applicability of this approach in mammals is limited, since the introduction of long dsRNA induces a sequence-nonspecific interferon response leading to a global inhibition of mRNA translation. Unlike fungi, plants and worms, there is no indication of replication of

siRNA in mammals. That is why a direct silencing using dsRNAs in mammals is limited. A way to overcome this is to use DNA-vector-mediated mechanisms to express substrates that can convert into siRNA *in vivo*. To date there are two expression systems to express siRNA *in vivo*: a first expression system in which the expression of a long hairpin is driven by promoters from RNA polymerase II, and a second one were the expression of a short hairpin (shRNA) is driven by promoters for RNA polymerase III. The one driven by RNA polymerase II allows inducible, tissue- or cell- specific RNA expression but they cannot be used in mammalian cells because of an interferon response. The ones using RNA polymerase III uses the U6 and H1 promoters. This last type of system does not activate an interferon response, and in addition the RNA polymerase III recognizes 4 or more T as a termination signal that terminates transcription in the absence of other cofactors. Lastly, viruses have been used to deliver with a high efficiency siRNAs into cells. Among the virus that are used are oncoretroviruses like the MoMLV and murine stem cell virus (MSCV), and the lentivirus vectors that are derived from HIV-1. Oncoretroviruses undergo proviral silencing during development, and Lentivirus have the capacity to infect actively dividing and non-dividing, post-mitotic cells, which makes them resistant to silencing (Dykxhoorn *et al.*, 2003).

In the zebra finch, the method using a lentivirus carrying the RNA polymerase III system with a U6 promoter and driving shRNA was used to successfully silence FoxP2 in Area X (Haesler *et al.*, 2007), where it could be assessed that expression of the gene was reduced and this reduction affected song learning.

In summary, it is possible to generate transgenic birds that express the transgene stably in the germline and to manipulate gene expression *in vivo*, which will improve the usefulness of this model.

## *1.13 Aims of this study*

The aim of this study was to gain further mechanistic insights into how FoxP2 relates to vocal learning in songbirds, and by extension possibly to human language as well. Specifically I hypothesized that FoxP2 interacts with FoxP1 and FoxP4 in vitro and in vivo, in regions relevant for vocal learning, affecting this behaviour. This assumption was based on in vitro studies showing homo- and hetero-dimerization as a prerequisite for transcriptional regulation and some reports in mammals and songbirds showing overlapping expression patterns of FoxP1 and FoxP2.

The first aim of this study was to identify and characterize the FoxP4 zebra finch gene because the pattern of expression of FoxP4 in the zebra finch had not been explored. Then I compared the pattern of expression of FoxP1, FoxP2 and FoxP4 in the zebra finch brain and identified regions and within the regions neurons, with overlapping expression, particularly focusing on regions of the song control system.

Since FoxP2 expression varies in different ages in Area X in the zebra finch I also analyzed the expression of FoxP1 and FoxP4 at different ages, as a prerequisite to co-regulation. To further explore the possible interactions between the three different FoxP proteins, and to assess the effect of the mutations, I used co-immunoprecipitation *in vitro* and *in vivo*.

Finally, I wanted to answer the question if FoxP1 and FoxP4 genes could also be important for vocal learning, since the zebra finch has been proved to be a suitable model for answering such a question. Since FoxP2 knock-down in Area X was shown to affect vocal learning with a phenotype of singing impairment similar to what can be seen in the KE family, I used lentivirally mediated knock down of FoxP1 and FoxP4 in Area X. This type of manipulation allows the study of FoxP1 and FoxP4 function in the neural circuits for learning, isolated from its involvement in the development of the brain. All genetically manipulated animals were tutored by adult birds and their songs recorded. Using software for the quantitative analysis of song the consequence of FoxP1 and FoxP4 knockdown on song learning success was evaluated.

# 1. Methods

## *2.1 Solutions and buffers*

<table>
<tr><td>10x Oligo annealing buffer<br>100 mM Tris HCl (pH7.5)<br>1M NaCl<br>10mM EDTA<br>in molecular biology grade $H_20$</td><td>0.5 M PB<br>7.10g $Na_2HPO_4$ in $H_20$</td></tr>
<tr><td rowspan="2">10x PBS<br>NaCl 80g (for 1370mM end concentration)<br>KCl 2g (for 27mM end concentration)<br>$Na_2HPO_4$ 14,2g (for 100mM end concentration)<br>$KH_2PO_4$ 2,4g (for 200mM end concentration)<br>1l of $ddH_2O$</td><td>PBST<br>1ml Tween 20 in 1l 1x PBS</td></tr>
<tr><td>PBS-Tx 0,3%<br>15ml 10% Triton X in 1l 1x PBS</td></tr>
<tr><td>10x white Laemmli-buffer<br>25mM Tris base<br>192mM Glycine<br>3.5mM SDS (1%)<br>in aqueous solution<br>Do not adjust pH, store at RT</td><td>for 1l of buffer prepare:<br>30.3g<br>144g<br>10g<br>$ddH_2O$ to 1 liter</td></tr>
<tr><td colspan="2">Cell lysis Buffer<br>MPER 10ml<br>1 tablet of Complete Mini Protease Inhibitor (Roche, Mannheim, Germany)<br>300µl NaCl 5M<br>1:2000 PMSF</td></tr>
<tr><td>Brain nuclear extraction buffer (non-ionic detergent)<br>50mM Tris pH 8.0<br>2mM EDTA pH 8.0<br>0.1% NP40<br>in aqueous solution</td><td>for 10ml of buffer prepare:<br>0.5ml of 1M Tris-HCl, pH 8.0<br>0.04ml of 0.5M EDTA, pH 8.0<br>0.01ml NP40 (very viscous! Use 10% stock solution in water and dilute 1:100)<br>$ddH_2O$ to 10ml</td></tr>
<tr><td>5x Blotting buffer<br>29.1g Tris<br>14.65g Glycine<br>18.75ml 10% SDS<br>ad 1l with ddH2O</td><td>DEPC water<br>Add 0,5ml DEPC to 500ml $H_2O$<br>Shake vigorously<br>Leave open under hood over night<br>Autoclave</td></tr>
<tr><td>10x MAB (for 2l)<br>Maleic Acid 232,2g (1M end concentration)<br>NACl 175,3g (1,5M end concentration)<br>NAOH ~160g<br>NAOH 1M x ml<br>Add $ddH_2O$ to ~2l and adjust pH to 7,5 with NAOH<br>Autoclave</td><td>1x MABT (for 2l)<br>200ml 10x MAB<br>2ml Tween-20<br>Add $ddH_2O$ to 2l</td></tr>
<tr><td>20x SSC pH 4,5<br>NaCl 87,65g (3M end concentration)<br>Tri-sodium dehydrate 44,1g (0,3M end</td><td>20x SSC pH 7,0<br>NaCl 350,6g (3M end concentration)<br>Tri-sodium dehydrate 176,4g (0,3M end</td></tr>
</table>

| | |
|---|---|
| concentration)<br>DEPC $H_2O$ to ~500ml and adjust pH to 4,5 with citric acid | concentration)<br>DEPC $H_2O$ to ~2l and adjust pH to 7,0 |
| **20x SSPE**<br>NaCl 350,6g (3M end concentration)<br>$NaH_2PO_4 – H_2O$ 55,2g (200mM end concentration)<br>EDTA 14,8g (20mM end concentration)<br>DEPC $H_2O$ to 2l | **10x TBE**<br>Tris 108g<br>Boric acid 55g<br>Dissolve in ~900ml $ddH_2O$<br>Add 40ml 0,5M $Na_2EDTA$ (pH8,0)<br>$ddH_2O$ to 1l |
| **Hybridization Buffer**<br>Formamide deionized (Sigma F9037)<br>20x SSC 4,5 pH with citric acid<br>Blocking reagent (Roche 11096176001) in 1x MABT (10% stock)<br>SDS<br>Yeast tRNA (Invitrogen 15401-029) (25 mg/ml stock)<br>Heparin (Polysciences Inc.01491) (0,mg/µl stock)<br>DEPC water | **for 50 ml:**<br>25ml (50% v/v end concentration)<br>12,5ml (5x end concentration)<br>10ml (2% end concentration)<br><br>1g (2% end concentration)<br>500µl (0,25 mg/ml end concentration)<br><br>25µl (0,1 mg/ml)<br><br>DEPC $H_2O$ to 50ml |
| **Stop Buffer (Hybridization)**<br>Tris HCl pH8,0 1M (Stock)<br>EDTA 1 mM 0,5 (Stock)<br>add | **for 200ml:**<br>2ml (for 10mM end concentration)<br>400µl (for 1mM end concentration)<br>$ddH_2O$ to 200ml |
| **NTMT**<br>Tris HCl pH9,5 (1M stock)<br>NACl (5M stock)<br>$MgCl_2$ (1M stock)<br>Tween-20 | **for 200ml:**<br>20ml (for 100mM end concentration)<br>4ml (for 100mM end concentration)<br>10ml (for 50mM end concentration)<br>200µl (for 0,10%)<br>Add $ddH_2O$ to 200ml |
| **TNE**<br>Tris HCl pH 7,5 (1M stock)<br>NaCl (5M)<br>EDTA (0,5M) | **for 20 ml:**<br>2ml (for 100mM end concentration)<br>20ml (for 500mM end concentration)<br>0,4ml (for 1mM end concentration)<br>Add $ddH_2O$ to 200ml |
| **HISS**<br>heat inactivated sheep serum(30min 56°C) | **Narcotic**<br>Meloxidyl 0,15mg/ml (stock)<br>For zebra finches: 0,5mg/kg body weight |
| **HEK293-T / Hela cell culture medium**<br>500ml DMEM (Invitrogen, Carlsbad, USA)<br>55ml Foetal Calf Serum (this corresponds to ~ 10 %)<br>6ml L-Glutamine (200mM; Invitrogen)<br>7ml ready-to-use Penicillin/Streptomycin-Mix (Penicillin 10.000 U/ml, Streptomycin 10.000µg/ml (Invitrogen) | |
| **TDMH (Taq polymerase reaction buffer)**<br>10x PCR-buffer without MgCl2 (Roche)<br>25mM $MgCl_2$<br>25mM dNTP´s<br>$H_20$ Molecular grade | **for 1896µl:**<br>494µl<br>394,6µl<br>35,08µl<br>976,4µl |
| **LB medium** | **Ethidium bromide stock solution** |

| | |
|---|---|
| 1% (w/v) yeast extract<br>0,5% (w/v) trypton<br>0,5% (w/v) NaCl<br>pH 7,2 | 10mg/ml in $ddH_2O$ |
| **Ampicillin stock**<br>100mg/ml in $ddH_2O$<br>Sterile filtered | **Zeocin stock**<br>25mg/ml in $ddH_2O$<br>Sterile filtered |
| **Kanamycin stock**<br>50mg/ml in $ddH_2O$<br>Sterile filtered | **Coomassie Stain (1l)**<br>Coomasie Brilliant Blue R-250 2,5g<br>Dissolve in:<br>methanol 450ml<br>acetic acid 100ml<br>$ddH_2O$ to 1l |
| **PMSF stock solution**<br>100mM in DMSO | **Blocking buffer (Western blots)**<br>5% dry milk in PBST |
| **4x SDS-PAGE-1**<br>Separating Gel:<br>1,5 M Tris pH to 8,8<br>0,4% SDS<br>in $ddH_2O$ | **4x SDS-PAGE-2**<br>Stacking gel:<br>0,5M Tris pH 6,8<br>0,4% SDS<br>in $ddH_2O$ |
| **0,5M $Na_2EDTA$ (pH8,0)**<br>$Na_2EDTA$ 186,12g<br>Dissolve in ~800ml $ddH_2O$<br>Adjust pH to 8,0 with NaOH<br>$ddH_2O$ to 1l<br>Autoclave | **Chromallum** (for 1l)<br>400ml $dH_2O$<br>5g Gelatine<br>$dH_2O$ to 1l<br>add 0,5g Chromium(III) potassium sulphate dodecahydrate |
| **Mowiol**<br>6g Glycerin<br>2,4g Mowiol<br>6ml $ddH_2O$<br>Stir at RT<br>Add 12ml 0,2M TRIS (pH 8,5)<br>Stir at 53°C over night<br>Centrifuge at 500 rpm for 20 minutes<br>Aliquot and store at -20°C | **Cryoprotectant**<br>30% ethylene glycol and 30% sucrose in PB<br>Recipe adapted from Nordeen laboratory publication<br>Dilute 100ml 0,5M PB in 150 ml $dH_2O$<br>Dissolve 150g Sucrose in diluted<br>Add 150ml ethylene glycol to PB/sucrose solution<br>$dH_2O$ to 500ml<br>stored at 4°C |
| **Washing Buffer (Co-IP)**<br>PBS pH 7,4 w/ 0,02% Tween 20 | **Elution Buffer (Co-IP)**<br>50mM Glycine pH 2,8 |

## *2.2 Enzymes*

All restriction enzymes were purchased from New England Biolabs (Ipswich, USA) and Fast Digest enzymes from Fermentas (St. Leon-Rot, Germany). Recombinant Taq polymerase was made in the laboratory. Pfu proof reading polymerase was purchase from Stratagene (La jolla, California, U.S.A; 600380); Phusion proof reading polymerase (Finnzymes) purchase from

(Thermo Scientific, F-549). The T4 Ligase enzyme was purchase from Promega (Madison, WI U.S.A).

## *2.3 Nomenclature*

For avian brain regions, I used the recently revised nomenclature proposed by the Avian Brain Nomenclature Forum (Reiner *et al.*, 2004) (http://avianbrain.org/). For FoxP2 nomenclature, I followed the convention proposed by the Nomenclature Committee for the Forkhead family of genes (i.e., FOXP2 in *Homo*, Foxp2 in *Mus*, and FoxP2 in all other species, proteins in roman type, and genes and RNA in italics) (Kaestner *et al.,* 2000).

## *2.4 Molecular Biology*

### 2.4.1 Sex determination of young zebra finches

The sexing protocol is based on the detection of a length polymorphism in the chromo box helicase DNA binding gene (CDH) located on both bird sex chromosomes called by "W" and "Z" (Griffiths *et al.*, 1998). I used a modified sexing protocol using saliva samples from nestling zebra finches (Adam *et al.*, 2010). DNA was extracted and sexing PCR was conducted using ~18 µl of genomic DNA as template. Primers in this reaction amplify discriminable fragments of the Z- and W-linked genes CHDZ and CHDW. Amplification generates two different bands in the heterogametic females (karyotype ZW) and a single band in the homogametic males (karyotype ZZ) (Griffiths *et al.*, 1996; Soderstrom *et al.*, 2007).

### 2.4.2 RNA extraction from zebra finch tissue

Tissue was removed from anesthetized animals and transferred into liquid N2. The frozen tissue was disrupted with a mortar and a pestle, both precooled in liquid N2. Tissue powder was weighed and stored at -80°C. RNA extraction was the performed using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufactures protocol.

For the quantification of FoxP1 and FoxP4 expression in Area X in injected birds, animals were given an overdose of isofluorane decapitated, their brains dissected out, and the hemispheres were separated using a razorblade. Each hemisphere was put in an embedding mold and covered with Tissue-Tek O.C.T. compound and immediately shock-frozen on liquid nitrogen or dry ice. Brains were stored then at -80°C until the brains were cut in the cryostat at a temperature of the knife of -10°C and the holder at -4°C. Alternating slices of 2x12µm and 200µm were cut. The 12µm slices were taken on superfrost plus slides (Thermo scientific) for *in situ* hybridization against GFP probe. On the 200µm slides I punch out Area X with a Razor sharp cutting edge (Harris Uni-Core tip) of different diameters (0.5-1,5mm). Each punch was then put on a pre-cooled 0,5ml Eppendorf and stored at -80°C until mRNA extraction. The remains of the 200µm tissue were put then in a well (24 well plates) containing 1,5ml of 4% PFA at 4°C and stored. Next day I looked for GFP signal in the PFA fixed slices in the surroundings of the punched area. For the RNA extraction from these small amounts of tissue material I used 200µl of TRIZOL for each punch. To digest remaining DNA I used Turbo DNAse from AMBION following the manufactures instructions. RNA yield was determined as in 2.4.20.

### 2.4.3 Cloning of FoxP4 from zebra finch

I used mice and human FoxP4 sequences to blast them against zebra finch EST in NCBI (2.22.2). Based on the sequences I retrieved from this search I generated primers specific for the FoxP4 zebra finch sequence. I used Zf_sFOXP4_for and Zf_eFOXP4b_rev (Table 2.17) to amplify the zebra finch FoxP4 full sequence from adult male zebra finch brain total RNA. For reverse transcription of RNA into cDNA I used Superscript III (Invitrogen, Carlsbad, USA) and followed the manufacturer's manual. I amplified the entire ORF of the FoxP4 zebra finch nucleotide sequence which is 2019 bp. All PCRs programs are listed in 2.4.13.1. All PCR products were examined on agarose gels, cleaned from nucleotides with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and cloned into the pGEMTeasy vector (Promega, Madison, USA, Table 2.15). Inserts from 15 independent FoxP4 clones were then sequenced on both strands with primers M13-f and M13-r (accession number in NCBI: JN160732). I named the construct as FoxP4 full (Table 2.16).

### 2.4.4 Cloning of FoxP1, FoxP2 and FoxP4 *in situ* probes

*FoxP1* and *FoxP2* probes were made using plasmids carrying the whole zebra finch sequence cloned in previous work (Haesler *et al.*, 2004) (accession numbers AY549148, AY549149, AY549150 and AY549151 for *FoxP2*; and AY549152 for *FoxP1*). Additionally I used the FoxP4 full sequence cloned in this study for amplifying different probes.

For the FoxP1 probe I used the nFoxP1for and the nFoxP1.2 rev to obtain a 301 bp product (nt 1690-1990). This probe lies in the C terminal part of the FoxP1 sequence, which is the part of the FoxP subfamily members that diverges the most.

For cFoxP2 probe I used cFoxP2_for and kaz-2rev primers to obtain a 353 bp product (nt 1784-2136). This probe lies in the C terminal part of the FoxP2 sequence, which is the part of the FoxP subfamily members that diverges the most. Other two additional probes for FoxP2 were made, both bear the poly-Q rich part of FoxP2 sequence, a region that FoxP1 and FoxP4 do not have. The nFoxP2 probe was done with Ig-2for and the FoxP2 R2 rev primers for amplifying a 506 bp long product (nt 277-782). The nsFoxp2 probe was done with the same for primer as the nFoxP2 and nsFoxP2rev for a amplifying a 319 bp long product (nt 277-595).

For FoxP4 probe I used fFoxP4 for and fFoxP4 rev primers for amplifying a 112bp PCR product (nt 304-415). For bFoxP4 probe I used the same forward primer and E_FoxP4_3 reverse primers for amplifying a 651bp PCR product (nt304-954). Finally, for the cFoxP4 probe I used cFoxP4 for and rev primers for amplifying a 334 bp PCR product (nt 1654-1987). This last FoxP4 probe lies in the C terminal part, which is the part of the FoxP subfamily members that diverges the most. Al primers used for cloning the different probes are listed in Table 2.17.

All PCR products were examined on 1-2% agarose gels, cleaned from nucleotides with Qiaquick PCR purification kit (Qiagen, Chatsworth, CA) and cloned into pGEMTeasy vector (Promega, Madison, WI). Inserts from different clones were then sequenced on both strands. The probes were generated from PCR-amplified sequences using M13 primers or linearized plasmids (using SalI and NcoI) using T7 and SP6 RNA polymerase in order to drive the transcription of the mRNA sense and anti-sense probes.

### 2.4.5 Cloning of FoxP1, FoxP2 and FoxP4 FLAG and V5 tagged expression constructs

The FoxP2-V5 construct was done in a previous study (Sebastian Haesler 2007). I used this construct for making the FoxP2 FLAG construct amplifying the FoxP2 ORF sequence with the same forward primer used for cloning FoxP2 V5 (Ig3) (which has a *BamHI* restriction site followed by a Kozak sequence and the beginning of FoxP2) and the eFoxP2FLAG_rev primer (that has the end of the FoxP2 gene without a stop codon, followed by the FLAG sequence with the stop codon and at the end a *EcoRI* restriction site) using the Phusion proof reading polymerase (as described in 2.4.13.1). After purification of the PCR product using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). The PCR product was then cut with *BamHI* and *EcoRI* and ligated into pCDNA3.1(-) linearized with the same enzymes and named FoxP2-FLAG.

For FoxP1 over expression constructs I used Mb_25 for primer (having a *BamHI* restriction site followed by a Kozak sequence and the start of FoxP1) and Mb_26 primer (that has the end of the FoxP1 sequence without the stop codon, followed by the FLAG sequence, stop sequence and the *EcoRI* restriction site) and amplify using Pfu proof polymerase from cDNA the whole FoxP1 ORF. Then I took the clone that had the correct FoxP1 sequence and the FLAG tag and did amplify the FoxP1 ORF with the Mb_25 for primer as before but I used the eFoxP1V5tag_rev primer (having the end of FoxP1 without the stop codon, followed by the V5 sequence, stop and the *EcoRI* restriction site) instead. Both constructs were then cut with *BamHI* and *EcoRI*, and ligated in pCDNA3.1(-) vector that was cut with the same enzymes. Constructs were named as Foxp1-V5 and FoxP1-FLAG.

For FoxP4 I used as template the FoxP4 full construct and amplified the ORF with the sFoxP4oe1 for primer (having a *BamHI* restriction site followed by a Kozak sequence and the start of FoxP4) and the eFoxP4FLAG_rev (that has the end of the FoxP4 sequence without the stop codon, followed by the FLAG sequence, stop sequence and the *EcoRI* restriction site). The purified PCR product was then cut with BamHI and EcoRI and ligated into pCDNA3.1(-) as previously described. This construct was named FoxP4-FLAG. For the V5 tagged FoxP4 I used the same forward primer and the eFOXP4h1rev primer (having the end of the FoxP4 sequence without a stop codon and the EcoRI restriction site).

This last primer was designed such that after cutting the PCR product with *BamHI* and *EcoRI* it can be ligated in-frame into the multiple-cloning-site (MCS) of pCDNA4/V5-His B

or pCDNA3.1(-) (Invitrogen, Carlsbad, USA). The construct was named FoxP4-V5. All constructs listed in Table 2.16 and primers used listed in Table 2.17.

### 2.4.6 Cloning of R553H and ΔFox Box FLAG and V5 tagged expression constructs

Both V5 over expression mutants were previously cloned (Martin Begeman). I used both constructs as template for their FLAG tagged versions using the primers used for the FoxP2-FLAG construct previously described, since the start and end sequence of the mutant versions are the same. Constructs were named R553H-FLAG and ΔFox Box- FLAG and are listed in Table 2.16.

### 2.4.7 Cloning constructs encoding short hairpin RNA for FoxP1 and FoxP4

A list of putative shRNA targets within the zebra finch FoxP1 and FoxP4 genes were generated using the web-based software from Ambion and MWG (Listed in 2.22.2) . Since shRNA´s were to be expressed from a plasmid via U6 promoter driven RNA polymerase III, it was absolutely crucial NOT to include more than 4 consecutive thymidines (uracils), which are recognized as a stop signal by the polymerase. All proposed targets that contained more than 3 thymidines in a row were excluded. In order to reduce the risk of cross-reactivity with other genes, all target sequences were checked for homology to chick expressed sequence tags (EST) with the internet-based BLAST tool "search for short nearly exact matches" (Listed in 2.22.2). They were further directly compared for their possible cross reactivity with their closest FoxP homologs. Only target sequences with at least 6 non-homologous bases were chosen. Target sequences within the known protein domains of either FoxP1 or FoxP4 were also avoided. In a last step, all chosen targets were checked for ambiguity in the sequence raw data of all available zebra finch clones, to rule out interference with single-nucleotide-polymorphisms. All target sequences have a GC-content of approximately 50%. Sequences are shown in Table 2.18 for FoxP1 and Table 2.19 for FoxP4. For each target sequence meeting the above mentioned criteria, a DNA sequence encoding the corresponding short hairpin RNA was generated. The general composition of the sequence was: sense ➔ hairpin

loop ➔ antisense. The sequence of the hairpin loop was GTGAAGCCACAGATG. A *BbsI* and a *BstBI* restriction site were added to the 5´and the 3´ end respectively, which allow cloning the DNA fragments into the short-hairpin expression vector pBudΔU6. A non-silencing control shRNA from *Thermotoga maritimia*, used in previous study was used as a control (Table 2.20). Tables 2.20.1 (for FoxP1) and Table 2.21.1 (for FoxP4) lists the sequence of all ssDNA fragments. For cloning of the DNA fragments encoding the different short hairpin RNAs into pBudΔU6, I first generated double stranded DNA fragments from single stranded synthetic oligonucleotides. Each pair of complementary strands was diluted in annealing buffer. The tubes were placed in boiling water to denature the DNA. Next the tubes and the water were slowly cooled down to RT. After that, the now double-stranded DNA was digested with enzymes *BbsI* and *BstBI* and ligated into pBudΔU6, cut with the corresponding enzymes before.

All hairpin constructs were tested for their knockdown efficiency *in vitro*. Functional U6-shFoxP1 and U6-shFoxP4 expression cassettes (U6 promoter + shRNA) were subcloned into the viral transfer vector pFUGW_linker with the enzymes *NheI* and *BstBI*. The U6-shControl control construct I used were already cloned in a previous study by Sebastian Haesler. This vector was subsequently used to generate lentiviral particles. I confirmed the sequence of all pFUGW-shFoxP1 and pFUGW-shFoxP4 constructs by sequencing with primers *Seq pFUGW-f* and *Seq pFUGW-r* (Table 2.17).

## 2.4.8 Preparation of plasmid DNA

All vectors were transformed into chemically competent TOP10 (Invitrogen, Carlsbad, USA) *E. coli* cells as described in 2.4.18 or DH5α (Invitrogen, Germany). Mini preparations of plasmid DNA were performed from 3ml *E. coli* overnight cultures. For small extractions I used Qiagen Mini-plasmid preparation kits (Hilden, Germany) as well as Invisorb Spin Plasmid Mini Two columns (Invitek, Ref 1010140300) as described by the manufacturer. Large scale extraction from 250ml *E. coli* overnight cultures was done with Cesium chloride by L. Vogt, MPI for Molecular Genetics according to (Sambrook and Russell, 2001).

## 2.4.9 Sequencing

I sent all constructs to LGC genomics (AGOWA) for sequencing. For sending samples I prepared 800ng plasmid, 2µl of the selected primer (20pMol) and added molecular grade $H_2O$ up to 10µl.

### 2.4.10 Sequence analysis

Sequence assembly and analysis was conducted with Chromas Lite program Version 2.01 (from Applied Biosystems and Amersham MegaBace DNA sequencers) as well as Vector NTI (Invitrogen) and ClustalW (all listed in 2.22.2).

### 2.4.11 *In situ* hybridization

Nonradioactive ISH was performed using both Digoxigenin (DIG)-labelled and Fluorescein (Fitc)-labelled mRNA probes.

Riboprobes were transcribed *in vitro* from T7 and SP6 promoter sides of the pGEM-T-easy cloning vector containing the FoxP1, FoxP2 and FoxP4 cDNA clones. The probes were generated from PCR-amplified sequences using M13 primers (as described in 2.4.13.1) or SalI / NcoI digested plasmid, using T7 (for SalI digested plasmid) and SP6 (for NcoI digested plasmid) RNA polymerase.

Series of at least 3 zebra finches per age (35, 50, 75 and >100PHD) were hybridized at the same time with a FoxP1, FoxP2 or FoxP4 master mix at the probes specific temperature.

Sections were first treated with neutral buffer 4% PFA for 5 minutes at room temperature. Then the sections were dehydrated through a row of ethanol washes (75%, 95% and 100%) and dried. Sections were acetylated treated with triethanolamine and acetic anhydride for 10 minutes, followed by two washes in 2x SSPE. Then slides were dehydrated through a row of ethanol washes (75%, 95% and 100%) and dried. Slides where then pre-hybridized in hybridization solution for at least 1 hour at 65°C. Slides were then hybridized with 2-4µl of probe for each 100µl hybridization solution in hybridization buffer (5× SSC, 2% blocking reagent, 50% formamide) overnight at desired temperature in an oil-bath. After hybridization the slides where washed with 2 rows of chloroform to remove the oil, followed

by washes in 2x SSC to take of the glass-covers and 5x SSC to wash remaining chloroform. Post-hybridization washes were done as follow, with 1× SSC containing 50% formamide for 30 minutes at hybridizations temperature, if needed followed by RNAse A (20 µg/ml) treatment in 10 mM Tris HCl (pH 7.5), 500 mM NaCl and 1mM EDTA for 30 min at 37°C. Sections were washed sequentially with 2× SSC and to rows 0.2× SSC for 20 min at hybridizations temperature. Thereafter, the sections were washed twice in MABT (100mM Malic Acid, 150 mM NaCl, 0,1 % Tween 20, pH 7,5) and incubated with alkaline phosphatase-conjugated goat anti-DIG Fab′ antibody (Roche) overnight at 4°C. Slides were washed with MABT again, followed by a wash in NTMT (100mM NaCl, 100mM Tris pH 9.5, 50mM MgCl and 0.1% Tween 20) for 10 minutes. Colorimetric detection was performed by a standard immunoalkaline phosphatase reaction, with NBT (Nitro blue tetrazolium chloride) / BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) (Roche) solution as the substrate. Antisense as well as sense probes were always run for each experiment.

### 2.4.12 Double *in situ* hybridization

I first established a method to do double *in situ* hybridization using NBT / BCIP staining for one probe, and using Fast Red (DAKO) as the second, fluorescent, dye for the second probe. With both I could detect a specific pattern of expression and no background with sense probes (data not shown). I also did not got staining in alkaline phosphatase quenching controls (data not shown), which shows that this enzyme can be quenched successfully and reliably. One advantage of making one probe in a normal NBT / BCIP staining is that the expression pattern can be seen, and therefore Area X was also visible.

Nonradioactive double in situ hybridization was performed using both Digoxigenin (DIG)-labelled and Fluorescein (FITC)-labelled mRNA probes. Probes were hybridized sequentially starting with the probe with the highest temperature as described for single in situ hybridization. After the last 0.2x SSC post- hybridization wash, I started over with pre-hybridization, followed by hybridization of the second probe at needed temperature. I did again all post- hybridization washes and RNAse treatment if needed. Thereafter, the sections were incubated with alkaline phosphatase-conjugated goat anti-DIG Fab′ antibody (Roche) overnight at 4°C, followed by NBT / BCIP staining as described before. After first probe was completely stained, I killed the alkaline phosphatase of the anti-DIG Fab´ antibody with a wash in MABT at 65°C for 30 min followed by a wash in 0.1 M glycine-HCl pH 2.2 also for 30 min. Sections were washed with MABT followed by incubation with the second alkaline

phosphatase-conjugated goat anti-FITC Fab' antibody (Roche) overnight at 4°C to detect the second probe. Slides were washed with MABT, followed by NTMT for 10 minutes. A colorimetric reaction with Fast Red (DakoCytomation, K0597) was used as a substrate for the second alkaline phosphatase incubating for 30 minutes at room temperature, and then I did DAPI staining. Sense and antisense probes were always run for each experiment. And the quenching of the Alkaline phosphatase was also controlled running slides with single *in situ* hybridization normally done, but before staining doing the quenching of alkaline phosphatase.

## 2.4.13 PCR

### 2.4.13.1 Normal PCR

The PCR was used (i) to amplify DNA fragments from existing plasmids or cDNA for subsequent cloning, (ii) and to screen *E. coli* colonies after transformation with newly generated plasmid constructs (Colony PCR). For (i) PCR was performed in different final volumes depending ranging from 25µl to 100µl, for (ii) PCR was performed in a final volume of 25µl. If PCR was done with the lab made polymerase in a final volume of 25µl, it would have the following constituents:

**For 25µl PCR reaction (1x):**
10µl TDMH
0,5 µl forward primer
0,5µl reversed primer
0,5µl Lab Taq
0,5-5 µl Template
$H_2O$ to 25µl

For the lab Taq I used the following program in a PTC-200 Peltier Thermal Cycler of MJ Research:

| Step | Time / min | Temperature / °C | cycles | Process |
|---|---|---|---|---|
| 1 | 5 | 94 | 1 | Initial denaturation |
| 2 | 0,5 | 94 | 33 cycles Steps 2-4 | Denaturation |
| 3 | 0,5 | 50-65* | | Annealing |
| 4 | 1-5** | 72 | | Elongation |
| 5 | 10 | 72 | 1 | Final elongation |
| 6 | ∞ | 4 | 1 | |

* Depending on the primers specific annealing temperature

** Depending on the length of the PCR product (approx. 1 min per 1000 bp).

If PCR was done with the Phusion proof reading polymerase in a final volume of 20µl, it would have the following constituents:

| For a 20µl PCR reaction with Phusion proof reading polymerase: | | |
|---|---|---|
| | **µl** | **End concentration** |
| **Phusion 5x Buffer** | 4 | 1x |
| **dNTP´s** | 0,40 | 250µM |
| **Primer 1** | 1 | 0,5µM |
| **Primer 2** | 1 | 0,5µM |
| **Phusion Polymerase** | 0,50 | 2U/µl |
| **Template** | 1-10 | |
| **Water** | Up to 20 | |

According to manufacturer's protocol, I adjusted the PCR program as follows:

| **Step** | **Time** | **Temperature / °C** | **cycles** | **Process** |
|---|---|---|---|---|
| 1 | 30s | 98 | 1 | Initial denaturation |
| 2 | 10s | 98 | 35 cycles Steps 2-4 | Denaturation |
| 3 | 30s | 85 | | Annealing |
| 4 | 30s/1kb | 72 | | Elongation |
| 5 | 10 | 72 | 1 | Final elongation |
| 6 | ∞ | 4 | 1 | |

If PCR was done with the Pfu proof reading polymerase in a final volume of 20µl, it would have the following constituents:

| For a 30µl PCR reaction with Pfu proof reading polymerase: | | |
|---|---|---|
| | **µl** | **End concentration** |
| **Pfu 10x PCR Buffer** | 3 | 1x |
| **dNTP´s** | 2,4 | 250µM |
| **Primer 1** | 0,9 | 0,3µM |
| **Primer 2** | 0,9 | 0,3µM |
| **Pfu Polymerase** | 0,60 | 2,5U/µl |
| **Template** | 1-10 | |
| **Water** | Up to 30 | |

According to manufacturer's protocol, I adjusted the PCR program as follows:

| Step | Time | Temperature / °C | cycles | Process |
|---|---|---|---|---|
| 1 | 2min | 95 | 1 | Initial denaturation |
| 2 | 30s | 95 | 30 cycles Steps 2-4 | Denaturation |
| 3 | 30s | 50 | | Annealing |
| 4 | 2,5min | 72 | | Elongation |
| 5 | 10 | 72 | 1 | Final elongation |
| 6 | ∞ | 4 | 1 | |

### 2.4.13.2 Real time PCR

For the quantification of FoxP1 and FoxP4 expression levels in Area X I used the real time PCR system Mx3005P and the MxPRO QPCR program (Stratagene; Agilent Technologies, U.S.A.). DNA quantification was performed with the Sybr Green MIX containing the Rox passive control. I determined FoxP1 and FoxP4 expression levels by relative quantification based on the normalization of expression levels to the *Hmbs* internal control gene shown to be a good housekeeping gene (Sebastian Haesler 2007). All primers were designed to yield PCR products of approximately 100bp length (for primer sequences see Table 2.12.1). These short amplicons are likely to achieve optimal amplification efficiency. $T_m$ was run in optimal annealing temperatures tested in gradient PCR. Sequence specificity of primers was assessed by cloning the single bands of the PCR at optimal temperature. The cloned fragments were then diluted and used for making the standards for assessing the absolute copy number in the samples. For determination of relative expression levels I used the comparative $C_t$ method. The Ct value of each PCR reaction is defined as the threshold cycle in the linear exponential phase of the amplification, at which the PCR product is first detected to increase significantly. Differences in expression levels can be calculated by comparing the different threshold $C_t$ values for each gene of the same cDNA. E.g. the expression level for FoxP1can be expressed as $\lambda C_{t\ FoxP1}$ by simply subtracting $C_{t\ FoxP1}$ - $C_{t\ control\ gene}$. In order to compare expression levels between two different cDNA samples from the same animal, normalized $C_t$ values ($\lambda C_t$) were calibrated to one cDNA. In this study, I always calibrated the knockdown treatment to the control treatment. Given that under ideal conditions, one amplicon is amplified once per cycle, the amount of a target gene relative to the internal control gene and calibrated to one cDNA is then $2^{-\lambda\lambda Ct\ FoxP1}$] with $\lambda\lambda Ct_{\ FoxP1} = \lambda C_{t\ FoxP1\ cDNA\ knockdown} - \lambda C_{t\ FoxP1\ cDNA\ control}$. For the $C_t$ method to be valid, it is important that all amplicons are amplified with similar efficiency. Last I tried to follow the "MIQE" guidelines for QPCR experiments (Bustin *et al.*, 2010; Bustin, 2010).

### 2.4.14 Agarose gel electrophoresis: analytical and preparative

Agarose gels for separation of PCR amplicons, restricted inserts and vectors were prepared in a concentration of 1-2% (w/v) in 1xTBE buffer. In 50ml of Agarose gels I added 7µl of EtBr before cooling. DNA and plasmid solutions were mixed with 6x loading dye prior loading onto the gel. Electrophoresis was carried out in 1x TBE buffer at 70V for the first 10 minutes, and afterwards up to 120V. The stained DNA was visualized by UV illumination of the gel.

### 2.4.15 DNA isolation and purification from agarose gels

After preparative gel electrophoresis, DNA bands of the desired size were cut out of the gel. The DNA was then isolated using the QIAquick Gel extraction kit (QIAGEN) according to manufacturer's instructions.

### 2.4.16 DNA restriction digest

NEB restriction enzymes and recommended buffers were used. 1-5 µg plasmid DNA or isolated PCR products were digested in a final volume of 50µl containing NEBuffer (10x stock), BSA (100x stock) and 10 to 50U of restriction enzymes (approx. 1o U per 1µg of DNA). The reactions mixtures were incubated at 37°C for 3 h.

Using Fast digest enzymes (Fermentas) I also cut 1-5µg of plasmid DNA or isolated PCR products in a final volume of 20-50µl containing Fast digest buffer (10x stock) and 1µl for the 20µl reaction and 5µl for the 50µl reaction. The reactions mixtures were incubated at 37°C for 5-30 minutes depending on the DNA quantity.

Digested PCR amplicons were purified QIAquick Gel purification kit (Qiagen) using the manufacturer's protocol of Clean up of enzymatic reactions using a microcentrifuge.

### 2.4.17 Ligation of DNA inserts into linearized vectors

All Lab Taq PCR products were after purification directly ligated into pGEMT easy (Promega).

Prior to ligation, both purified insert and vector DNA were digested with the appropriate restriction enzymes.

For ligation, vector and insert DNA were combined in a 1:3 (1:6 for inserts bigger than 1,5kb) ng ratio in a total volume of 20µl including 10µl 2x Rapid Ligase Buffer and 1 U T4-DNA Ligase (Promega). Samples were incubated at room temperature for at least 30min. Then, the ligation mix was applied for transformation of chemically competent *E. coli* TOP10 cells.

### 2.4.18 Transformation of chemically competent E. coli cells

For heat shock transformation 100µl aliquots of competent *E. coli* TOP 10 (Invitrogen) were thawed on ice and either 2-5µl of ligation reaction or 10 to 100ng of purified vector DNA were added. After 30 min incubation on ice, cells were heat shocked in a water-bad at 42°C for 30s and immediately put on ice for 5 min. Then I added 200-400µl of SOC medium and incubated at 37°C for 45 minutes shaking at 400rpm. Afterwards the cells were platted on LB-agar plates containing the appropriate antibiotic for selection. Plates were incubated at 37°C overnight.

### 2.4.19 Colony PCR

Colony PCRs were used to check grown bacteria colonies for the integration of the correct DNA insert. For this purpose, PCRs were carried out containing some cell material from a single colony and primers in the insert or vector. During the first round of denaturation, the cells are disrupted and the plasmid DNA becomes accessible as a template. Amplicons after the PCR should be of the desired size if bacteria have the insert.

For the PCR, a master mix of the Lab Taq polymerase was made as described above. 24µl of the PCR master mix were added to each PCR tube. In other PCR tubes I added 10µl of $ddH_2O$. Single clones were picked with a sterile pipette tip from the agar plate, dipped into the 10µl $ddH_2O$ and then I put a dot in a space of a gridded agar plate (to have the clone for further usage). Then I took 1µl of the water were I dipped the pipette tip and added it to the

PCR mix. A PCR standard program for Lab Taq was run in a thermo cycler as described above. Afterwards, the PCRs were analyzed by agarose gel electrophoresis.

### 2.4.20 Spectrophotometric DNA and mRNA concentration determination

DNA as well as mRNA yield was determined by UV spectroscopy at 260 / 280 nm with Nanodrop (PEQLab Nanodrop, Spectrophotometer ND-1000) in 2µl samples.

For measuring the mRNA quantities for the RT-PCR I used the Quant-iT$^{TM}$ RNA assays kit (Invitrogen, Q32855) following manufacturer's protocol and determined the yield by fluorescence using the Qubit ® fluorometer (Invitrogen).

### 2.4.21 Luciferase assays

One day prior to transfection, I seeded 2-6x$10^4$ HeLa cells in each well of a 96-well plate (Nunclon, Cat.No.136101, Denmark), using 200µl DMEM medium from GIBCO with antibiotics. Plates were incubated at 37°C in a $CO_2$ incubator for 24h.

I prepared a first master mix that contained 25µl of OptiMEM (GIBCO), 30ng of pGL4.13 (Luciferase gene driven by the SV40 promoter which is known to be regulated by FoxP subfamily members) and 30ng of pGL4.75 (Renilla gene driven by the CMV promoter that is not affected by FoxP subfamily members as a normalization control) for each well (listed in 2.15). Then I put the master mix for each triplicate into 1,5ml Eppendorf tubes (75µl total mix I). Then I added 250ng total over expression vector for each well (750ng total plasmid DNA in an Eppendorf).

Then I prepared a second master mix containing 1 µl of Lipofectamine™ 2000 (Invitrogen) into 25 µl OptiMEM Medium for each well and incubate for 5 min at room temperature.

Then I took 75µl of the second master mix and added it to the first mix and incubated at room temperature fir 20 minutes.

In the meanwhile I changed the medium of the HeLa cells to 100µl of antibiotic free medium.

After the 20 minutes of incubation I added 50µl of the transfection mix to each well.

After 4-6h I changed the medium to 75µl of antibiotic medium and incubated for 48h at 37°C in a $CO_2$ incubator.

I then measured luminescence using the Dual Glo Luciferase Kit (Promega) following manufacturer's protocol in an Elisa plate reader (Tecan, GENios; Switzerland).

I calculated the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Normalize this ratio to the ratio of a control well. I present Luciferase results as Luciferase Renilla Ratios calculated from the normalized ratios.

## *2.5 Protein Biochemistry*

### 2.5.1 Protein extraction from culture cells

For extracting proteins from 6 well plate culture cells I washed the cells with 1ml of 1x PBS for each well (Gibco). After removing the PBS of each well I added 250µl of M-PER lysis medium in each well and incubated on ice for 15 minutes. I then used a 16cm Cell scraper (Sarstedt, Germany) and detach the cell layer completely. The medium was then taken with a 1ml pipette and put in a 1,5ml Eppendorf tube in ice. I then centrifuged at 4°C and 4000rpm for 10 minutes. The supernatant was then taken and put in a new 1,5ml Eppendorf tube and labelled. Proteins were then stored at -20°C for a few weeks.

### 2.5.2 Protein extraction from zebra finch tissue

Tissue powder was generated as described in 2.4.2. A small sample (0.1-0.3 g) of the powder was transferred into a 1.5ml Eppendorf tube containing 600µl of brain nuclear extraction buffer. The tube was vortexed vigorously. The tissue was disrupted by pipetting up and down approximately 15 times, followed by incubation on ice for 20min. While on ice, the tube was vortexed from time to time (~3x). Next, the sample was centrifuged 5min at 1500xg at 4°C. The supernatant, which contains mainly the cytoplasmic fraction of the sample, was pipetted off for subsequent Western blotting. The remaining pellet was re-dissolved in brain nuclear extraction buffer and incubated on ice for another 20min. After a second centrifugation step (5min at 1500xg at 4°C) the supernatant was discarded and the pellet, containing mainly cell nuclei, re-dissolved in 200µl M-PER. Nuclei were incubated for 15 minutes in ice and centrifuged at 4°C and 4000rpm for 10 minutes. The supernatant was then

put in a new Eppendorf tube and labelled. All samples were processed as described in Western blot for western blotting.

### 2.5.3 Western blot

Protein samples were prepared in a total volume of 30µl: The protein was diluted in 2xLaemmli containing 0.1M DTT. The sample should contain at least 7µl of 2xLaemmli. Samples were denatured at 95°C for 5min, cooled briefly on ice and loaded on a denaturing acrylamide gel (Sambrook and Russell, 2001). Electrophoresis was performed according to (Sambrook and Russell, 2001). After the gel run, the gel was blotted onto a Polyvinylidene fluoride (PVDF)-membrane with the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell from BioRad (Munich, Germany) according to the manufacturer's manual for 25min at 15V. After that, blots were blocked in PBST/5% dry milk or Roti- Immunoblock (for goat antibodies) overnight at 4°C. Before incubation with the antibody, the membranes were briefly washed in PBST. Antibodies against the protein of interest were then diluted in 2ml PBST/1% BSA. Table 2.13.1 lists all antibodies and dilutions used in this study. Blots were transferred into the antibody solution and incubated overnight at 4°C. After that, membranes were washed 3 times for 5min in PBST and subsequently incubated for 30min with the corresponding HRP-conjugated secondary antibody diluted in PBST (Table 2.13.2). Next, they were washed 3 times for 5min in PBST. The blots were then wetted with 1ml of the final detection solution from the Western lightning kit (Perkin Elmer, Rodgau, Germany). Chemiluminescense was detected by exposure to an X-ray film (Kodak, Stuttgart, Germany). Films were developed in a Curix 60 developing machine (Agfa, Cologne, Germany).

### 2.5.4 Co- immunoprecipitation

For Co-Immunoprecipitation (Co-IP) using proteins from over expression I used 150µl, from brain proteins I used 200µl.

I used Dynabeads® Protein G (Invitrogen, Cat.No.100.04D). I first did a pre-clearing step (all steps on ice). For this I first resuspended Dynabeads on a roller for 5 minutes and then transferred 50µl to a 1,5ml Eppendorf tube. I then placed the tube in a magnet to separate the Dynabeads from the solution and took out the solution with a 200µl pipette. I then

removed the tube from the magnet and added the protein extract and incubated at 4°C for 30min with rotation.

In the meanwhile I took other 50µl of resuspended Dynabeads and put them again in a 1,5ml Eppendorf tube. I then placed the tube in a magnet (Invitrogen) to separate the Dynabeads from the solution and took out the solution with a 200µl pipette. I then added the primary antibody (FLAG or FoxPs specific antibody) (Table 2.13.1) in 200µl Washing buffer and incubated with rotation for 10 minutes at room temperature. I then placed the tube in the magnet and removed the supernatant. I then removed the tube from the magnet and washed with 200µl cold Washing buffer pipetting up and down and finally put the tube again in the magnet and removed the washing buffer. I took the protein lysate Dynabeads mix and put the tube containing it in a magnet. The supernatant was then taken and put in the tube containing the Dynabeads with the primary antibody and gently resuspended by pipetting. I then incubated with rotation for 10 minutes at room temperature. I then placed the tube in the magnet again a put the supernatant in a fresh tube (supernatant sample). I then washed the Dynabeads-Antibody-Antigen complex 3 times each with 200µl of washing Buffer. Then I resuspended the Dynabeads with 100µl of washing buffer and transferred the suspension to a new tube. I then place the tube in a magnet and removed the washing buffer. I then added 15µl of Elution buffer and 15µl of 2x Laemmli. Samples were denatured at 95°C for 5min, cooled briefly on ice and before loading the sample they were put again in the magnet and the supernatant was then loaded to the electrophoresis gel and treated as described above for western blotting using the other antibody for detection (V5 or FoxPs specific antibody).

## 2.6 Knock down efficiency of hairpin constructs in vitro

Since the optimal sequence of a short hairpin RNA (shRNA) targeting the *FoxP1* and *FoxP4* message RNA with maximum efficiency cannot be predicted, different shRNA constructs (Table 2.20 for FoxP1 and 2.21 for FoxP4) were tested experimentally *in vitro* to identify those resulting in maximal knockdown. Knockdown efficiency of shRNA constructs *in vitro* was determined by co- transfecting each hairpin construct (pBudΔU6 constructs table 2.16) together with FLAG-tagged FoxP1 or FoxP4 into HeLa cells. $1.5x10^5$ HeLa cells were seeded into each well of a 6-well plate (Corning, Corning, USA). One day later, 4µg of total DNA was used in a ratio of 1:8 of over expression to short hairpin to transfected the cells

using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) as described in the manufacturer's protocol. 48h post transfection total protein was extracted and analysed by western blot.

In addition the short hairpins that worked were tested for their cross reaction against the other FoxP subfamily members. The same method as for testing the targeting efficiency was used to test the cross reaction. In this study only the short hairpins that would have an optimal targeting efficiency and would not have any cross reactions to the other FoxP members would be taken for *in vivo* studies.

## 2.7 Generation of Lentivirus

Recombinant lentivirus was generated as described in (Lois *et al.*, 2002) with the following specifications and modifications. HEK293-T cells (kindly provided by D. Vanhecke, MPI Molecular Genetics, Berlin, Germany) were used for transfection of viral constructs and titration of virus. Four cell culture plates (10cm diameter CELL+ from Sarstedt, Nümbrecht, Germany) each containing $8x10^6$ cells with 12ml HEK293-T medium, were transfected with 40 µg viral transfer vector, 20 µg envelop vector pVsVg and 30 µg packaging vector λ8.9 using 225,2 µl Lipofectamine 2000 (Invitrogen, Carlsbad, USA). For transfection cells were kept in antibiotic-free cell culture medium. Approximately 4-6 hours post transfection, the culture medium was changed.

Collection of virus

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

overnight at 4°C. Finally, virus solutions were aliquoted into 2µl aliquots in Eppendorf tubes, shock frozen in liquid nitrogen and stored at -80°C.

Titration of the virus by infection of HEK293-T cells

The virus titer was determined by infection of $4x10^5$ HEK293-T cells, seeded 12hours prior to titration per well of a coated 6-well plate (CELL+, Sarstedt) with various dilutions of virus. For infection, 1µl of undiluted, 1:10, 1:100 or 1:1000 diluted virus solution was added directly to the culture medium containing antibiotics. Infection was quantified after 72h by flow cytometry with a FACScalibur (Beckton Dickinson, Heidelberg, Germany). All virus constructs generated in this study encode the green fluorescent protein (GFP), thus the 530nm channel of the FACS was used to determine the number of infected cells. Usually the percentage of green cells in the 1:10 and 1:100 dilutions were used to calculate the titer. The percentage of GFP positive cells was divided by the total number of cells present in the dish before infection (here $4x10^5$) and multiplied with the dilution factor. Titers of virus solution were usually in the range of $1\text{-}3x10^6$/µl.

## 2.8 Surgery and stereotactic injection of virus and retrograde tracers

The birds received painkiller Meloxidyl (active substance is meloxicam, in a dose 0.5 mg per kg body weight) with a pipette through their beak 1 hour before operation. Isoflurane evaporation was mixed with oxygen at the level of 1-2% and was delivered to the beak through a pipe system at 1l/min flow rate. After operation and through the next 3 days birds were given Meloxidyl again, but this time with food once per day to eliminate possible discomfort caused by pain sensation after surgery.

The stereotactic coordinates for targeting Area X in juveniles with FoxP1, FoxP4 and control virus were:

| | |
|---|---|
| medial / lateral: | ±1.4 / ±1.6 |
| Anterior / posterior: | 3.6 / 4.0 |
| dorso / ventral: | 3.8 / 4.0 |

The stereotactic coordinates for targeting Area X in adult birds with retrograde tracers for labelling HVC to Area X projections were:

| | |
|---|---|
| medial / lateral: | ±1.5 |
| Anterior / posterior: | 3.8 / 4.0 |
| dorso / ventral: | 4.0 / 4.2 |

The stereotactic coordinates for targeting RA in adult birds with retrograde tracers for labelling RA to Area X projections were:

| | |
|---|---|
| medial / lateral: | ±2.4 |
| Anterior / posterior: | 1.5 / 1.8 |
| dorso / ventral: | 1.8 / 2.0 |

All injections targeting Area X penetrated the brain perpendicular to the surface of the brain (90° vertical injection angle relative to the horizontal plane). For RA all injections penetrated with an angle tilted 9° relative to the vertical plane (to avoid passing through HVC, for it lies above RA).

A small opening in the skull above the expected site of injection was made with a curette (delicate bone scraper, FST 100075-16) and pulled off with sharp forceps (Dumont). Then, by slow injection using a hydraulic micromanipulator (Narishige, Japan) approximately 200nL of retrograde tracer solution or tracers was delivered through the injection needles with a plunger that had been constructed from pulled capillary tubes (Drummond® Wiretrol), tips of which had been cut to 20–50μm inner diameter.

I used green and red fluorescent latex microspheres (Lumafluor, Naples, USA)(0.2% diluted in 0.1M phosphate buffer saline, Molecular Probe, Karlsruhe, Germany). After the injections the piece of the skull from the opening was returned back and the skin was closed with collodion glue. The animals resumed normal activity 5-10 min following surgery and were returned to their home cages. After ~ 5 days they were sacrificed with and isofluorane overdose and perfused.

## 2.9 Behavioural paradigm and song analysis

The general procedure for studying the behavioural consequence of locally reduced FoxP1 and FoxP4 levels in Area X was as in a previous study (Sebastian Haesler 2007). Young birds from around post hatch day (PHD) 7-14 were sexed to identify the males. All adult males, including the father, were removed from the cage at latest by PHD20 to achieve vocal isolation before the onset of the sensory learning phase. On PHD23 lentiviral injections into the brains of male zebra finches were performed. Animals were put after recovery (30 minutes after waking up) from the micro-surgery, animals were brought back to their home cages. On PHD30 training of the birds with an adult male as tutor started. Tutors and young animals were kept together in sound-isolated recording boxes. Song was recorded during all this period using Sound Analysis Pro [SAP+ (Tchernichovski *et al.*, 2000)]. By day PHD95 or later animals were perfused and their brains dissected for further analysis.

## 2.10 Histology

Animals were perfused with 4% paraformaldehyde (in 1x PBS). Brains were taken out and post fixed overnight in 4% paraformaldehyde. Then the brains were cut sagitally with a vibratome (Leica, Wetzlar, Germany) at a thickness of 40µm. Brain slices were stored in 1x PBS at 4°C in the dark. Acetylcholinesterase (AChE) staining (as Puelles, 2007) was done in every fourth slice to better see Area X. Slices were mounted on Chromallum treated slides and mounted with Mowiol. Birds that were on target in Area X, GFP was detected in Area X, were taken for the song analysis. The resting slices were put into Cryoprotectant and stored at -20°C. I calculated the targeted area using ImageJ and quantifying GFP targeted area as well as Area X area of each bird's hemisphere as in Tramontin *et al.,* 1998.

## 2.11 Immunohistochemistry

Cryostat 14µm thick brain slices were first fixed in 4% PFA in 1x PBS for 5 minutes. After fixation the slices were permeabilized with 3 washes, each 5 minutes, in 0.3% Triton X in PBS. Slices were then blocked with 1x Roti-Immunblock (Carl Roth, Germany) in PBS for 1h at room temperature and then incubated with the first antibody diluted in PBS (for dilutions of antibodies

see Table 2.13.1). Next, the slices were washed 3 times with 0.3% Triton X in PBS followed by incubation with the corresponding fluorescently labelled secondary antibody in PBS. Slices were washed 3 times with PBS and were then stained for DAPI for minute before mounting. After another triple wash, the slices were mounted on slides using MOWIOL mounting medium (Calbiochem, San Diego, USA). For the triple immunohistochemistry against FoxP1, FoxP2 and FoxP4, antibodies were incubated step wise, starting with FoxP2 (using a secondary Alexa 488), then FoxP4 (using a secondary Alexa 568) and at the end FoxP1 (using a biotinylated secondary followed by Streptavidin 647) blocking with 1x Roti-Immunoblock after each secondary. I used 3 animals per age (50, 75 and >100PHD) and two animals (35PHD). All antibodies used are listed in Table 2.13.1 and 2.13.2.

## 2.12 Microscopy

For triple immunohistochemistry and for co-localization of FoxP1 and GFP of the control injected animals I used a Zeiss Axiovert 200M Digital Research Microscopy System equipped with a special light source and controlled by the Slidebook Digital Microscopy software package (Intelligent Imaging Innovations) was used for fluorescence image acquisitions. I took 4 photos at 40x of each animal and quantified manually co-localization of all FoxPs using AxioVision 4.6 program. For statistical analysis of the populations of Area X the lme function of the 'nlme' package in R from 'asbio' package was used (Pinheiro et al. 2009, R Development Core Team 2009).

For *in situ* hybridization (both single and double), for immunohistochemistry detecting up to 3 channels and for assessing GFP on injected animals I used a Zeiss Axiovert S 100 microscope equipped with an AxioCam HRc camera from Zeiss for fluorescence and bright field image acquisitions.

For quantifying the area of Area X I took photos on a Leica Z16 APO macroscope equipped with a Leica DFC 420 C camera using the Leica LAS V3.7 program for image acquisitions. Area of GFP and Area X was measured using ImageJ program.

## 2.13 Antibodies

Primary antibodies listed in the table below were used for immunoblotting (IB), immunofluorescence (IF) and immunoprecipitation (IP).

2.13.1 Table of primary antibodies

| Antigen | Origin / company | Animal | Type | Dilution |
|---|---|---|---|---|
| FoxP1 | Abcam 32010 | mouse | Monoclonal | IB 1:2000. IF 1:2000, IP 1:2000 |
| FoxP2 | Abcam 1307 | goat | Polyclonal | IB 1:2000/4000, IF 1:2000, IP 1:2000 |
| FoxP2 | Abcam 16046 | rabbit | Polyclonal | IB 1:2000/4000, IF 1:2000, IP 1:2000 |
| FoxP4 | Provided by E. Morrisey | rabbit | Polyclonal | IB 1:1000, IF 1:500, IP 1:1000 |
| FLAG | Stratagene 20072 | mouse | Monoclonal | IB 1:2000, IF 1:2000, IP 1:2000 |
| Actin | Sigma A2066 | rabbit | Monoclonal | IB 1:2000 |
| V5 | Invitrogen R96025 | mouse | Monoclonal | IB 1:2000, IF 1:2000, IP 1:2000 |
| IgG | Santa Cruz sc-2027 | rabbit | - | IP 1:200 |
| IgG | Santa Cruz sc-2025 | mouse | - | IP 1:200 |
| Dig-AP | Roche 11093274910 | mouse | Monoclonal | IF 1:2000 |
| Fitc-AP | Roche 11426338910 | mouse | Monoclonal | IF 1:2000 |

2.13.2 Table of secondary antibodies.

| Secondary Antibody | Origin | Conjugated | Dilution |
|---|---|---|---|
| Donkey anti Mouse | Invitrogen | Alexa 488 | IF 1:200 |
| Donkey anti Mouse | Invitrogen | Alexa 568 | IF 1:200 |
| Horse anti Mouse | Vector | Biotinylated | IF 1:200 |
| Streptavidin | Invitrogen | Alexa 647 | IF 1:200 |
| Donkey anti Rabbit | Invitrogen | Alexa 568 | IF 1:200 |
| Donkey anti Goat | Invitrogen | Alexa 488 | IF 1:200 |
| Donkey anti Rabbit | GE Healthcare | HRP | IB 1:2000 |
| Sheep anti goat | GE Healthcare | HRP | IB 1:2000 |
| Sheep anti mouse | GE healthcare | HRP | IB 1:2000 |

## 2.14 Bacterial strains

| Strains | Genotype | Purpose | Source |
|---|---|---|---|
| *E. coli* Top 10 | F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL($Str^R$) endA1 $\lambda^-$ | High – efficiency cloning and plasmid propagation | Invitrogen |
| DH5α | $F^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-$ $m_K^+$), λ– | High – efficiency cloning and plasmid propagation | Invitrogen |

## 2.15 Plasmids

| Plasmid | Description | Tag (position) | Resistance | Source |
|---|---|---|---|---|
| pcDNA4V5/HisB | Eukaryotic expression vector | V5 C terminal | Ampicillin | Invitrogen |
| pcDNA3.1(-) | Eukaryotic expression vector | -- | Ampicillin/ Neomycin | Invitrogen |
| pFUGW_Linker | viral transfer vector contains a ubiquitin C promoter-driven GFP cassette and the human U6 promoter for expression of short hairpin RNA´s | -- | Ampicillin | Custom made based on pFUGW (Lois *et al.*, 2002). |
| pBudΔU6 | Short hairpin expression construct based on pBudCE4.1 from Invitrogen | -- | Zeocin | Custom made |
| pGemT easy | Cloning vector | -- | Ampicillin | Promega |
| pVsVg | Viral vector envelope vector expressing the vesicular stomatitis virus glycoprotein (VSVg) | -- | Ampicillin | Custom made |
| λ8.9 | HIV-1 packaging vector | -- | Ampicillin | Custom made based on pCMVdeltaR9 (Naldini *et al.*, 1996) |
| pGL4.13 | Vector contains the luc2 (from *Photinus pyralis*) reporter gene and the SV40 early enhancer/promoter for use as an expression control or a co-reporter vector | -- | Ampicillin | Promega |
| pGL4.75 | Vector contains the hRluc (from *Renilla reniformis*) | -- | Ampicillin | Promega |

| | | | | |
|---|---|---|---|---|
| | reporter gene and a CMV immediate-early enhancer/promoter and can be used as an expression control or a co-reporter vector. | | | |

## 2.16 Constructs

| Name of construct | Vector | Restriction sites | Amino acids | Mutations/deletions | Tag at C terminal |
|---|---|---|---|---|---|
| nFoxP1 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| FoxP1-FLAG | pcDNA3.1(-) | *BamHI / EcoRI* | 1-683 | -- | FLAG |
| FoxP1-V5 | pcDNA4 | *BamHI / EcoRI* | 1-683 | -- | V5 |
| shFoxP1_1 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP1_2 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxp1_3 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP1_1 | pFUGW_linker | *NheI/BstBI* | -- | -- | -- |
| shFoxP1_2 | pFUGW_linker | *NheI/BstBI* | -- | -- | -- |
| shFoxp1_3 | pFUGW_linker | *NheI/BstBI* | -- | -- | -- |
| shControl | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shControl | pFUGW_linker | *NheI/BstBI* | -- | -- | -- |
| nFoxP2 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| nsFoxP2 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| cFoxP2 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| FoxP2-FLAG | pcDNA3.1(-) | *BamHI / EcoRI* | 1-711 | -- | FLAG |
| FoxP2-V5 | pcDNA4 | *BamHI / EcoRI* | 1-711 | -- | V5 |
| R553H-FLAG | pcDNA3.1(-) | *BamHI / EcoRI* | 1-711 | R549H Zebra finch version of the R553H mutation | FLAG |
| R553H-V5 | pcDNA4 | *BamHI / EcoRI* | 1-711 | R549H Zebra finch version of the R553H mutation | V5 |
| ΔFoxBox- | pcDNA3.1(-) | *BamHI /* | 1-630 | Δ aa 500-580 | FLAG |

| | | | | | |
|---|---|---|---|---|---|
| FLAG | | *EcoRI* | | | |
| ΔFoxBox-V5 | pcDNA4 | *BamHI / EcoRI* | 1-630 | Δ aa 500-580 | V5 |
| FoxP4 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| bFoxP4 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| cFoxP4 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| FoxP4 full | pGem T easy | pGEM T easy MCS | 1-668 | -- | -- |
| FoxP4-FLAG | pcDNA3.1(-) | *BamHI / EcoRI* | 1-668 | -- | FLAG |
| FoxP4-V5 | pcDNA4 | *BamHI / EcoRI* | 1-668 | -- | V5 |
| shFoxP4_2 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_5 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_7 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_8 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_10 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_16 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_18 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_19 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_20 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_21 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_22 | pBudΔU6 | *Bsp119I / BstBI* | -- | -- | -- |
| shFoxP4_7 | pFUGW_linker | *NheI/BstBI* | -- | -- | -- |
| shFoxP4_19 | pFUGW_linker | *NheI/BstBI* | -- | -- | -- |
| EM_qPCR FoxP1_5 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| GFP_EM_1 | pGem T easy | pGEM T easy MCS | -- | -- | -- |
| EM_qPCR FoxP4_3 | pGem T easy | pGEM T easy MCS | -- | -- | -- |

## 2.17 Primers

| Name | Sequence 5´to 3´ | Annealing | Construct used for |
|---|---|---|---|
| nFoxP1for | AACATACAGACCAGCCACACC | 60°C | C terminal FoxP1 probe |
| nFOXP1.2 rev | TGTGGTTGGCTGTTGTCACT | 60°C | C terminal FoxP1 probe |
| Mb25_for [2] | CGCGGATCCGCCACCATGATGCAAGAATCTGGG | 60°C | FLAG / V5 FoxP1 over-expression |
| Mb26_rev [2] | GCGGAATTCCTACTTATCGTCGTCATCCTTGTAATC TTCTATGTCCTC | 60°C | FLAG FoxP1 over-expression |
| eFoxP1V5tag _rev | GCGGAATTCCTACGTAGAATCGAGACCGAGGAGA GGGTTAGGGATAGGCTTACCTTCTATGTCCTCATTT ACAGGTTC | 60°C | V5 FoxP1 over-expression |
| qRT_FoxP1_ 3_for | CGTTAAAGGGGCAGTATGGA | 60°C | qRT-PCR FoxP1_5 |
| qRT_FoxP1_ 1_rev | GCCATTGAAGCCTGTAAAGC | 60°C | qRT-PCR FoxP1_5 |
| cFOXP2_for | CCACCAGCTTAGGCTATGGA | 60°C | C terminal FoxP2 probe |
| kaz-2 [3] | TCATTCCAGATCTTCAGATAAAG | 60°C | C terminal FoxP2 probe |
| ig-2 [4] | ATGATGACTCCCCAGGTGATC | 60°C | n/ns FoxP2 probe |
| nsfoxp2rev | GCTGCTCTTTTGCTTGCTTT | 60°C | ns FoxP2 probe |
| FOXP2 R2 [5] | TCAGCAGGACTTAAGCCAGCT | 60°C | n FoxP2 probe |
| Ig3 [4] | CGCGGATCCGCCACCATGATGCAGGAATCTGCGAC AG | 55°C | FLAG/V5 FoxP2, R553H and ΔFoxBox over expression |
| eFoxP2FLAG _rev | GCGGAATTCCTACTTATCGTCGTCATCCTTGTAATC TTCCAGATCTTCAGATAAAGGCTC | 55°C | FLAG FoxP2, R553H and ΔFoxBox over expression |
| fFOXP4_for | ATGATGTCCCCGCAGATG | 60°C | FoxP4/ bFoxP4 probe |
| fFoxP4_rev | GCGTTACCTGTTGCAGCATT | 60°C | FoxP4 probe |
| E_FOXP_4_3 rev | TTTGACAAACTGCCCCAAGT | 60°C | bFoxP4 probe |
| cFoxp4 for | ATTTCAGGACTCGGTTACGG | 60°C | cFoxP4 probe |
| cFoxp4 rev | GCAGCTCCTCCTCCAGGT | 60°C | cFoxP4 probe |
| sFOXP4oe1 | GGATCCGCCACCATGATGGTTGAATCCGCCTCG | 55°C | V5/FLAG FoxP4 over expression |
| eFOXP4FLA G_rev | GCGGAATTCCTACTTATCGTCGTCATCCTTGTAATC GGACAAGTCTTCCACCGGCAGCTC | 55°C | FLAG FoxP4 over expression |
| eFOXP4h1rev | GAATTCGGACAAGTCTTCCACCGGCAGCTC | 55°C | V5 FoxP4 over expression |
| qRTPCR_Fox P4_7f | TGACAGGGAGTCCCACCTTA | 65°C | qRT-PCR FoxP4_3 |
| qRTPCR_Fox P4_7r | AGCTGGTGTTGATCATGGTG | 65°C | qRT-PCR FoxP4_3 |

| | | | |
|---|---|---|---|
| Zf_sFOXP4_for | TGCTAGCGAGTCATGATGGT | 65°C | Cloning of full FoxP4 |
| Zf_eFOXP4b_rev | TTAGGACAAGTCTTCCACCGG | 60°C | Cloning of full FoxP4 |
| actin-SH2-for [6] | CGAGCGCAAGTACTCCGTGT | 60°C | DNA contamination in mRNA |
| actin-SH2-rev [6] | GCCGGACTCGTCGTACTCCT | 60°C | DNA contamination in mRNA |
| Seq pFUGW-f [6] | GGTACAGTGCAGGGGAAAGA | 55°C | Sequencing sh in pFUGW |
| Seq pFUGW-r [6] | GTCCTGATCCTTCCGCCC | 55°C | Sequencing sh in pFUGW |
| Hmbs-SH2-for [6] | GCAGCATGTTGGCATCACAG | 64°C | qRT-PCR |
| Hmbs-SH2-rev [6] | TGCTTTGCTCCCTTGCTCAG | 64°C | qRT-PCR |
| EM_GFP_RT_1f | AGAACGGCATCAAGGTGAAC | 65°C | qRT-PCR |
| EM_GFP_RT_1r | TGCTCAGGTAGTGGTTGTCG | 65°C | qRT-PCR |

[1] Design by Nshdejan Arpik
[2] Design by Martin Begemann
[3] Design by Kazuhiro Wada
[4] Design by Ingrid Ghattas
[5] Design by Jana Petri
[6] Design by Sebastian Haesler

## 2.18 Short hairpin target sequences for FoxP1

The next table shows all target sequences resulting of the search for possible shRNA sites in the zebra finch FoxP4 sequence using the short hairpin design tool of Ambion and MWG.

2.18.1 Table of targeting sequences for short hairpins against FoxP1

| shRNA | Target sequence in FoxP4 5´→3´ | FoxP2 homology | FoxP4 homology | Offset* |
|---|---|---|---|---|
| shFoxp1-1 | AACAGTATACCTCTATAC | 61%<br>8 differences | 61%<br>8 differences | 1756 |
| shFoxP1-2 | TGCATGTCAAAGAAGAAC | 88%<br>2 differences | 72%<br>5 differences | 1916 |
| shFoxP1-3 [1] | CCATTAGACCCAGATGAAA | 63%<br>9 differences | 57%<br>10 differences | 1932 |
| shFoxP1-4 [1] | AAGACCTCCTTAATCATCAAC | 66,6%<br>12 differences | 66,6%<br>11 differences | 816 |
| shFoxP1-5 [1] | AAAGAGCGCCTGCAAGCCATG | 71%<br>7 differences | 76%<br>5 differences | 1095 |
| shFoxP1-6 [1] | AAGATCAGTGGTAACCCTTCT | 61% | 57% | 1659 |

| | | | | |
|---|---|---|---|---|
| | | 8 differences | 9 differences | |
| shFoxP1-7 [1] | AAACATACAGACCAGCCACAC | 66% 7 differences | 61% 12 differences | 1688 |
| shFoxP1-8 [1] | AAATCCCACTCTGGGCAATTT | 57% 13 differences | 61% 13 differences | 1790 |
| shFoxP1-9 [1] | AATGGAGCATACGAACAGTAA | 66% 7 differences | 66% 7 differences | 1844 |
| shFoxP1-10 | TGAAGGCCCACTATCCTTA | 63% 9 differences | 63% 10 differences | 1953 |
| shFoxP1-11 | AGAAGAACCATTAGACCCA | 63% 7 differences | 63% 10 differences | 1926 |
| shFoxP1-12 | TGGAGCATACGAACAGTAA | 73% 5 differences | 73% 5 differences | 1847 |
| Control [2] | No targeting sequence | -- | -- | -- |

[1] Design by Nshdejan Arpik
[2] Design by Sebastian Haesler.

## 2.19 Short hairpin target sequences for FoxP4

The next table shows all target sequences resulting of the search for possible shRNA sites in the zebra finch FoxP4 sequence using the short hairpin design tool of Ambion and MWG.

2.19.1 Table of targeting sequences for short hairpins against FoxP4

| shRNA | Target sequence in FoxP4 5´→3´ | FoxP1 homology | FoxP2 homology | Offset* |
|---|---|---|---|---|
| FoxP4_EM_1 [1] | AAAGATGACAGGGAGTCCCAC | 57% 8 differences | 80% 4 differences | 1616 |
| FoxP4_EM_2 [1] | AAATATGATTTCAGGACTCGG | 23% 11 differences | 23% 11 differences | 1646 |
| FoxP4_EM_3 [1] | AATGCAAGTTACCAGGCTGCG | 66% 6 differences | 80% 3 differences | 1680 |
| FoxP4_EM_4 [1] | AACAGCAACGGCAGCAACAGC | 80% 4 differences | 61% 7 differences | 1815 |
| FoxP4_EM_5 [1] | AAGAGCACTTCGGACACGTTT | 66% 7 differences | 61% 8 differences | 1155 |
| FoxP4_EM_6 [1] | AAGTTCTGCACCCCCATCTCT | 76% 6 differences | 61% 8 differences | 1302 |
| FoxP4_EM_7 [1] | AACCAGAATGTGACGATCCCC | 42% 13 differences | 23% 13 differences | 1938 |
| FoxP4_EM_8 [2] | GCACTTAATGCAAGTTACC | 42% 8 differences | 73% 4 differences | 1674 |
| FoxP4_EM_9 [2] | ATGCAAGTTACCAGGCTGC | 68% 5 differences | 84% 2 differences | 1681 |
| FoxP4_EM_10 [2] | GCCCCACCATGATCAACAC | 26% 10 | 36% 9 differences | 1732 |

| | | differences | | |
|---|---|---|---|---|
| FoxP4_EM_11 [1] | GAGCAGCTTCCCCCTGCTCAG | 42%<br>9 differences | 76%<br>5 differences | 1709 |
| FoxP4_EM_12 [1] | AAGTTACCAGGCTGCGCTGGC | 61%<br>8 differences | 76%<br>5 differences | 1685 |
| FoxP4_EM_13 [1] | AACACCAGCTCGGCCAGCGCC | 28%<br>12<br>differences | 28%<br>13<br>differences | 1746 |
| FoxP4_EM_14 [1] | AACGGCAGCAACAGCCCCCGC | 28%<br>14<br>differences | 38%<br>13<br>differences | 1821 |
| FoxP4_EM_15 [1] | AAGGAGGAGCCGGCCGAGGCG | 38%<br>9 differences | 42%<br>8 differences | 1878 |
| FoxP4_EM_16 [1] | AATGTGACGATCCCCGACGAC | 23%<br>14<br>differences | 23%<br>14<br>differences | 1944 |
| FoxP4_EM_17 [2] | TATGATTTCAGGACTCGGT | 19%<br>13<br>differences | 19%<br>12<br>differences | 1649 |
| FoxP4_EM_18 [2] | GAATGTGACGATCCCCGAC | 42%<br>15<br>differences | 26%<br>13<br>differences | 1943 |
| FoxP4_EM_19 [2] | CCCGTGCACGTGAAGGAGGAG | 57%<br>7 differences | 71%<br>8 differences | 1866 |
| FoxP4_EM_20 [2] | GCTTGCACAGAATCACGAG | 68%<br>6 differences | 57%<br>9 differences | 1328 |
| FoxP4_EM_21 [2] | GGAGGAGCTCGGAGAAGTT | 73%<br>6 differences | 63%<br>9 differences | 1288 |
| FoxP4_EM_22 [2] | GTTCTGCACCCCCATCTCT | 52%<br>5 differences | 42%<br>8 differences | 1304 |

[1] Designed with the Ambion tool
[2] Designed with the MWG tool
* This is the distance from start ATG of FoxP4 sequence in bp.

## 2.20 ssDNA sequences encoding shRNA for FoxP1

The next table shows the oligos for the short hairpins that were selected and tested for their down regulation activity against FoxP1 *in vitro*. Those were selected because they had the most differences if compared to FoxP2 and FoxP4 nucleotide sequences. Cross reaction against FoxP2 and FoxP4 genes was also tested *in vitro*, to be sure that the short hairpin was specific for FoxP1 of the zebra finch. The sequence of the control short hairpin was designed from *Thermotoga maritimia* and has no sequence matches in Zebra Finches.

2.20.1 Table of DNA oligos of short hairpins against FoxP1 and control that were tested.

| Name | Sequence (5´ to 3´, target sequence in FoxP1 shown in purple) |
|---|---|
| shFoxP1-1for [1] | TTTGAACAGTATACCTCTATACgtgaagccacagatgGTATAGAGGTATACTGTTCTTTTT |
| shFoxP1-1rev [1] | CGAAAAAGAACAGTATACCTCTATACcatctgtggcttcacGTATAGAGGTATACTGTT |
| shFoxP1-2for [1] | TTTGTGCATGTCAAAGAAGAACgtgaagccacagatgGTTCTTCTTTGACATGCACTTTTT |
| shFoxP1-2rev [1] | CGAAAAAGTGCATGTCAAAGAAGAACcatctgtggcttcacGTTCTTCTTTGACATGCA |
| shFoxP1-3for [1] | TTTGCCATTAGACCCAGATGAAAgtgaagccacagatgTTTCATCTGGGTCTAATGGTTTTT |

| | |
|---|---|
| shFoxP1-3rev [1] | CGAAAAACCATTAGACCCAGATGAAAcatctgtggcttcacTTTCATCTGGGTCTAATGG |
| shFoxP1-4for [1] | TTTGGACCTCCTTAATCATCAACgtgaagccacagatgGTTGATGATTAAGGAGGTCTTTTT |
| shFoxP1-4rev [1] | CGAAAAAGACCTCCTTAATCATCAACcatctgtggcttcacGTTGATGATTAAGGAGGTC |
| shFoxP1-5for [1] | TTTGAGAGCGCCTGCAAGCCATGgtgaagccacagatgCATGGCTTGCAGGCGCTCTTTTTT |
| shFoxP1-5rev [1] | CGAAAAAAGAGCGCCTGCAAGCCATGcatctgtggcttcacCATGGCTTGCAGGCGCTCT |
| shFoxP1-6for [1] | TTTGGATCAGTGGTAACCCTTCTgtgaagccacagatgAGAAGGGTTACCACTGATCTTTTT |
| shFoxP1-6rev [1] | CGAAAAAGATCAGTGGTAACCCTTCTcatctgtggcttcacAGAAGGGTTACCACTGATC |
| shFoxP1-7for [1] | TTTGACATACAGACCAGCCACACgtgaagccacagatgGTGTGGCTGGTCTGTATGTTTTTT |
| shFoxP1-7rev [1] | CGAAAAAACATACAGACCAGCCACACcatctgtggcttcacGTGTGGCTGGTCTGTATGT |
| shFoxP1-8for [1] | TTTGATCCCACTCTGGGCAATTTgtgaagccacagatgAAATTGCCCAGAGTGGGATTTTTT |
| shFoxP1-8rev [1] | CGAAAAAATCCCACTCTGGGCAATTTcatctgtggcttcacAAATTGCCCAGAGTGGGAT |
| shFoxP1-9for [1] | TTTGTGGAGCATACGAACAGTAAgtgaagccacagatgTTACTGTTCGTATGCTCCATTTTT |
| shFoxP1-9rev [1] | CGAAAAATGGAGCATACGAACAGTAAcatctgtggcttcacTTACTGTTCGTATGCTCCA |
| shFoxP1-10for [1] | TTTGTGAAGGCCCACTATCCTTAgtgaagccacagatgTAAGGATAGTGGGCCTTCATTTTT |
| shFoxP1-10rev [1] | CGAAAAATGAAGGCCCACTATCCTTAcatctgtggcttcacTAAGGATAGTGGGCCTTCA |
| shFoxP1-11for [1] | TTTGAGAAGAACCATTAGACCCAgtgaagccacagatgTGGGTCTAATGGTTCTTCTTTTTT |
| shFoxP1-11rev [1] | CGAAAAAAGAAGAACCATTAGACCCAcatctgtggcttcacTGGGTCTAATGGTTCTTCT |
| shFoxP1-12for [1] | TTTGTGGAGCATACGAACAGTAAgtgaagccacagatgTTACTGTTCGTATGCTCCATTTTT |
| shFoxP1-12rev [1] | CGAAAAATGGAGCATACGAACAGTAAcatctgtggcttcacTTACTGTTCGTATGCTCCA |
| shcontrol_for [2] | TTTGTTCTCCGAACGTGTCACGTgtgaagccacagatgACGTGACACGTTCGGAGAATTTTT |
| shcontrol_rev [2] | CGAAAAATTCTCCGAACGTGTCACGTcatctgtggcttcacACGTGACACGTTCGGAGAA |

[1] Designed by Nshdejan Arpik
[2] Designed by Sebastian Haesler.

## 2.21 ssDNA sequences encoding shRNA for FoxP4

The next short hairpins were selected following the same criterion as for FoxP1short hairpins. Cross reaction against FoxP1 and FoxP2 genes was also tested *in vitro*, to be sure that the short hairpin was specific for FoxP4 of the zebra finch.

2.21.1 Table of DNA oligos of short hairpins against FoxP4 that were tested.

| Name | Sequence (5´ to 3´, target sequence in FoxP4 shown in purple) |
|---|---|
| shFoxP4_2for | TTTGATATGATTTCAGGACTCGGgtgaagccacagatgCCGAGTCCTGAAATCATATTTTTT |
| shFoxP4_2rev | CGAAAAAATATGATTTCAGGACTCGGcatctgtggcttcacCCGAGTCCTGAAATCATAT |
| shFoxP4_5for | TTTGGAGCACTTCGGACACGTTTgtgaagccacagatgAAACGTGTCCGAAGTGCTCTTTTT |
| shFoxP4_5rev | CGAAAAAGAGCACTTCGGACACGTTTcatctgtggcttcacAAACGTGTCCGAAGTGCTC |
| shFoxP4_7for | TTTGCCAGAATGTGACGATCCCCgtgaagccacagatgGGGGATCGTCACATTCTGGTTTTT |
| shFoxP4_7rev | CGAAAAACCAGAATGTGACGATCCCCcatctgtggcttcacGGGGATCGTCACATTCTGG |
| shFoxP4_8for | TTTGGCACTTAATGCAAGTTACCgtgaagccacagatgGGTAACTTGCATTAAGTGCTTTTT |
| shFoxP4_8rev | CGAAAAAGCACTTAATGCAAGTTACCcatctgtggcttcacGGTAACTTGCATTAAGTGC |
| shFoxP4_10for | TTTGGCCCCACCATGATCAACACgtgaagccacagatgGTGTTGATCATGGTGGGGCTTTTT |
| shFoxP4_10rev | CGAAAAAGCCCCACCATGATCAACACcatctgtggcttcacGTGTTGATCATGGTGGGGC |
| shFoxP4_16for | TTTGTGTGACGATCCCCGACGACgtgaagccacagatgGTCGTCGGGGATCGTCACATTTTT |
| shFoxP4_16rev | CGAAAAATGTGACGATCCCCGACGACcatctgtggcttcacGTCGTCGGGGATCGTCACA |
| shFoxP4_18for | TTTGGAATGTGACGATCCCCGACgtgaagccacagatgGTCGGGGATCGTCACATTCTTTTT |
| shFoxP4_18rev | CGAAAAAGAATGTGACGATCCCCGACcatctgtggcttcacGTCGGGGATCGTCACATTC |
| shFoxP4_19for | TTTGCGTGCACGTGAAGGAGGAGgtgaagccacagatgCTCCTCCTTCACGTGCACGTTTTT |
| shFoxP4_19rev | CGAAAAACGTGCACGTGAAGGAGGAGcatctgtggcttcacCTCCTCCTTCACGTGCACG |
| shFoxP4_20for | TTTGGCTTGCACAGAATCACGAGgtgaagccacagatgCTCGTGATTCTGTGCAAGCTTTTT |
| shFoxP4_20rev | CGAAAAAGCTTGCACAGAATCACGAGcatctgtggcttcacCTCGTGATTCTGTGCAAGC |
| shFoxP4_21for | TTTGGGAGGAGCTCGGAGAAGTTgtgaagccacagatgAACTTCTCCGAGCTCCTCCTTTTT |

| | |
|---|---|
| shFoxP4_21rev | CGAAAAAGGAGGAGCTCGGAGAAGTTcatctgtggcttcacAACTTCTCCGAGCTCCTCC |
| shFoxP4_22for | TTTGGTTCTGCACCCCCATCTCTgtgaagccacagatgAGAGATGGGGGTGCAGAACTTTTT |
| siFoxP4_22rev | CGAAAAAGTTCTGCACCCCCATCTCTcatctgtggcttcacAGAGATGGGGGTGCAGAAC |

## 2.22 Others

### 2.22.1 Mammalian cell lines

| | | |
|---|---|---|
| HeLa: | Human cervix carcinoma cells | - epithelial- like cells |
| HEK293-T: | Human embryonal kidney cells | - fibroblast |

### 2.22.2 Software and internet resources

| | |
|---|---|
| ExPASy (Expert Protein Analysis System | - www.expasy.org |
| | - alignments, protein parameters |
| | - DNA- protein translation tool |
| NCBI (Ntl. Center for Biotech. Information) | - www.ncbi.nlm.nih.gov |
| | - BLAST, literature |
| | - ORF finder |
| | - DNA, mRNA and protein sequences |
| | - Blast against different genomes |
| | - Primer design |
| PRISM ® Software (GraphPad Software) | - statistical analysis, bar diagrams |
| UCSC | - http://genome.ucsc.edu/ |
| | - Blat against different species |
| | - GC rich regions |
| | - TSS |
| Allen Brain Atlas (Allen Institute of Brain research) | - www.brain-map.org |
| | - expression patterns of different genes |
| CLUSTALW (European Bioinformatics Institute) | - www.ebi.ac.uk/Tools/msa/clustalw2/ |

| | |
|---|---|
| | - align of DNA and protein |
| LGC genomics | - www.agowa.de |
| | - sequencing of plasmids and PCR |
| Primer3 | - http://frodo.wi.mit.edu/primer3/ |
| | - primer designing tool |
| MWG | - www.eurofinsdna.com |
| | - Primer ordering |
| | - short hairpin designing tool |
| Ambion | - www.**ambion**.com |
| | - short hairpin designing tool |
| Reverse complement | - www.bioinformatics.org/sms/rev_comp.html |
| | - Reverse complement of sequences |
| MacMaster Biophotonics Facility | - www.macbiophotonics.ca/imagej/ |
| | - download of ImageJ and plug ins |
| Pfam (Welcome Trust Sanger Institute) | - http://pfam.sanger.ac.uk/ |
| | - prediction of protein domains in amino acid sequences |
| The DINAMelt web server (The RNA Institute) | - www.bioinfo.rpi.edu/applications/mfold |
| | - quick-fold tool for the prediction of the 3D short hairpin structure |
| ProP 1.0 server | www.cbs.dtu.dk/services/ProP/ |
| | - prediction of pro protein cleavage sites |

# 3. Results

## 3.1 Expression pattern of FoxP1, FoxP2 and FoxP4 in the zebra finch

### 3.1.1 Cloning of FoxP1, FoxP2 and FoxP4 genes

The entire *FoxP4* sequence from mRNA of an adult brain male zebra finch was cloned (Taeniopygia guttata) (Accession number NCBI JN160732). Full sequences of *FoxP1* (AY54952) and *FoxP2* (AY549148, AY549149, AY549150, and AY549151) were already cloned by Haesler *et al.*, 2004. The entire Open Reading Frame (ORF) of *FoxP4* is 2007 bp long and 668 amino acids (AA) long, which is shorter than *FoxP2* with 2136 bp/711 AA and *FoxP1* with 2049 bp/683 AA long. The fact that *FoxP4* was expressed in adult zebra finch brain so it could be cloned suggests that *Foxp4* expression persists into adulthood in zebra finches, whereas Takahashi *et al.*, 2008 showed a decrease in the adult rat brain.

### 3.1.2 Comparisons of FoxP1, FoxP2 and FoxP4 sequences

Table 3.1 shows the overall very high similarity of FoxP1, 2 and 4 (between 67% and 70% for pair wise comparisons between any two members. Comparison of the zebra finch FoxP4 to the human and mice nucleotide sequences revealed high conservation (79% identical to human, 78% to mouse).

**Table 3. 1 Comparison of FoxP1, FoxP2 and FoxP4 zebra finch and FoxP4 Homo and Mus nucleotide sequences**

| | Full FoxP1 Taeniopygia | Full FoxP2 Taeniopygia | Full FoxP4 Taeniopygia | Full FoxP4 Homo | Full FoxP4 Mus |
|---|---|---|---|---|---|
| Full FoxP1 Taeniopygia | 100 | 70 | 68 | 64 | 64 |
| Full FoxP2 Taeniopygia | 70 | 100 | 67 | 65 | 64 |
| Full FoxP4 Taeniopygia | 68 | 67 | 100 | 79 | 78 |
| Full FoxP4 Homo | 64 | 65 | 79 | 100 | 88 |
| Full FoxP4 Mus | 64 | 64 | 78 | 88 | 100 |

Aligning the FoxP4 amino acid sequence to FoxP2 and FoxP1 showed the following conserved domains, starting from the N terminal to the C terminal part of the protein (Figure 3.1): First FoxP2 and FoxP1 have a putative Pro-protein convertase cleavage site starting at amino acid 32 (RXXR) (Figure 3.1) (de Zoeten *et al.*, 2009). The function of this cleavage

site is not known, but in FoxP4 it is absent. In zebra finches, FoxP2 has a polyglutamine tract, starting at amino acid 152, absent in FoxP1 and FoxP4. Interestingly, FoxP1 in humans has an FoxP1 isoform that has a polyglutamine tract too (Haesler *et al.*, 2004; Wang *et al.*, 2003). The zinc finger domain, starting at amino acid 338 of the FoxP2 sequence, exists in all three members. This motif is involved in dimerization (Wang *et al.*, 2003) and can bind in principle to DNA as well (Banham *et al.*, 2001), which has not been shown however for any FoxP member so far. A leucine zipper starts at amino acid 382 of the FoxP2 sequence and is also present in all members. This motif is involved in homo- and hetero-dimerization of FoxP members (Shanru Li *et al.*, 2004; Wang *et al.*, 2003). A Co-repressor C-terminal Binding protein 1 motif starts at amino acid 414 of the FoxP2 sequence, which is also present in all three P family members in zebra finches. This is different in mice and humans were FoxP4 lost the CtBP-1 binding site. It might be that zebra finch FoxP4 may have a functional CtBP-1 domain judging by the amino acid sequence and the conservation of the core sequence (Shanru Li *et al.*, 2004). The Forkhead box starting at amino acid 500 of the FoxP2 sequence is the most conserved part of the protein and is present in all three members. Mutation in this domain compromise DNA binding and isoforms lacking this domain do not bind to DNA. In addition, the Forkhead domain contains a nuclear localization signal and mutation of the FoxP box compromise nuclear localization in vitro (Vernes *et al.*, 2006). Engrailed-Homology 1 (EH1) motif, in the Forkhead box of FoxP1, that starts at amino acid 538 (Fetterman *et al.*, 2008). This motif could be used by transducin-like enhancer of split (TLE) proteins that would act as co repressors of FoxP1 (Chen and Courey, 2000) a further way of regulating these proteins.

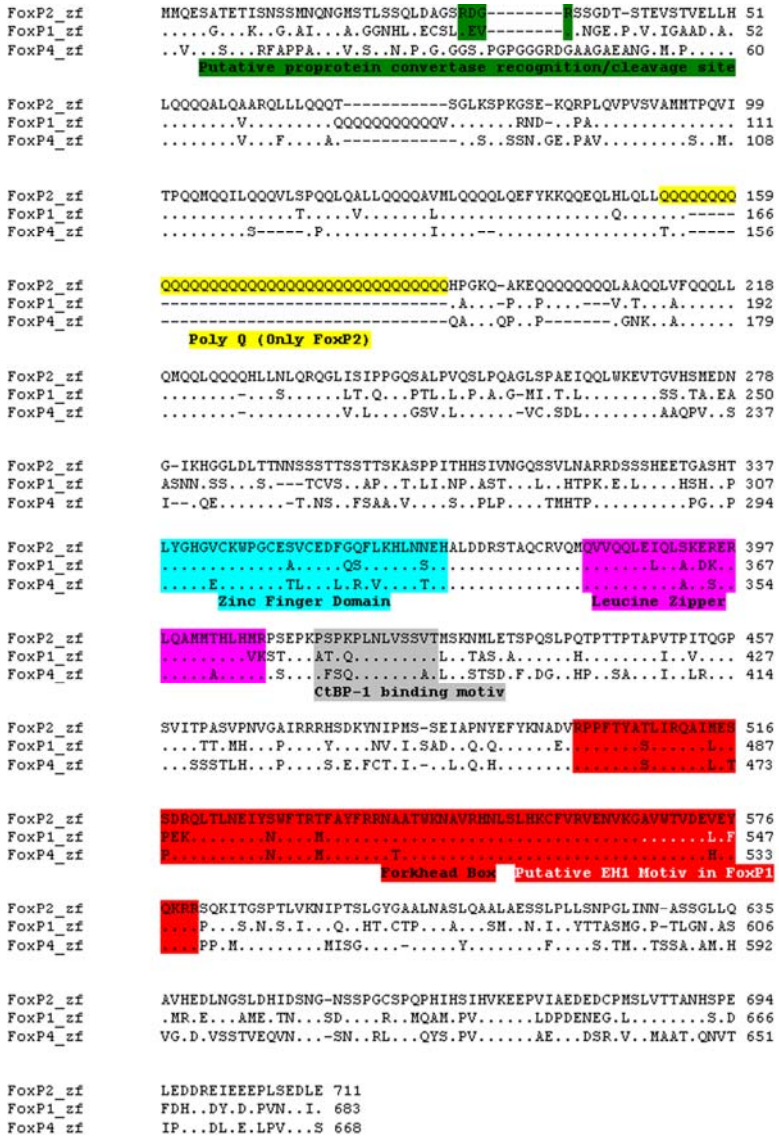



**Figure 3. 1 Alignment of the amino-acid sequences of zebra finch FoxP1, FoxP2 and FoxP4 proteins. The different domains are colour coded. Starting from the N-terminal to the C-terminal: In green a Pro-protein Convertase cleavage site found in FoxP2 and FoxP1; yellow the poly-glutamine tract, only found in FoxP2; Blue the zinc finger domain found in all FoxP members studied; Magenta the leucine finger, also found in all FoxP members studied; in Gray the co-repressor C- terminal Binding protein, also found in all FoxP members studied; in Red the Forkhead Box, also found in all FoxP members studied; in white letters in the Forkhead box an putative Engrailed Homology 1 (EH1) motif found only in FoxP1.**

At the protein level FoxP4 shares more sequence similarity with FoxP2 (67%) than with FoxP1 (58%) (Table 3.2). The similarity between domains is high and ranges from 73-100%.

**Table 3. 2 Comparison of Foxp1, FoxP2 and FoxP4 whole protein sequences, and comparisons of the different domain protein sequences.**

| | Ful FoxP1 Taeniopygia protein | Ful FoxP2 Taeniopygia protein | Ful FoxP4 Taeniopygia protein |
|---|---|---|---|
| **Ful FoxP1 Taeniopygia protein** | 100 | 67* | 58 |
| **Ful FoxP2 Taeniopygia protein** | 67* | 100 | 67 |
| **Ful FoxP4 Taeniopygia protein** | 58 | 67 | 100 |
| **FoxP1 zinc finger** | 100 | 86* | 73 |
| **FoxP2 zinc finger** | 86* | 100 | 80 |
| **FoxP4 zinc finger** | 73 | 80 | 100 |
| **FoxP1 Leucine zipper** | 100 | 77 | 77 |
| **FoxP2 Leucine zipper** | 77 | 100 | 88* |
| **FoxP4 Leucine zipper** | 77 | 88* | 100 |
| **FoxP1 Forkhead box** | 100 | 88 | 92* |
| **FoxP2 Forkhead box** | 88 | 100 | 90 |
| **FoxP4 Forkhead box** | 92* | 90 | 100 |
| **FoxP1 CtBP1 domain** | 100 | 80* | 73 |
| **FoxP2 CtBP1 domain** | 80* | 100 | 73 |
| **FoxP4 CtBP1 domain (1)** | 73 | 73 | 100 |

(1) Sequence seems to have a completely functional binding domain, unlike mouse FoxP4

* Highest similarity

### *3.1.3 Expression of FoxP1, FoxP2 and FoxP4 genes in the adult zebra finch brain*

To assess the expression pattern of *FoxP4* and compare it to the published FoxP1 and FoxP2 expression patterns in the zebra finch song system and particularly Area X (Haesler *et al.*, 2004; Teramitsu *et al.*, 2004), we designed various probes in different regions of all *FoxP* genes (accession numbers are in Table 3.3). To avoid cross hybridizations of probes for similar genes, probes should have less than 85% similarity with the sequence of other genes (Kubikova *et al.*, 2009). The probes used in this thesis had similarities well below this threshold (Table 3.3). The probe that had the highest similarities was the nsFoxP2 probe with 81% similarities to the *FoxP1* sequence. I did not see differences in the pattern of expression for the different probes, but in the case of the *FoxP2* probes, the *nsFoxP2* and *nFoxP2* tended to give stronger signals than the *cFoxP2* (data not shown).

**Table 3. 3 Comparison of the different *FoxP* probes used to the full nucleotide sequences of the other FoxP sub family members.**

| | Accesion number | Full FoxP1 Taeniopygia | Full FoxP2 Taeniopygia | Full FoxP4 Taeniopygia |
|---|---|---|---|---|
| **nFoxp1 (probe)** | JN800730 | 100 | 66 | 49 |
| **cFoxP2 (probe)** | JN800731 | 64 | 100 | 55 |
| **nsFoxP2 (probe)** | JN800735 | 81 | 100 | 65 |
| **nFoxP2 (probe)** | JN800734 | 69 | 100 | 54 |
| **FoxP4 (probe)** | JN800732 | 80 | 80 | 100 |
| **bFoxP4 (probe)** | JN800733 | 80 | 72 | 100 |
| **cFoxP4 (probe)** | JN800729 | 43 | 55 | 100 |

To assess the pattern of expression of these three *FoxP* genes, *in situ* hybridization with different probes were performed on adjacent sagittal sections through the brain of an adult zebra finch that had not sung prior to sacrifice. The specific pattern of expression of *FoxP1*, *FoxP2* and *FoxP4* in the zebra finch brain is shown in Figure 3.2.

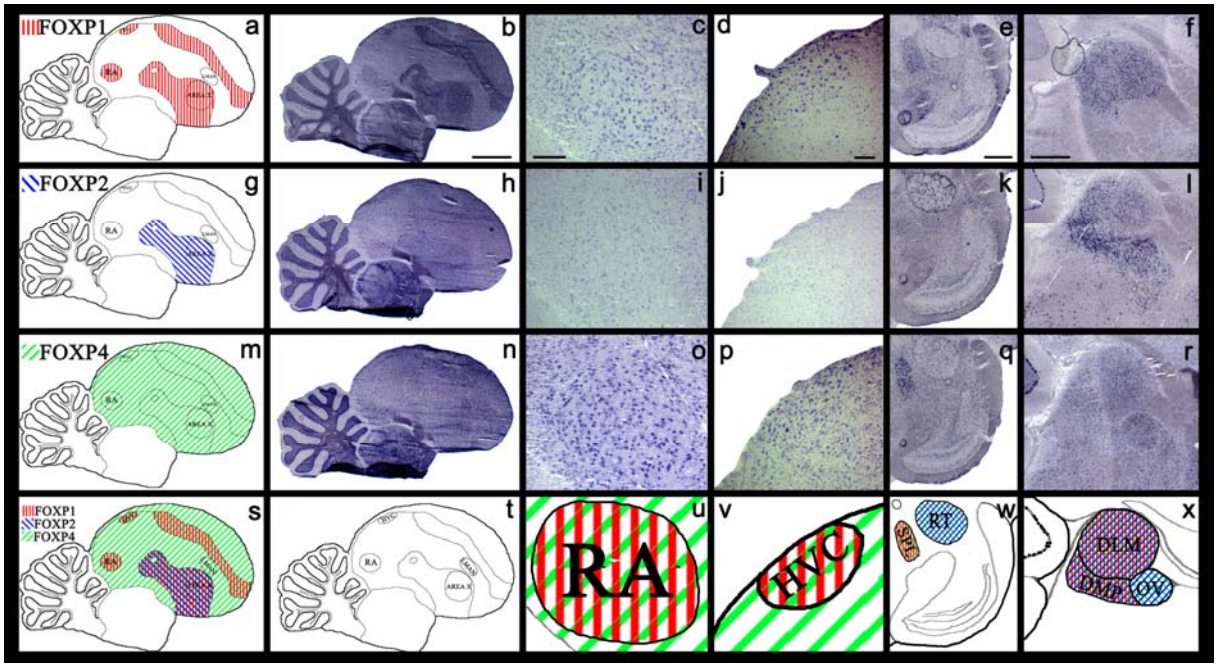



**Figure 3. 2 Representative bright-field photos and schematic summaries of in situ hybridizations against FoxP1, FoxP2 and FoxP4 with DIG-labelled Riboprobes on 12µm sagittal adjacent sections of male adult (>100 PHD) zebra finch. Schematic figures summarize the different patterns of expression of FoxP1 "a", FoxP2 "g" and FoxP4 "m" and all three genes "s" in the telencephalon, shown in b, h, n; schematic figure of whole zebra finch brain "t"; schematic figures of the overlapping regions in RA "u", HVC "v", optic tectum "w" and thalamus "x". Corresponding photomicrographs for the summaries depicted in ("u", "v", "w" and "x") are shown in the columns above for each gene (FoxP1 first row, FoxP2 second row, FoxP4 third row). Each area is characterized by particular combinations of FoxP1, 2, 4 expression (see also Table 3.4) nucleus spiriformis lateralis (SPL), nucleus rotundus (RT) nucleus dorsolateralis anterior thalami, pars medialis (DLM) Nucleus dorsomedialis posterior thalami (DMP) Scale bar in b = 2mm; c = 500µm; d= 500µm; e, f = 5µm.**

There were no regional differences observed with the different probes generated against the same gene, and all antisense probes tested gave good signals at optimal hybridization temperature. The results shown are mostly from probes directed against the C terminal part of the genes where sequences divergence most, minimizing cross-hybridizations (Table 3.3). Within the zebra finch telencephalon, each *FoxP* member had a unique expression pattern (Figure 3.2), underscoring probe specificity.

In the telencephalon of adult male zebra finches the only region that expressed all three *FoxP* genes was the striatum. Striatal Area X, a nucleus important for vocal learning, likewise

expressed *FoxP1*, *FoxP2* and *FoxP4* (Figure 3.2 b, h and n). At this age (>100PHD) the only gene that was expressed at higher levels in Area X than in the surrounding anterior striatum was *FoxP1* (Figure 3.2 b), whereas *FoxP2* expression in Area X is down-regulated, compared to some juvenile ages (Haesler *et al.*, 2004). *FoxP4* did not seem differentially expressed in Area X and the anterior striatum at this age.

Telencephalic song nucleus RA expressed only *FoxP1* (Figure 3.2 c) and *FoxP4* (Figure 3.2 o), but not *FoxP2* (Figure 3.2 i). In song nucleus HVC, *FoxP1* and *FoxP4* (Figure 3.2 d and p) were expressed at higher levels than *FoxP2* (Figure 3.2 j).

Every FoxP member studied had a different pattern of expression throughout the zebra finch brain. The most broadly expressed FoxP member was *FoxP4* (Figure 3.2 m, n). In the telencephalon of male zebra finches *FoxP4* was expressed in nidopallium, mesopallium, hyperpallium, arcopallium, striatum and pallidum (Figure 3.2 m, n; Table 3.4). *FoxP4* was also widely expressed through thalamic regions (Figure 3.2 r; Table 3.4) as well as optic tectum regions (Figure 3.2 q; Table 3.4). Very few regions were negative to *FoxP4*, among them tractus opticum and chiasma opticum (Table 3.4). Differences in the expression pattern existed between *FoxP1* (Figure 3.2 e), *FoxP2* (Figure 3.2 k) and *FoxP4* (Figure 3.2 q) in optic tectum, where nucleus spiriformis lateralis (SPL) was positive for *FoxP1* and *FoxP4* but not for *FoxP2*. The inverse was true for nucleus rotundus (RT), with low or absent *FoxP1* expression, but strong expression of *FoxP2* and *FoxP4*. Thalamic nucleus ovoidalis expressed *FoxP2* and *FoxP4* (Figure 3.2 l and r) but not *FoxP1* (Figure 3.2 f). In the cerebellar Purkinje cells, there was strong expression of *FoxP2* (Figure 3.2 l) and *FoxP4* (Figure 3.2 r) but no *FoxP1* expression (Figure 3.2 f). Another region that showed strong expression of all *FoxP* members was the Inferior olive (Table 3.4.). These results exemplify the specificity of the probes for these genes and possible important combinatorial regulatory functions.

Although the main interest of this thesis centered on the function of the different FoxP genes in song control nuclei of the zebra finch, expression was scanned across all brain regions. Table 3.4 summarizes these findings.

**Table 3. 4 Expression pattern of FoxP1, FoxP2 and FoxP4 in the adult zebra finch brain.**

| Abbreviation | Brain nuclei and regions | *FoxP1* | *FoxP2* | *FoxP4* |
|---|---|---|---|---|
| A | Arcopallium | - | - | + |
| APH | Area parahippocampalis | - | - | + |
| B, Bas | Nucleus basorostralis pallii | - | - | + |
| BSTL | Lateral part of the bed nucleus of the stria terminalis | - | + | + |
| BSTM | Medial part of the bed nucleus of the stria terminalis | - | - | + |

| | | | | |
|---|---|---|---|---|
| Cb (Purkinje cells) | Cerebellum | - | + | + |
| CDL | Area corticoidea dorsolateralis | - | - | + |
| CO | Chiasma opticum | - | - | - |
| CoA, CA | Commissura anterior | - | - | + |
| CP | Commissura posterior | - | - | + |
| DLM | Nucleus dorsolateralis anterior thalami, pars medialis | + | + | + |
| DM | Dorsomedial nucleus of the midbrain | - | - | + |
| DMA | Nucleus dorsomedialis anterior | + | + | + |
| DMP | Nucleus dorsomedialis posterior Thalami | + | + | + |
| DSD | Decussatio supraoptica dorsalis | - | - | + |
| E | Nucleus entopallis or entopallium | - | - | + |
| EM | Nucleus ectomammillaris | - | - | + |
| FA | Tractus fronto-arcopallialis | - | - | + |
| FPL, LFB | Fasciculus prosencephali lateralis (lateral forebrain bundle) | - | - | + |
| GLV, GLv | Nucleus geniculatus lateralis, pars ventralis | + | - | + |
| GP | Globus pallidus | + | + | + |
| HA | Hyperpallium apicale | - | - | + |
| HB | Habenula | - | + | + |
| HD | Hyperpallium densocellulare | + | - | + |
| HP, Hp | Hippocampal formation | - | - | + |
| **HVC** | formal name, located in nidopallium | + | - | + |
| IM | Nucleus isthmi, pars magnocellularis | - | - | + |
| Ipc | Nucleus isthmi, pars parvocellularis | - | - | + |
| ICo | Nucleus intercollicularis | - | + | + |
| L | Area L pallii, Field L | - | - | + |
| LMAN | Nucleus lateralis magnocellularis nidopallii anterioris | - | - | + |
| M | Mesopallium | + | - | + |
| MLd | Nucleus mesencephalicus lateralis, pars dorsalis | - | + | + |
| N | Nidopallium | - | - | + |
| NC | Nidopallium caudale | - | - | + |
| NF | Nidopallium frontale | - | - | + |
| NIf | Nucleus Interfacialis nidopallii | - | - | + |
| nXII | Hypoglossal nucleus or XII nucleus | | - | |
| OI | Nucleus olivaris inferior | + | + | + |
| OM | Tractus occipito-mesencephalicus | - | - | + |
| Ov | Nucleus ovoidalis | - | + | + |
| Pt | Pretectal nucleus | - | - | + |
| **RA** | Nucleus robustus arcopallii | + | - | + |
| RT | Nucleus rotundus | - | + | + |
| SP | Nucleus subpretectalis | | - | |

| | | | | |
|---|---|---|---|---|
| SPL | Nucleus spiriformis lateralis | + | - | + |
| StL | Striatum laterale | + | + | + |
| StM | Striatum mediale | + | + | + |
| TeO | Tectum opticum | + | + | + |
| TFM | Tractus thalamo-frontalis et frontalis-thalamicus medialis | - | - | + |
| TrO | Tractus opticum | - | - | - |
| VP | Ventral pallidum region of the basal forebrain | - | - | + |
| **X** | Area X | + | + | + |

### *3.1.4 Expression of Foxp1, FoxP2 and FoxP4 at different ages in the zebra finch brain*

To address the question whether *FoxP4* is regulated during the song learning phase, as is the case for *FoxP2* (Haesler *et al*., 2004), *in situ* hybridization on brains of different ages (35, 50, 75 and >100 PHD) were performed with all three genes (Figure 3.3).

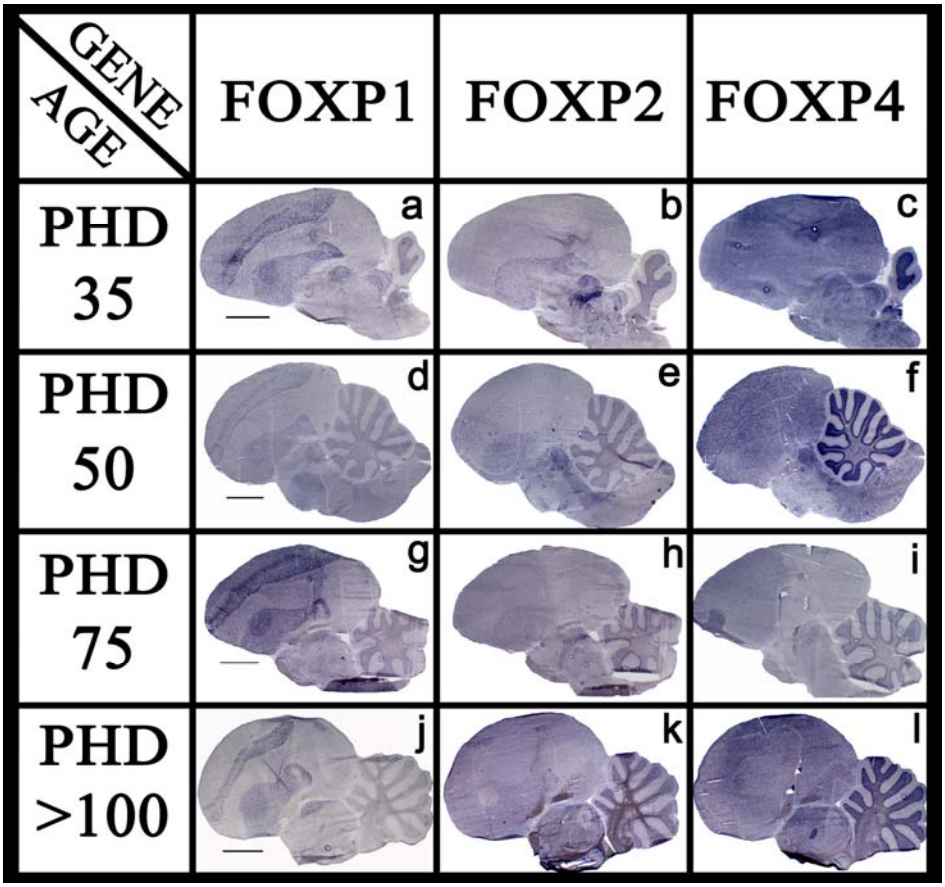



**Figure 3. 3 In situ hybridization of FoxP1 ("a", "d", "g" and "j"), FoxP2 ("b", "e", "h" and "k") and FoxP4 ("c", "f", "I" and "l") on adjacent sagittal 12µm sections of different ages (35, 50, 75 and >100 PHD). At all ages, FoxP1 is expressed at higher levels in Area X ("a", "d", "g" and "j") than in the surrounding striatum. FoxP2 and FoxP4 expression is stronger at ages 50 ("e", "f") in Area X relative to**

**the surrounding striatum, which corresponds to the learning phase of the zebra finches. Scale bars on "a", "d", "g", and "j" = 2mm.**

*FoxP1* appears to not be regulated by age or song learning phases, because across all ages there was a consistently stronger expression in Area X relative to the surrounding striatum (Figure 3.3 a, d, g and j), consistent with Haesler *et al.*, 2004. For *FoxP2* there was an up-regulation of gene expression at 50 PHD (Figure 3.3 e). *FoxP4* was also only up-regulated at 50 PHD in Area X if compared with surrounding striatum (Figure 3.3 f) in the other ages tested the expression in Area X is as strong as in the surrounding striatum. In *FoxP2* and *FoxP4* up-regulation occurs between the ages at which zebra finches actively learn how to imitate song which might be associated to vocal plasticity.

### *3.1.5* Characterization of *FoxP1*+ and *FoxP4*+ cells in HVC

To gain more insight into the possible function of FoxP1 and Foxp4, expression in different populations of HVC neuron was assessed. HVC projection neurons were retrogradely labelled with different tracers by injections into RA (red fluorescent microspheres) or Area X (green fluorescent microspheres). (for a timeline of experiments se Figure 3.4).

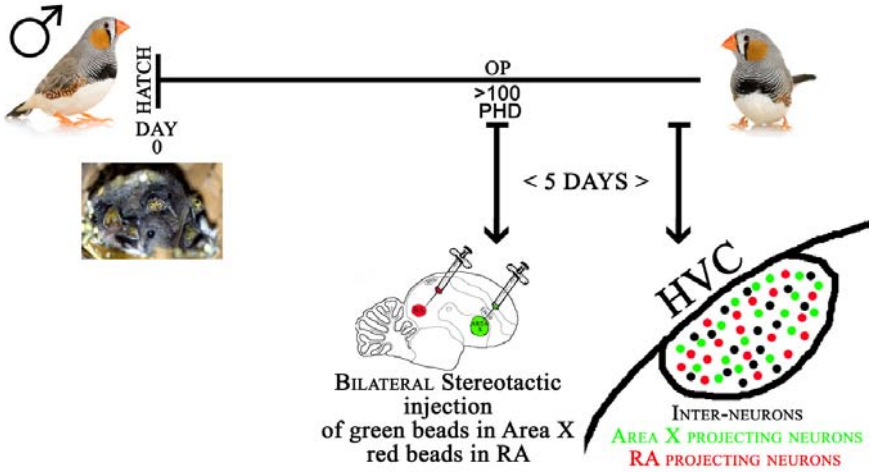



**Figure 3. 4 Timeline of retrograde tracer injections. Adult zebra finches (>100PHD) were bilaterally microinjected with green latex beads (in Area X) and red latex beads (in RA). After 3-5 days of the operation birds were sacrificed by Isofluorane overdose and the brains were shock frozen over liquid nitrogen and kept at -80°C. Afterwards 12µm slices were cut with the cryostat and slides with targeting in both nuclei (having red and green beads labelling HVC, and beads in the desired target nuclei) were used for *in situ* hybridization.**

Both $HVC_X$ and $HVC_{RA}$ neurons were found to express *FoxP1* (Figure 3.5 a, b, c, d and e) or *FoxP4* (Figure 3.5 f, g, h, i and j). It is thus likely that FoxP1 and FoxP4 can co-occur in the same type of HVC projection neuron.

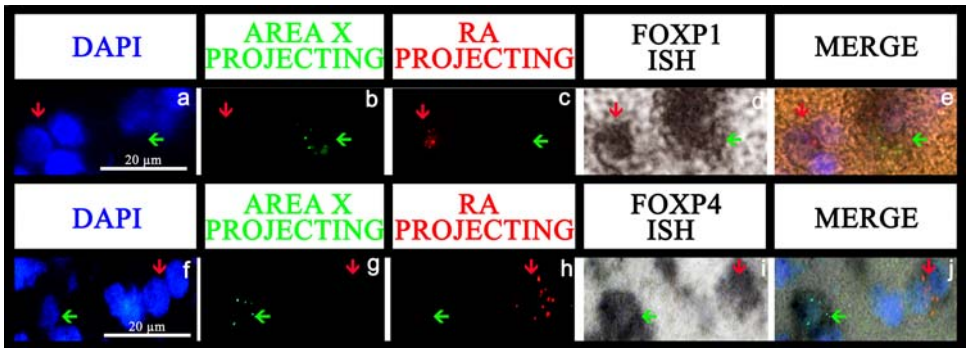



**Figure 3. 5 *In situ* hybridization with *FoxP1* and *FoxP4* in brains injected with retrograde tracers into RA (red) and Area X (green). High magnification photos at 63 x of HVC show that both genes are expressed in RA as well as in Area X projecting neurons in HVC. In ("a"and "f") DAPI nuclear staining (blue fluorescence) white bar 20µm; ("b" and "g") retrograde tracers injected in Area X (green); ("c" and "h") retrograde tracers injected to RA (red); "d" Bright field of in situ hybridization with *FoxP1;* "i" bright field in situ hybridization with *FoxP4*; in "e", "j" merged photo of RA tracers (red), Area X tracers (green), DAPI, *FoxP1* or *FoxP4 in situ* hybridization. Green arrowheads showing Area X projecting neurons and red arrowheads showing RA projecting neurons in HVC that also expressed *FoxP1* or *FoxP4*.**

### *3.1.6 Double in situ* hybridization; are *FoxP* subfamily members in the same cells?

Previous findings indicate that FoxP1, FoxP2 and FoxP4 is either expressed in complementary patterns, e.g. different tissues of the lung (Shanru Li *et al.*, 2004) or different layers of the mouse cortex (Hisaoka *et al.*, 2009) or can co-occur in the same region, e.g. in the striatum of primates and songbirds (Takahashi *et al.* 2002, 2008a, 2008b; Teramitsu *et al.*, 2004; Haesler *et al.*, 2004). Indirect evidence that FoxP1 and FoxP2 might be expressed by the same cells in striatum come from two studies in different mice and songbirds (Tamura, *et al.*, 2004; Haesler *et al.*, 2004), ,that showed that DARPP-32, but not NPY, PV and ChAT, co-localize with FoxP1 and FoxP2. This suggests that they occur in the same type of neurons in the striatum.

To address this, double *in situ* hybridization and immunohistochemistry were employed (discussed in 3.1.8), focussing on Area X.

*In situ* hybridization with all combinations of probes were performed in 50 PHD male zebra finches that had no sung before sacrifice, because at this age, FoxP2 is expressed in

Area X at higher levels than in adults (Figure 3.3d, e and f) facilitating the visualization of Area X in the slices.

*FoxP1* and *FoxP2*, were co-localized in many cells (Figure 3.6 a, b, c, d and e), but could also occur alone (Figure 3.6 g, h, I and j). In regions that are known to express only one Foxp2, e.g. the cerebellar Purkinje cells, no *FoxP1* staining was observed (Figure 3.6 l, m, n and o). In mesopallium, the opposite was true, with strong *FoxP1* expression but lack of but *FoxP2*. In the thalamus, at the border between nucleus ovoidalis (OV) and Nucleus dorsolateralis anterior thalami (DLM), *FoxP2* but not FoxP1 was expressed in nucleus ovoidalis, whereas in DLM the inverse was the case (Figure 4.6 v, w, x and y).

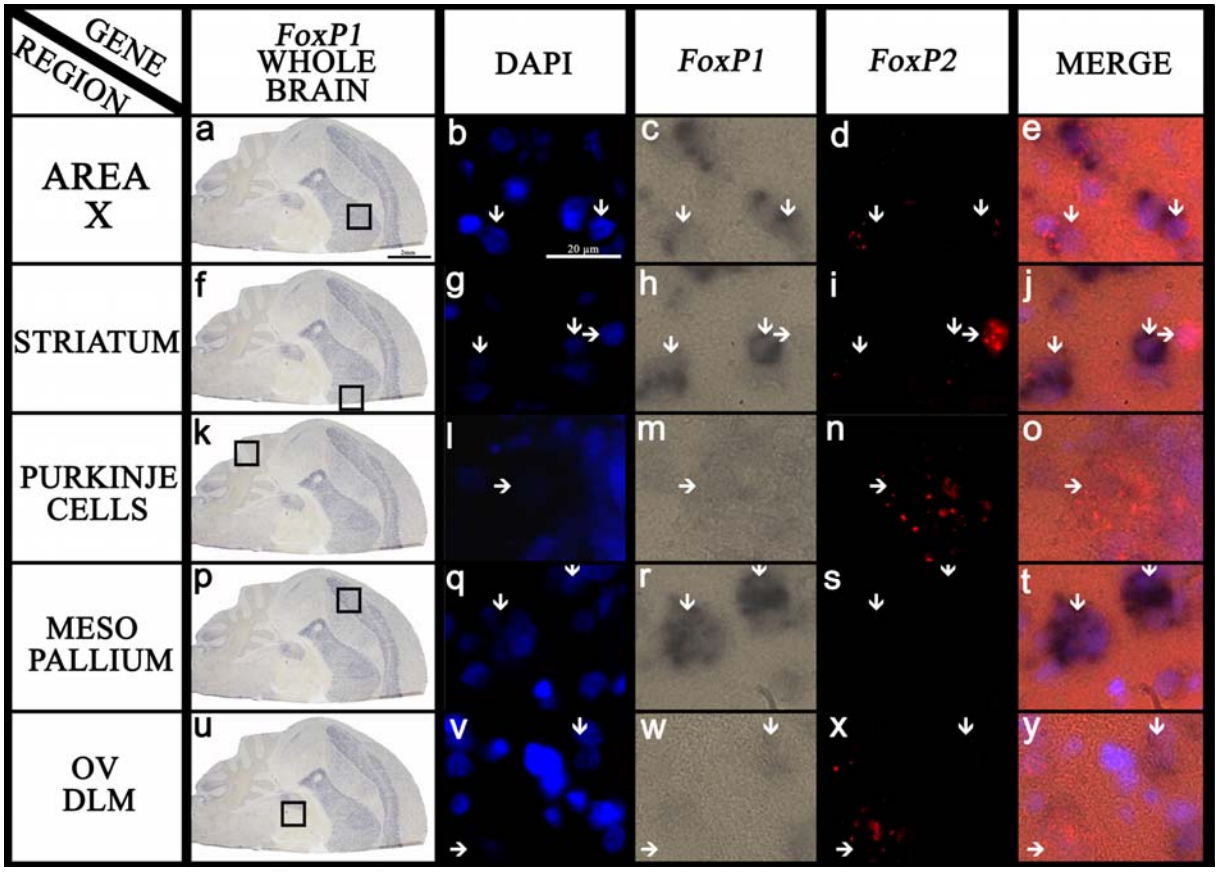



**Figure 3. 6 Double in situ hybridization against *FoxP1* (blue precipitate) and *FoxP2* (red fluorescence). In different regions of 50 PHD male zebra finches, sagittal 12µm brain sections. FoxP1 expression patterns are shown in "a", "f", "k", "p" and "u". The box indicates the regions of higher magnifications in all panels to the right of the first column. Arrowheads pointing down show cells positive for *FoxP1* and *FoxP2*, arrowheads pointing to the right are *FoxP2+* but *FoxP1-* and arrowheads pointing up are *FoxP1+* but *FoxP2-*. The frame outlined in u includes part of nucleus ovoidalis (bottom left corner in "v", "w", "x"and "y") and nucleus DLM (upper right in "v", "w", "x" and "y"). Neurons in n. ovoidalis were *FoxP2+* but *FoxP1-* (arrow head pointing right in "w", "x" and "y") whereas in DLM they were not co-localized and can be either FoxP1+ and FoxP2- (arrowhead pointing down in "w", "x" and "y") or FoxP1- and FoxP2+ (not shown).**

In Area X (Figure 3.7 b, c, d and e) the majority of cells expressed FoxP1 and foxP4. This was also the case in RA (Figure 3.2 l-o), HVC (Figure 3.7 g-j) and mesopallium (Figure

3.7 q, r, s and t). In the cerebellar, Purkinje cells, FoxP4 was expressed (Figure 3.7 x), but FoxP1 was not (Figure 3.7 w).

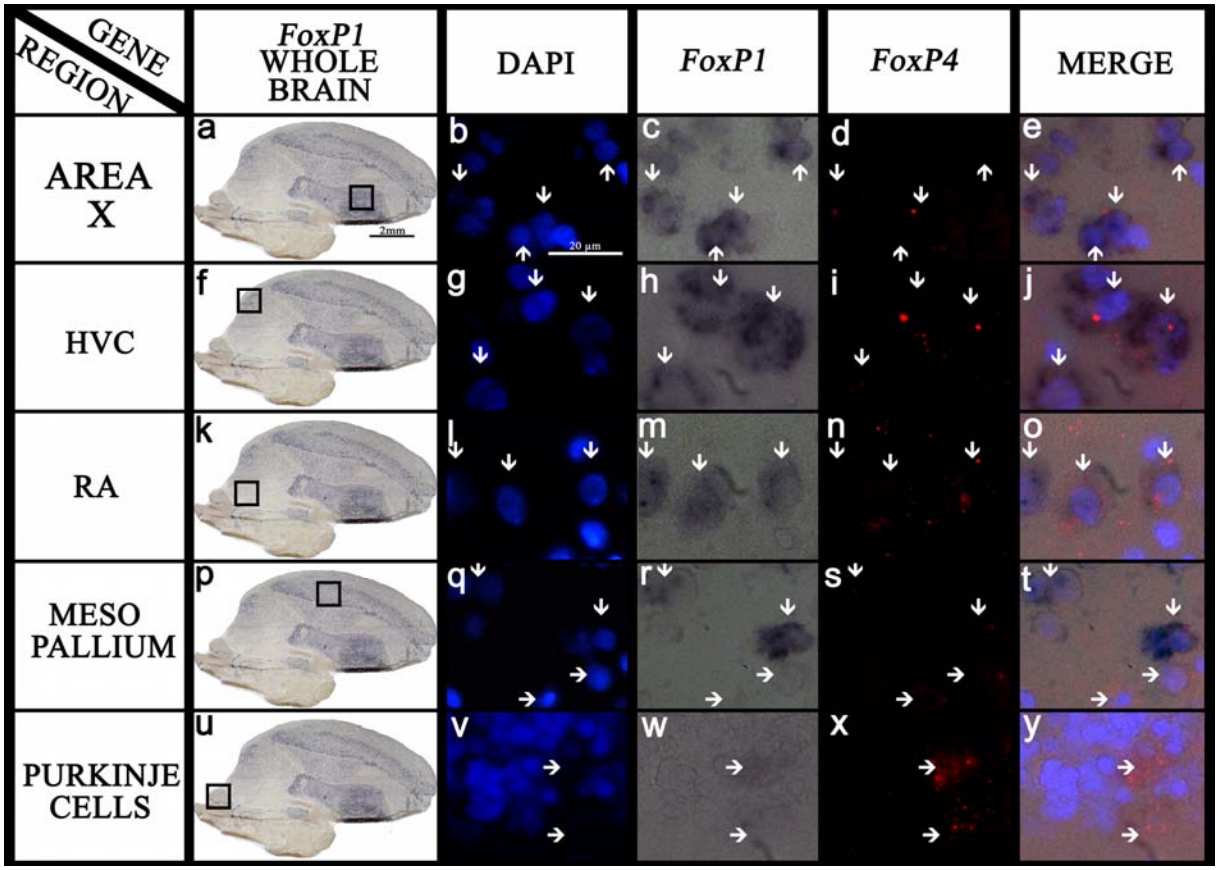



**Figure 3. 7 Double in situ hybridization against *FoxP1* (blue precipitate) and *FoxP4* (red fluorescence), in sagittal 12µm brain section of 50 PHD zebra finches. Conventon as in Figure 3.6 Arrowheads pointing down show cells positive for *FoxP1+* and *FoxP4+*; arrowheads pointing to the right are *FoxP1-* but *FoxP4+* cells; and arrowheads pointing up *FoxP1+* but *FoxP4-* cells.**

Co-expression could also occur between *FoxP2* and *FoxP4*, e.g. in Area X (Figure 3.8 b, c, d and e) or Purkinje cells (Figure 3.8 g, h, i and j).

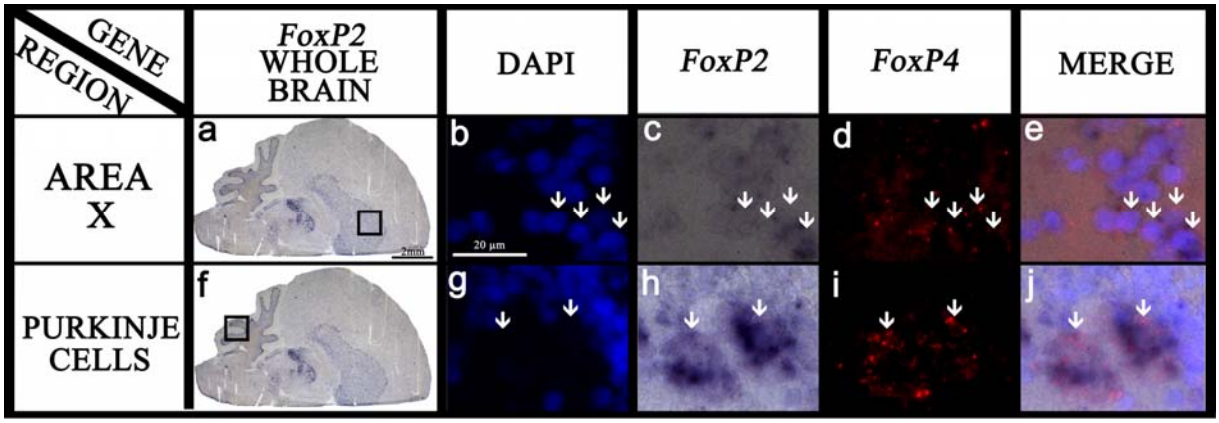



**Figure 3. 8 Double in situ hybridization against *FoxP2* (blue precipitate) and *FoxP4* (red fluorescence), in sagittal 12µm brain section of 50 PHD zebra finches. Conventon as in Figure 3.6 Arrowheads pointing down show cells positive for *FoxP2+* and *FoxP4+*.**

To further investigate the co-expression of FoxP1, 2 and 4 in different neurons, immunohistochemistry was employed,

### 3.1.7 Specificity of FoxP1, FoxP2 and FoxP4 antibodies

Antibodies were tested for specificity using western blots of transiently over expressed zebra finch versions of FoxP1, 2 and 4, in HeLa cells. Results showed that all antibodies (one for Foxp1, two for FoxP2 and one for FoxP4) were specific and did not cross-react with the other FoxP family members (Figure 3.9).

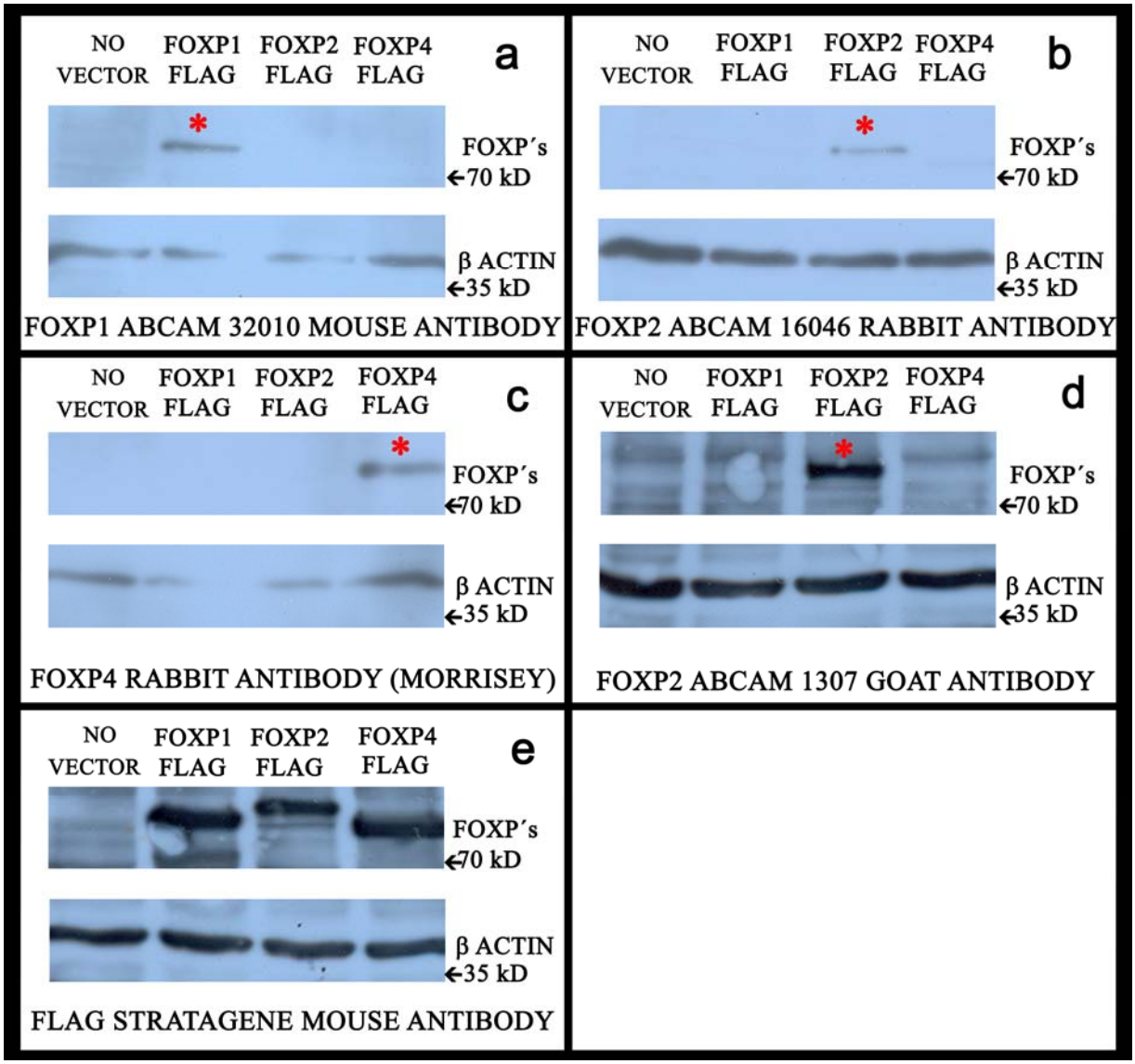



**Figure 3. 9 Western blots testing the specificity of the different antibodies using protein extractions of HeLa cells transfected with transient over-expression vectors carrying either FoxP1 (panel "a"), FoxP2 (panel "b" and "d") or FoxP4 "c" or FLAG-tagged sequences "e". Antibodies used were Abcam FoxP1 monoclonal mouse antibody (panel "a"); Abcam FoxP2 polyclonal rabbit antibody (panel "b"); Foxp4 polyclonal rabbit antibody (panel "c"); Abcam FoxP2 polyclonal goat antibody (panel "d"); and Stratagene FLAG monoclonal mouse antibody (panel "e"). In all cases, the specificity of the antibody is**

**evident because of a single band in the expected lane (*). Panel e is blotted with an anti-FLAG antibody and shows the over-expressed proteins FoxP1, FoxP2 and FoxP4.**

Because each FoxP subfamily member antibody was made in different animals triple immunohistochemistry was feasible.

### 3.1.8 Neurons in Area X express different combinations of FoxP1, FoxP2 and FoxP4

The question whether neurons expressed all three FoxP subfamily members or particular subsets was addressed using triple immunohistochemistry. For these experiments FoxP1 mouse monoclonal antibody of AbCam (32010), FoxP2 goat polyclonal from AbCam (1307) and FoxP4 rabbit polyclonal antibody (Morrisey) were used.

Results of triple immunohistochemistry were consistent with the *in situ* hybridization reported above. FoxP1-like immunoreactivity, as well as immunoreactivity against FoxP2 and FoxP4 were found in the same regions where mRNA was detected via *in situ* hybridizations (Figure 3.10, Figure 3.2 a-x). Likewise, particular combinations of two FoxP proteins were detected in cells in the same regions where double in situ hybridizations had detected combinations of particular FoxP mRNAs (Figure 3.10, Figure 3.6-8). In addition, triple immunohistochemistry revealed that in song nucleus Area X all three FoxPs were expressed, either singly (Figure 3.10 k-o) or in all possible combinations (Figure 3.10 k-o). In contrast, song nuclei HVC and RA, most neurons expressed FoxP1 and FoxP4 together (Figure 3.10 p-y).

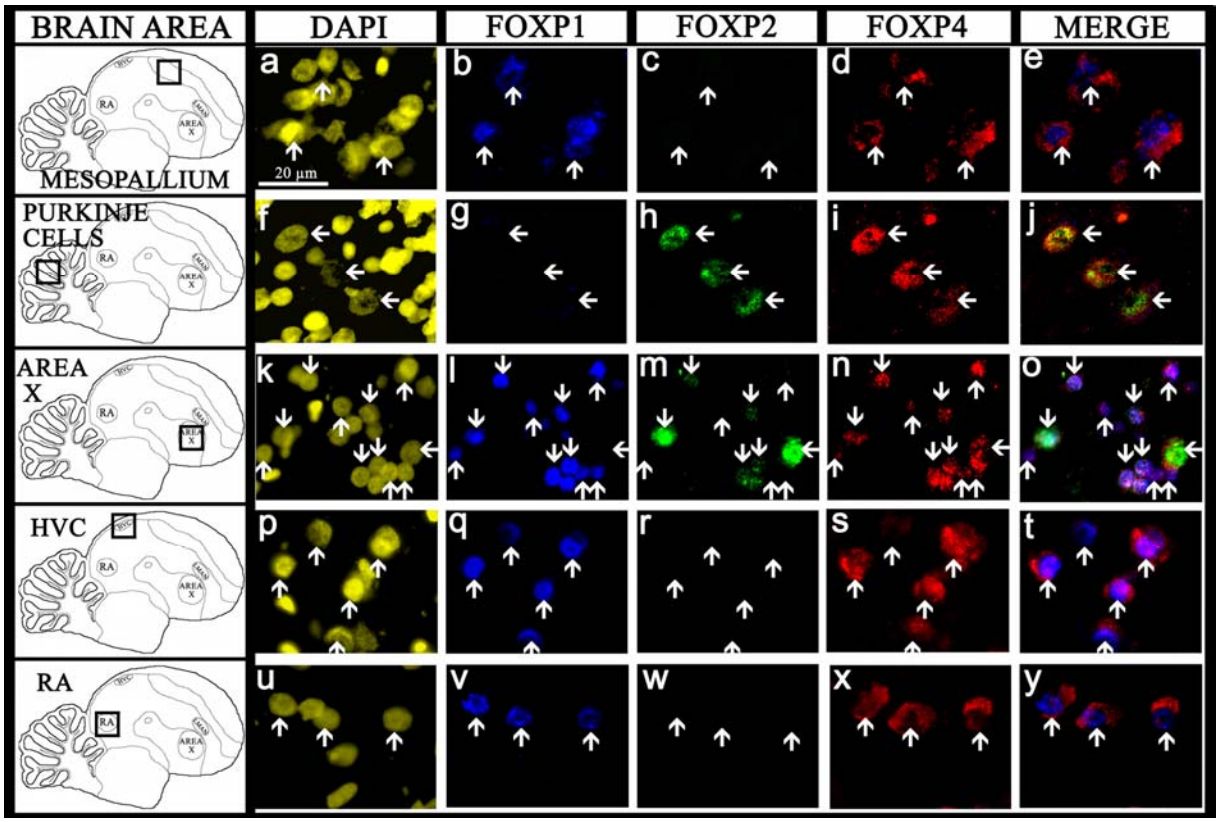



**Figure 3. 10 Triple immunohistochemical reactivity against FoxP1, FoxP2 and FoxP4 in sagittal 12µm brain sections of zebra finches. DAPI (blue false-colour-coded as yellow, panels "a", "f", "k", "p" and "q"), FoxP1 (Biotinylated secondary antibody and Streptavidin 647, deep red, fluorescence, false-colour-coded as blue panels "b", "g", "l", "q" and "v"), FoxP2 (Alexa 488 as the secondary antibody, green fluorescence , panels "c", "h", "m", "r" and "w"), and FoxP4 (Alexa 568 as the secondary antibody, red fluorescence, panels "e", "j", "o", "t" and "y"). Merged images are seen in the last column. In all cases arrows pointing down are FoxP1+/FoxP2+/FoxP4+ neurons, arrows pointing up are FoxP1+/FoxP2-/FoxP4+ neurons, arrows pointing left are FoxP1-/FoxP2+/FoxP4+ neurons. In mesopallium the majority of neurons are FoxP1+/FoxP2-/FoxP4+, in this region FoxP2 is not expressed; in cerebellum, Purkinje cells are Foxp1-/FoxP2+/FoxP4+; in Area X there are different populations expressing different combinations of FoxP1, FoxP2 and FoxP4; in HVC and RA neurons express are FoxP1+/FoxP2-/FoxP4+. Scale bar in panel "a" is 20µm, same magnification for all panels.**

Since multiple FoxP family members were found to be co-localized in the same cells, it is likely that they regulate transcription in vivo via homo- and hetero-dimerization as shown previously *in vitro* (Shanru Li *et al.* 2004). Particularly interesting is the fact that in Area X cells were found to express all possible combinations of FoxPs. To start addressing whether this reflects different cell populations expressing particular combinations of FoxPs stably over time, or whether the expression of FoxPs in Area X cells was dynamically regulated, different developmental time points were analyzed, both in Area X and the surrounding striatum (35, 50, 75 and >100 PHD, n=2 for 35 PHD, n=3 for all others). The density of the cells that expressed FoxP1, FoxP2 or FoxP4, not taking into account which other FoxPs they were co-localized with, did not differ significantly across the investigated ages, neither in Area X (Figure 3.11 a) nor in the adjacent striatum (Figure 3.11 b).

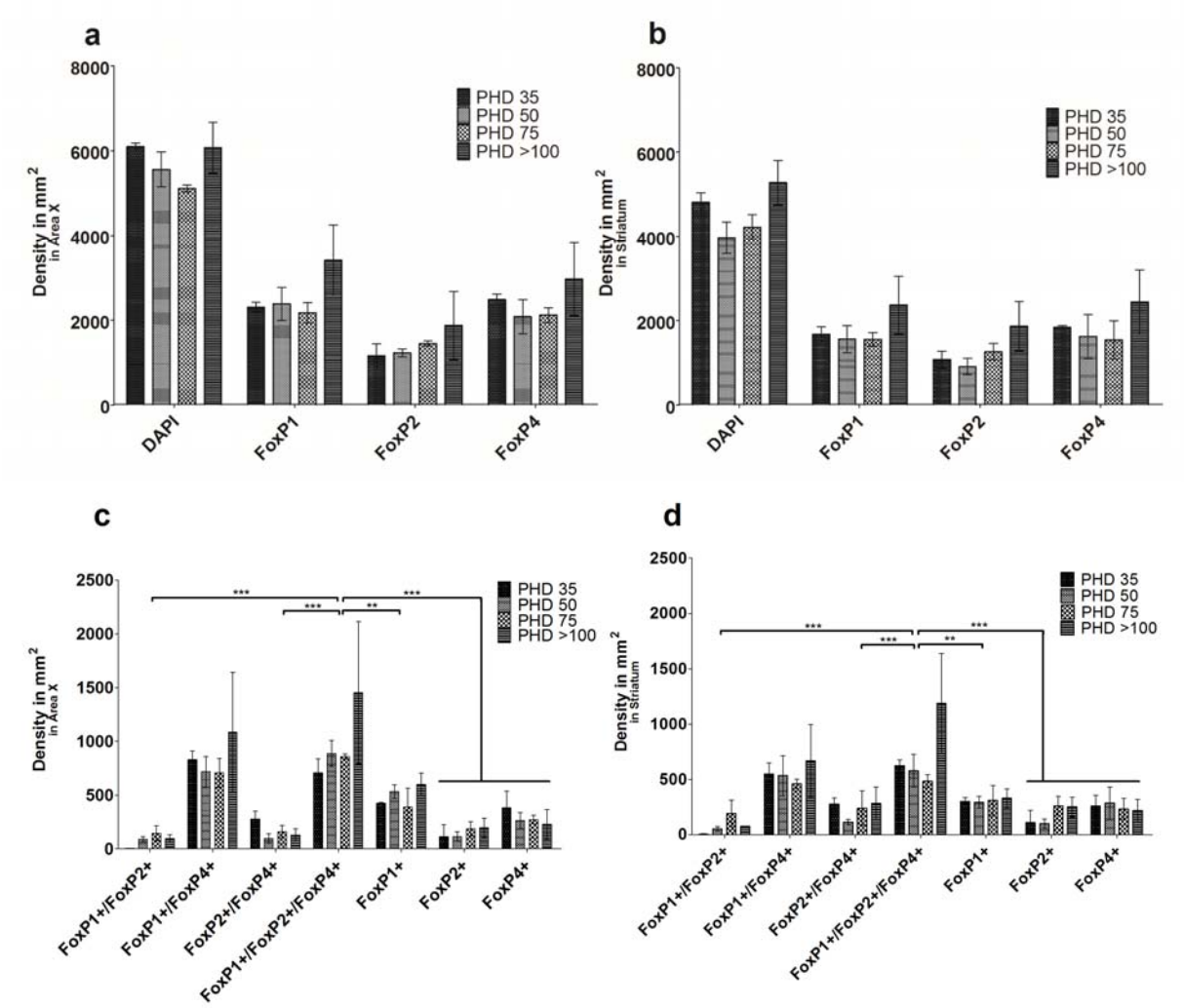



**Figure 3. 11 Density of neurons in Area X (panel "a" and "c") and striatum ventral to Area X (panel "b" and "d") immunopositive for FoxP1, FoxP2 or FoxP4 ("a" and "b"). Panels "c" and "d" depict the density of neurons that express either only one FoxP, (e.g. were immuno-negative for the other two), two FoxPs (immuno-negative for the third) or all three FoxPs (bars refer to mean of means ±standard error of the mean [SEM]; Linear mixed effect modelling; **P < 0,005; *** P < 0,0001; showed only for the FoxP1/FoxP2/FoxP4 population).**

Analyzing the density of cells that expressed different combinations of FoxP1, FoxP2 and FoxP4 in Area X (Figure 3.11 c) and striatum (Figure 3.11 d) at different ages showed that the density of cells in Area X was slightly higher than the one in striatum, but not significant so. Cells expressing all three FoxP2 or a combination of FoxP1 and FoxP4 were relatively abundant in both regions, whereas the FoxP1+/FoxP2+/FoxP4- population was among the most rarely encountered. There were no significant differences between the two regions (Linear mixed effect modelling; P > 0,05).

Comparison of the density of cells expression different combinations of FoxPs across the different ages also did not reveal significant differences (Linear mixed effect modelling; P > 0,05) between developmental ages. This suggests that the proportion of cells that express a particular combination of FoxPs remains stable across time, with a trend towards higher

density in the FoxP1+/FoxP2-/FoxP4+ and FoxP1+/FoxP2+/FoxP4+, both in Area X and striatum in adults.

Since the proportion of cells expression particular combinations of FoxPs did not differ among developmental ages, a linear mixed general model was used to analyze the different populations across different ages. In this model, populations were a fixed factor and individual birds were a random factor. A highly significant effect was found for populations FoxP1+/FoxP2-/FoxP4+ and FoxP1+/FoxP2+/FoxP4+ and for population FoxP1+/FoxP2-/FoxP4-, both in Area X and Striatum (Table 3.5).

**Table 3. 5 Linear mixed effect modelling with populations of Area X and striatum**

| Population | P-value Area X | Area X | P-value Striatum | Striatum |
|---|---|---|---|---|
| FoxP1 / FoxP2 | 0.3391 | n.s. | 0.2445 | n.s. |
| FoxP1 / FoxP4 | 0.0000 | *** | 0.0000 | *** |
| FoxP2 / FoxP4 | 0.5906 | n.s. | 0.1762 | n.s. |
| FoxP1 / FoxP2 / FoxP4 | 0.0000 | *** | 0.0000 | *** |
| FoxP1 | 0.0034 | ** | 0.0378 | * |
| FoxP2 | 0.5686 | n.s. | 0.4190 | n.s. |
| FoxP4 | 0.1335 | n.s. | 0.1070 | n.s. |

A Tukey's post hoc test for pair-wise comparisons was used to compare all different populations (Aho 2009, R Development Core Team 2009) for Area X and for striatum (Table 3.6). In Area X, the density of FoxP1+/FoxP2+/FoxP4+ cells was significantly higher than all other populations, except for the FoxP1+/FoxP2-/FoxP4+ population (Table 3.6). There were only two comparisons where two populations differed in magnitude in Area X but not in the striatum, namely FoxP1+/FoxP2-/FoxP4+ cells were more abundant than FoxP1-/FoxP2+/FoxP4+ and also more abundant than cells only expressing FoxP4 (in red in Table 3.6).

**Table 3. 6 Differences between all populations calculated with Tukey's post hoc test for pair wise comparison for Area X and Striatum populations expressing different combinations of FoxP1, FoxP2 and FoxP4**

| Comparison of populations | p-value Area X | p- value Striatum | Area X | Striatum |
|---|---|---|---|---|
| FoxP1 with FoxP1 / FoxP2 / FoxP4 | 0.004766 | 0.003154 | ** | ** |
| FoxP2 with FoxP1 / FoxP2 / FoxP4 | 1e-06 | 3.5e-05 | *** | *** |
| FoxP4 with FoxP1 / FoxP2 / FoxP4 | 2.3e-05 | 0.000641 | *** | *** |
| FoxP1/FoxP2 with FoxP1 / FoxP2 / FoxP4 | 0 | 2e-06 | *** | *** |
| FoxP1 / FoxP4 with FoxP1 / FoxP2 / FoxP4 | 0.817006 | 0.583424 | n.s. | n.s. |
| FoxP2 / FoxP4 with FoxP1 / | 1e-06 | 0.000256 | *** | *** |

| | | | | |
|---|---|---|---|---|
| FoxP2 / FoxP4 | | | | |
| FoxP1 with FoxP1 / FoxP4 | 0.185222 | 0.301391 | n.s. | n.s. |
| FoxP2 with FoxP1 / FoxP4 | 0.000124 | 0.015024 | *** | * |
| FoxP4 with FoxP1 / FoxP4 | 0.00321 | 0.118516 | ** | n.s. |
| FoxP1/FoxP2 with FoxP1/FoxP4 | 1.5e-05 | 0.001234 | *** | ** |
| FoxP2 / FoxP4 with FoxP1 / FoxP4 | 0.00011 | 0.064326 | *** | n.s. |
| FoxP1 / FoxP2 with FoxP1 | 0.058629 | 0.404352 | n.s. | n.s. |
| FoxP1 / FoxP2 with FoxP2 | 0.997704 | 0.98632 | n.s. | n.s. |
| FoxP1 / FoxP2 with FoxP4 | 0.752846 | 0.704389 | n.s. | n.s. |
| FoxP1/FoxP2 with FoxP2 / FoxP4 | 0.998341 | 0.845419 | n.s. | n.s. |
| FoxP2 / FoxP4 with FoxP1 | 0.195618 | 0.990725 | n.s. | n.s. |
| FoxP2 / FoxP4 with FoxP2 | 1 | 0.998291 | n.s. | n.s. |
| FoxP2 / FoxP4 with FoxP4 | 0.961442 | 0.999975 | n.s. | n.s. |
| FoxP1 with FoxP2 | 0.208266 | 0.87019 | n.s. | n.s. |
| FoxP1 with FoxP4 | 0.750218 | 0.999175 | n.s. | n.s. |
| FoxP2 with FoxP4 | 0.96725 | 0.985523 | n.s. | n.s. |

In summary, both in Area X and striatum, cells expressing all three FoxPs or a combination of FoxP1 and FoxP4 were significantly more abundant than cells expressing other combinations and this was stable across ages. In Area X and the striatum the relative abundance of cells expressing the different combinations of FoxP2 were similar. The only difference concerned the population that expressed FoxP1/FoxP4, which were more numerous in Area X than both the FoxP1/FoxP2 expressing cells and the Foxp4 expression cells, which was not the case in the striatum.

## 3.2 FoxP1, FoxP2 and FoxP4 interaction

Since the majority of cells were found to express FoxP1, FoxP2 and FoxP4, direct protein interactions were assessed next. In mice it was shown that all FoxP subfamily members are able of homo- and hetero-dimerization *in vitro* (Shanru Li *et al.*, 2004). For assessing this with the zebra finch FoxP proteins two different approaches were used: an *in vitro* approach, using co-immunoprecipitation (Co-IP) of transiently transfected HeLa cells with different combinations of FoxP1, FoxP2 and FoxP4 zebra finch proteins, tagged either with V5 or FLAG; second, an *in vivo* approach for which specific antibodies were used to Co-IP hetero-dimers from protein extracts of brain of adult zebra finches. The first approach demonstrated that all zebra finch FoxPs are able to interact *in vitro*, and the second showed that this also is true in brains.

### 3.2.1 Hetero- and homo- dimerization of zebra finch FoxP1, FoxP2 and FoxP4 *in vitro*

All FoxP zebra finch proteins have a conserved leucine zipper (Figure 3.1). This is a characteristic feature of the FoxP subfamily in all species investigated and may serve for homo- and hetero- dimerization of FoxP proteins. To perform Co-IPs of the different FoxP zebra finch proteins, each protein was tagged with a V5 or FLAG tag (Figure 3.12 a and b). The expected tagged proteins could be detected. After Co-IP the expected tag proteins could be also detected, thus showing that FoxP1, FoxP2 and FoxP4 can also homo- and hetero-dimerize (Figure 3.12 c).

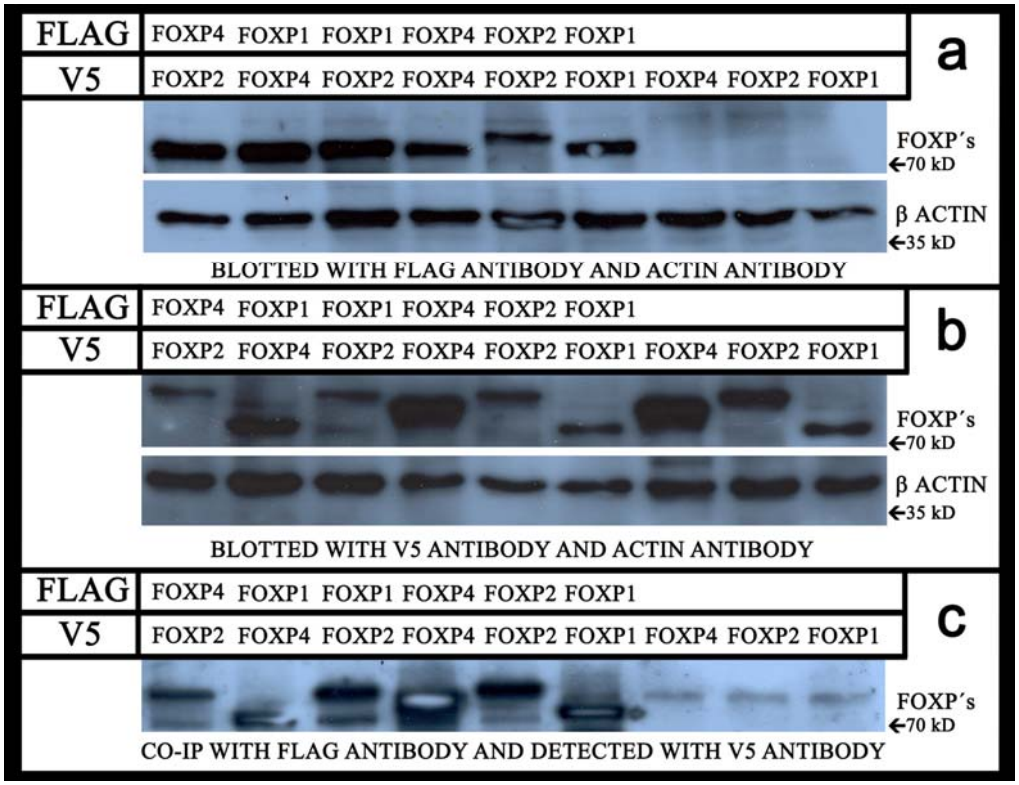



**Figure 3. 12 Hetero- and homo- dimerization of FoxP1, FoxP2 and FoxP4 proteins. HeLa cells were transiently transfected with combinations of expression vectors encoding FoxP1, FoxP2, and FoxP4 proteins that had been tagged with FLAG or V5. The FLAG monoclonal antibody (Invitrogen) was used to immunoprecipitate proteins from cell extracts. Immunoprecipitated proteins were resolved on SDS-polyacrylamide gels or Western blots, which were probed with the anti-V5 monoclonal antibody to reveal co-immunoprecipitated proteins. In all Western blots ("a", "b" and "c") the first protein listed above each lane was tagged with FLAG, while the second protein was tagged with V5. From left to right, the cells were transfected by plasmids encoding FLAG-tagged FoxP4 and V5-tagged FoxP2 (FoxP2/FoxP4), FLAG-tagged FoxP1 and V5-tagged FoxP4 (FoxP1/FoxP4), FLAG-tagged FoxP1 and V5-tagged FoxP2 (FoxP1/FoxP2), FLAG-tagged FoxP4 and V5-tagged FoxP4 (FoxP4/FoxP4), FLAG-tagged FoxP2 and V5-tagged FoxP2 (FoxP2/FoxP2), FLAG-tagged FoxP1 and V5-tagged FoxP1 (FoxP1/FoxP1), V5-tagged FoxP4 (FoxP4), V5-tagged FoxP2 (FoxP2) and V5-tagged FoxP1 (FoxP1). In panel "a" western blot of a sample of the protein extract used for Co-IP blotted with FLAG and actin antibody; in panel "b" western blots of a sample of the same protein extract used for Co-IP blotted with V5 and actin antibody; in panel "c" Co-immunoprecipitation assays of Foxp1, Foxp2, and FoxP4. Results are configured as listed before, with the first protein being FLAG-tagged and the second protein being V5-tagged. The FLAG monoclonal antibody was used for immunoprecipitation, while the anti-V5 monoclonal antibody was used on Western**

**blots for immunodetection of co-immunoprecipitated proteins. The faint bands visible in the last three lanes of "c" are non-specific V5 background staining, visible in all other lanes blotted with V5 as well ("b" and "c").**

Western Blots using V5 and FLAG antibodies to detect over-expressed V5-tagged FoxP1, FLAG-tagged FoxP1 and untransfected HeLa cells confirmed the specificity of the antibodies (Figure 3.13 a and b). In both cases the "No vector" controls gave no bands if detected with FLAG or V5 antibodies. Furthermore, anything could be detected after doing the same procedure than in the Co-IP with those protein extracts (Figure 3.13 c).

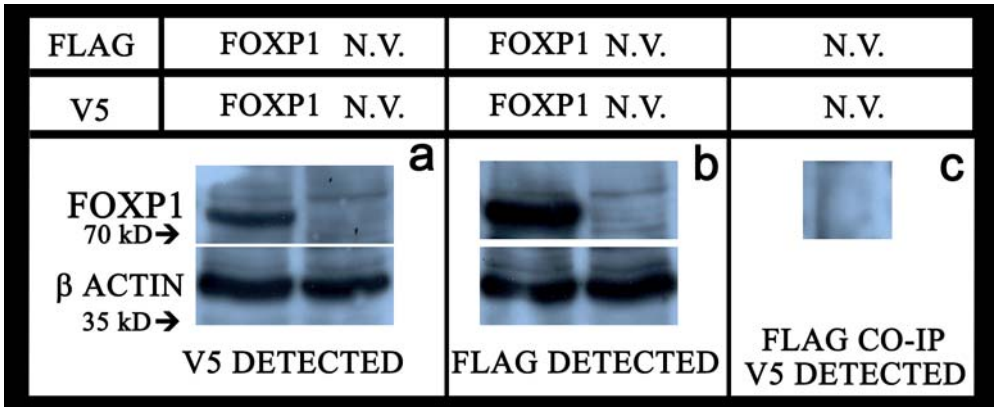



**Figure 3. 13 No vector control for the *in vitro* co-immunoprecipitation. HeLa cells were transfected with expression vectors encoding for FoxP1 that had been tagged with FLAG or V5 (panel a and b first lane from the left). No vector HeLa cells were run in the next lane to the right and detected with V5 (panel "a") and FLAG (panel "b"). The FLAG monoclonal antibody (Invitrogen) was used to immunoprecipitate proteins from the no vector cell extract. HeLa immunoprecipitated proteins were resolved on SDS-polyacrylamide gels or Western blots, which were probed with the anti-V5 monoclonal antibody to reveal co-immunoprecipitated proteins (panel "c"). In all Western blots (panel "a", "b" and "c") the first protein listed above each lane was tagged with FLAG, while the second protein was tagged with V5. The cells were transfected by plasmids encoding FLAG-tagged FoxP1 and V5-tagged FoxP1. The FLAG monoclonal antibody was used for immunoprecipitations, while the anti-V5 monoclonal antibody was used on Western blots for immunodetection of co-immunoprecipitated proteins.**

To further proof that the FoxP subfamily members can build dimers protein extracts in two different conditions were done: one denatured condition (Figure 3.14 panel a and b, all "D" lanes), were proteins were boiled at 95°C for 5 minutes, which would also denature the dimers present in the sample; and a native condition (Figure 3.14 panel a and b, all "N" lanes), were there was no boiling and the dimers should be preserved. Two methods were used for the detection of the proteins: one gel was stained with Coomasie (Figure 3.14 panel a), which allowed us to see all proteins; and a second one was blotted and detected with the FLAG and actin antibodies (Figure 3.14 panel b), which allowed us to see which bands are the FoxP proteins.

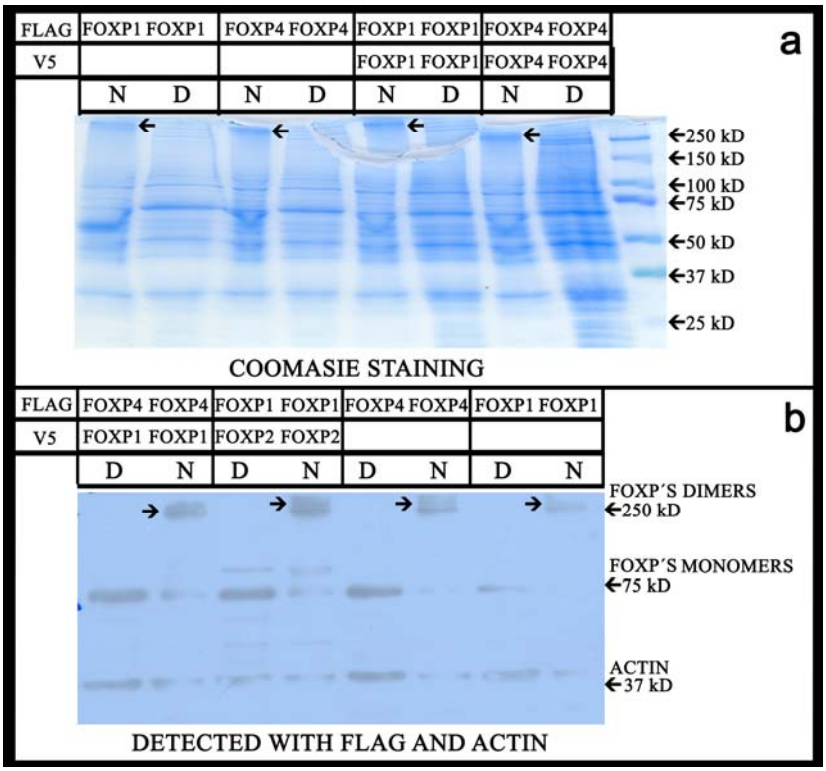



**Figure 3. 14 Assessing FoxP1, FoxP2 and FoxP4 dimers. Coomasie stained gel (in panel "a") and Western blot detected with FLAG and actin antibodies (panel "b"). Denatured ("D") and native ("N") of the same protein extracts were run side by side to see the interaction of the FoxP subfamily members. In panel "a" from left to right the first two lanes are from a sample of protein extract of FoxP1-FLAG, then FoxP4-FLAG, FoxP1-FLAG / FoxP1-V5, FoxP4-FLAG / FoxP4-V5 and protein ladder. In panel "b" from left to right the first two lanes are from a sample of protein extract of FoxP4-FLAG / FoxP1-V5, then FoxP1-FLAG / FoxP2-V5, then FoxP4-FLAG and last FoxP1-FLAG. In panel "a" and "b" arrowheads point to "dimers".**

In the coomasie gel (Figure 3.14 a) a band at around 250 kD could be seen, only present in the native ("N") extracts (arrowheads Figure 3.14), but not in the denatured conditions. When detecting with a specific antibody against the tag of the protein, the same band at 250kD was also detected (Figure 3.14 b), present only in the native ("N") conditions, but not in the denatured. This suggests that FoxP proteins can interact. The fact that the band at the size expected for monomers is weaker than in denatured condition, suggest that most of the proteins are interacting with the other FoxP subfamily members (Figure 3.14 b). However, the expected size for a FoxP homo- and hetero- dimer would be around 150kD, since the monomers are around 75kD. But the size of the band seen in the native condition in all cases (Figure 3.14 a and b), are more than 250kD. This would suggest that either FoxP proteins interact with other co-factors in HeLa cells or that FoxP could be tetramers.

### 3.2.2 Hetero- dimerization of zebra finch FoxP1, FoxP2 and FoxP4 *in vivo*

To show *in vivo* that zebra finch FoxP proteins can interact, extracted proteins from the telencephalon of male adult zebra finches were used and Co-IP with specific antibodies was performed (Figure 3.15). First detection of the zebra finch FoxP proteins from the nuclear telencephalon protein extracts was assessed. FoxP1, FoxP2 and FoxP4 proteins could be detected in brain protein extracts (Figure 3.15 a, b and c). As a positive control for Co-IP and detection, in parallel lanes, protein extracts of transient over-expression in HeLa cells of the FoxP protein to be detected was run.

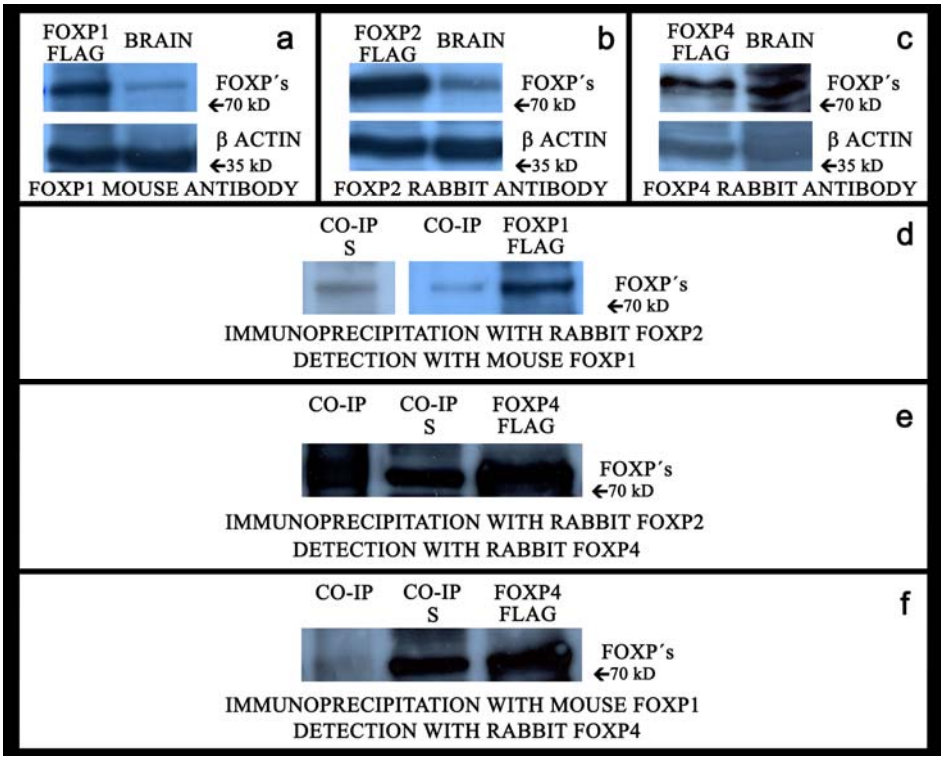



**Figure 3. 15 *In vivo* heterodimerization of FoxP1, FoxP2 and FoxP4 in brain protein extract. Detection of endogenous FoxP1, FoxP2 and FoxP4 in brain nuclear protein extract of adult male zebra finch telencephalon (panels "a", "b" and "c"). First lane from left to right in "a", "b" and "c" show proteins of over expression in HeLa cells as positive controls of detection, second lane show detection of the desired protein from brain tissue. Co-IP made from the same brain protein extracts (panels "d", "e" and "f"). Proteins were immunoprecipitated with rabbit polyclonal FoxP2 antibody or mouse monoclonal FoxP1 antibody and detected with either FoxP1 mouse monoclonal antibody or rabbit polyclonal FoxP4 antibody. Protein of over expression of FoxP1 and FoxP4 in HeLa cells were run in the lane next to the Co-IP as positive controls of detection. Supernatant after the Co-IP was also run in the same gel and detected with rabbit FoxP2 antibody (Co-IP S) or rabbit polyclonal FoxP4 antibody. A band of the expected size was seen after Co-IP for FoxP1 and FoxP4 immunoprecipitated with the rabbit FoxP2 antibody (Co-IP lane in panels d and e). FoxP4 was not detected after immunoprecipitating with FoxP1 mouse monoclonal antibody (Co-IP lane in panel f). In all cases there was still detection in the supernatant fraction (lane Co-IP S in panels "d", "e" and "f") which could be due to homo-dimerization of the detected protein that was not Co-IPed.**

We wanted to assess all three possibilities of different heterodimerization in brain tissue, namely FoxP1/FoxP2, FoxP1/FoxP4 and FoxP2/FoxP4. For this, proteins were Co-IPed with either FoxP2 or FoxP1 antibodies and detected with FoxP1 or FoxP4 antibodies (Figures 3.15 d, e and f). FoxP1 / FoxP2 (Co-IP lane, Figure 3.15 d) and FoxP2 / FoxP4 (Co-

IP lane, Figure 3.15 e) heterodimers could be detected from brain protein extracts, but not FoxP1 / FoxP4 heterodimers (Co-IP lane, Figure 3.15 f). The supernatant fraction after the immunoprecipitation shows how much protein was still present (Co-IP S lanes; Figure 3.15 d, e and f). It is possible that FoxP1 was not precipitated because dimerization with other proteins obscured the antigenic site. Switching the order of the antibodies for immunoprecipitation and detection to see if this would change t, but it did not. For instance, the FoxP1 / FoxP2 hetero-dimer was pulled down immunoprecipitating with the FoxP1 antibody first and then detecting with the FoxP2 antibody (Co-IP lane, Figure 3.16), but FoxP4 was not detectable after FoxP1 pull-down (data not shown).

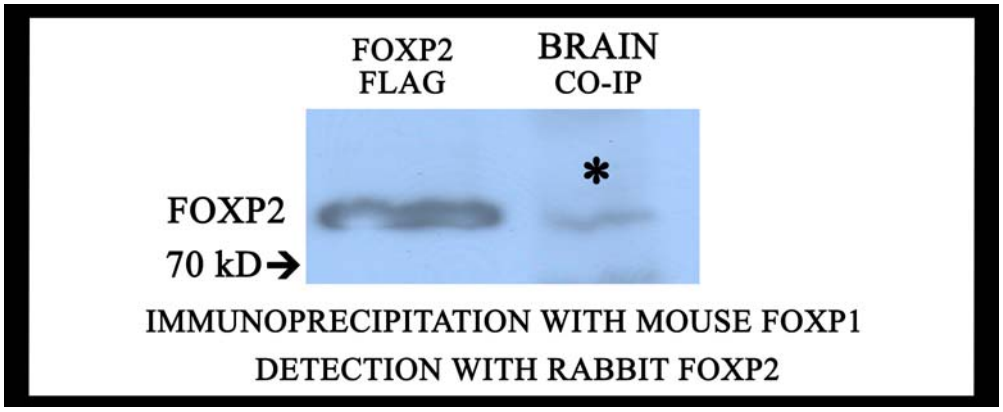



**Figure 3. 16 *In vivo* heterodimerization of FoxP1 and FoxP2 proteins. Nuclear extracts of the same whole brain protein extract (Figure 3.15) were immunoprecipitated using mouse monoclonal FoxP1 antibody and detection was done using rabbit FoxP2 polyclonal antibody. Protein of over expression of FoxP2 in HeLa cells was run in the lane next to co-immunoprecipitation (*).**

To test why the FoxP1/FoxP4 heterodimer was not pulled down using specific antibodies, but was pulled down in our *in vitro* Co-IP experiments that used V5 and FLAG-tagged antibodies (Figure 3.12) we tried pulling down this heterodimer with FoxP-specific antibodies from protein extracts of HeLa transiently over expressing different combinations of FoxP proteins (Figure 3.17). We confirmed over expression using the FLAG-tag (Figure 3.17 a) and V5- tag (Figure 3.17 b).

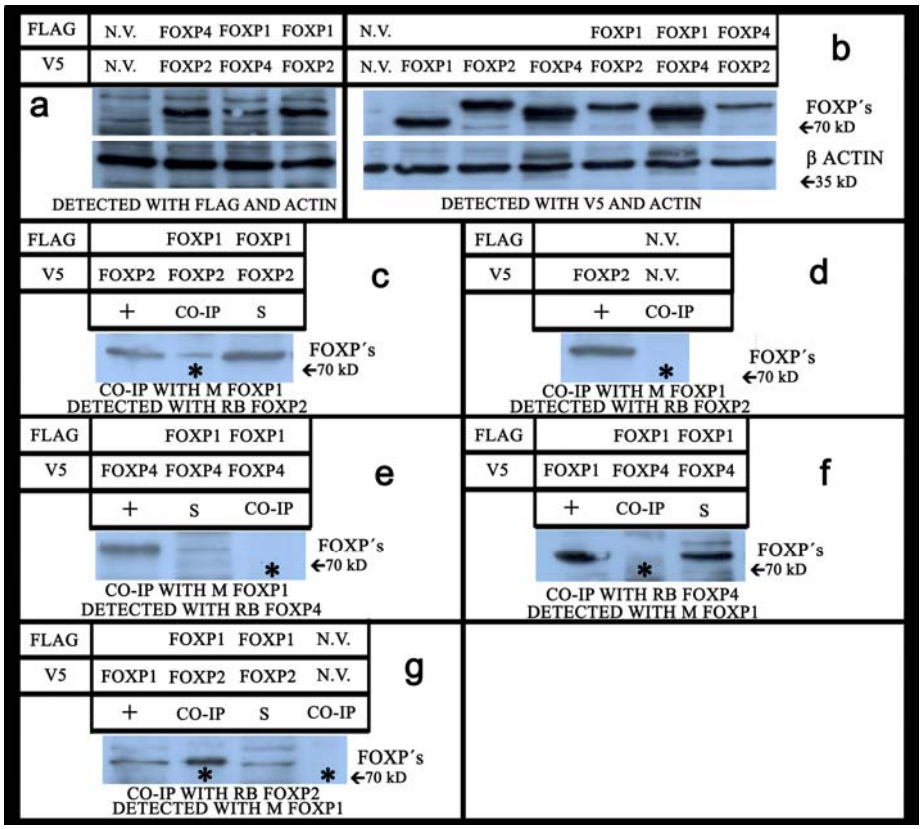



**Figure 3. 17 Testing whether the inability to detect FoxP1/P4 heterodimers *in vivo* (Fig 3.15) resulted from epitope masking, by analysis of different combinations of over expressed FoxPs in HeLa cells, comparing detection via antibodies against V5 and FLAG tags or against protein-specific epitopes. "a" and "b" Confirmation of over expression of different combinations of FoxPs in HeLa cells via detection of FLAG "a" or V5 "b" antibodies. Each FoxP was detectable regardless of whether another FoxP was also expressed by the same cells or not. "a"-"g" Different combinations of FoxP over expression, with subsequent IP and detection of potential heterodimer-partner shows that FoxP1/FoxP2 was detected by specific antibody ("c" and "g") whereas FoxP1/FoxP4 was not ("e" and "f"). A + sign in the first lane of ("a"-"g") refers to over expressed single protein as positive control. "d" Untransfected HeLa cells did not show a specific FoxP2 band after Co-IP with FoxP1.**

Again, the Foxp1/FoxP2 heterodimer was pulled down either with the FoxP1 antibody (Figure 3.17 c, Co-IP lane (*)) or the FoxP2 antibody (Figure 3.17 g; Co-IP lane (*)), since a band was seen in the Co-IP fraction. In both cases there was still some protein left in the supernatant. Also in the N.V. controls there was no band with both antibodies used (Figure 3.17 d (*); g N.V. lane (*)). Altogether these results clearly show that the FoxP1 and FoxP2 specific antibodies are able to specifically pull down the FoxP1/FoxP2 heterodimer from *in vivo* and *in vitro* samples. The FoxP1/FoxP4 hetero-dimer was not pulled down either with the FoxP1 antibody (Figure 3.17 e; Co-IP lane (*)) or the FoxP4 antibody (Figure 3.17 f; Co-IP lane (*)), since a band was not seen in the Co-IP fraction and protein was evident in the supernatant fraction (Figure 3.17 e and f; "S" lane). These results suggest that the FoxP1/FoxP4 heterodimer cannot be pulled down either by the FoxP1 or the FoxP4 antibodies, but *in vitro* results suggest that FoxP1 and FoxP4 can interact (Figure 3.12; Figure 3.14 FoxP1/FoxP4 sample). Altogether these results suggest that the FoxP1/FoxP4

heterodimer was not pulled down with the specific antibodies because the epitope was not available. This could be because of the spatial conformation of this special hetero-dimer does not allow the binding of the antibody. The tag of all proteins is in the C terminal part of the protein, which might be accessible in both cases, but in the case of the specific antibodies the epitope might be hidden.

Lastly, we performed IgG controls to assess whether IgGs pulled down any proteins that could be confused with FoxPs. Two types of antibodies were used, antibodies made in rabbit (FoxP2 and FoxP4 antibodies) and mouse (V5, FLAG and FoxP1 antibodies), for all Co-IP experiments. Therefore IgG secondary antibodies of those two species were tested and Co-IP experiments were done with them using protein extracts of over expression FoxP proteins in HeLa cells (Figure 3.18).

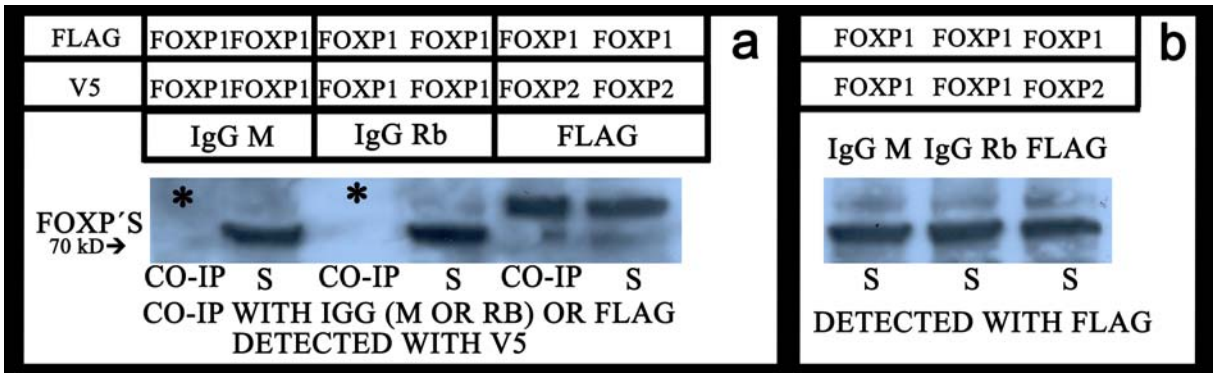



**Figure 3. 18 IgG controls for co-immunoprecipitation with rabbit and mouse antibodies. HeLa cells were transiently transfected with expression vectors encoding for FoxP1 and FoxP2 tagged with FLAG or V5. In "a" IgG control for mouse and rabbit antibodies, FLAG as a positive control for co-immunoprecipitation. Proteins extracts were co-immunoprecipitated either with IgG from mouse (M), rabbit (Rb) or FLAG antibody, all Co-IP fractions were detected with V5. Co-IP and supernatant of each sample was run side by side. The order from left to right is FoxP1-FLAG/FoxP1-V5 (IgG control for mouse antibody); FoxP1-FLAG/FoxP1-V5 (IgG control for the rabbit antibody); FoxP1-FLAG/FoxP2-V5 (FLAG positive control). In "b" supernatant fractions of all Co-IP of panel "a" were also detected against FLAG antibody, in all cases there is still protein. There is no bands in the IgG controls (*), no un-specific binding of the antibodies.**

These negative controls for co-immunoprecipitation experiments show that all antibodies used did not pull down proteins detectable with FoxP or FLAG antibodies, since no bands were detected in the Co-IP fraction of IgGs (Figure 3.18 a (*)).

Altogether these results suggest that all FoxP subfamily members can interact in the zebra finch brain.

## 3.3. Function of the interaction of FoxP1, Foxp2 and FoxP4

The, results presented here show that the most cells express more than one FoxP subfamily member and that they can form homo- and hetero-dimers as described in mice (Shanru Li *et al.*, 2004). We used the luciferase reporter system to compare in transcriptional regulation of cells expressing one or more FoxP sub family members. FoxP dimerization is needed for transcriptional regulation as well as DNA-binding (Shanru Li *et al.*, 2004), but the significance of formation of FoxP subfamily members complexes is not known. We used a Dual Glo luciferase assays where SV40 promoter in the pGL4.13 vector driving Luciferase (*Photinus pyralis* synthetic protein) and the Renilla luciferase (*Renilla reniformis*) was under the control of the CMV promoter in the pGL4.75 vector. The SV40 promoter has a putative core consensus (TATTTRT) for FoxP DNA-binding. Previous work showed that human and murine FoxP1, FoxP2 and FoxP4 (Vernes *et al*., 2006) (Wang *et al.*, 2003) down regulate this promoter individually. Therefore, we adapted the dual lucifarese method to HeLa, instead HeK cells (Figure 3.19).

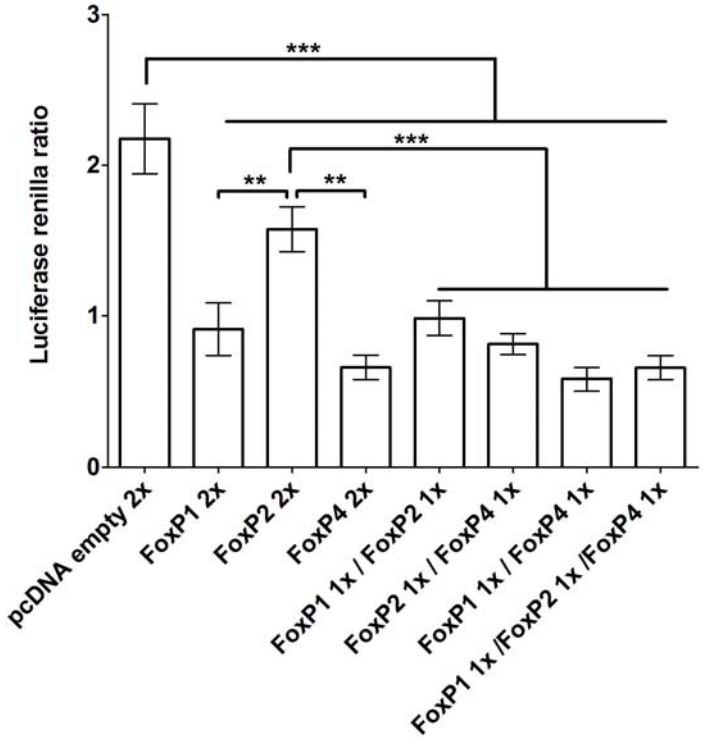



**Figure 3. 19 Luciferase assays determine transactivation properties of FOXP1/2/4 variants and different combinations of FoxP1/2/4. FoxP1, FoxP2 and FoxP4, as well as their combinations significantly (One Way ANOVA; Tukey´s Multiple comparison Test; *** P < 0,0001) repress the pGL4.13-promoter transcriptional activity through a specific DNA-binding site in the SV40 promoter. FoxP1 and FoxP4 repress significantly more than Foxp2 (** p< 0,005). All combinations were significantly different from empty vector values (*** P< 0,0001). All combinations that had FoxP2 over expression were significantly different (***) from FoxP2 expressed alone at the same concentration of the total FoxP concentration. Bars show mean of means +/- SEM of 5 independent experiments performed in triplicate presented as luciferase / renilla ratio, corrected for transfection by pGL4.75 Renilla luciferase activity. 1x = 125 ng of**

**overexpressing vector pro well, 2x = 250 ng of overexpressing vector pro well. The control transfection value was obtained with the empty expression vector (pcDNA3.1).**

Cells were co-transfected with the luciferase under the SV40 promoter, Renilla under the CMV promoter and vectors for over expression of FoxP1, FoxP2 or FoxP4 alone or in combination. We standardized the amount of over expression vector transfected (250ng vector DNA/well of a 96 well plate). Like in mouse and human, zebra finch FoxP1, FoxP2 and FoxP4, repress the SV40 promotor (***, P<0.0001) (Vernes *et al.*, 2004; Shanru Li *et al.*, 2004). Also, like its mammalian homologues, zebra finch FoxP4 repressed transcription stronger than its homologues (Wang *et al.*, 2003; Shanru Li *et al.*, 2004; Vernes *et al.*, 2006). Cells expressing combinations of the different FoxP subfamily members show also a significant repression of the SV40 promoter (***, P<0.0001). There were no differences between cells expressing FoxP1 or FoxP4 alone or in any of the combinations tested. We did observe a higher transcriptional repression in cells expressing FoxP2 in addition to another FoxP subfamily member than when FoxP2 was expressed alone FoxP2 (**, P<0.005).

## 3.4 Interaction consequences of FoxP2 mutant variant with FoxP1, FoxP2 and FoxP4 proteins

There are four FoxP2 mutations known (R553H, R328X, Q17L and Q40-Q44) to lead to a speech phenotype (McDermott *et al.*, 2005), three (R553H, R328X and Q17L) of which have been further studied (Vernes *et al.*, 2006). This study analyzed cells expressing only the mutant FoxP2 over expressed *in vitro* in HeK cells, while in patients, both the mutant and the wild type allele are expressed. They looked at these mutations as if in humans they would be homozygous and dimerization of mutant-normal FOXP2 would not happen. Cells expressing only the mutant variant were studied. But in humans cells express one affected and one normal FOXP2 allele. Another study focused on the R553H mutation when expressed with wild type FoxP2 (Mizutani *et al.*, 2007). We analyzed the interaction between R549H FoxP2 zebra finch mutation (accession number JN800736) (Figure 3.20) (analogous to the R553H of humans, and hence refer to as "R553H"), or a zebra finch FoxP2 version without the Forkhead box (refer further on as ΔFoxBox; accession number JN800737) (Figure 3.20) and other FoxP subfamily members.

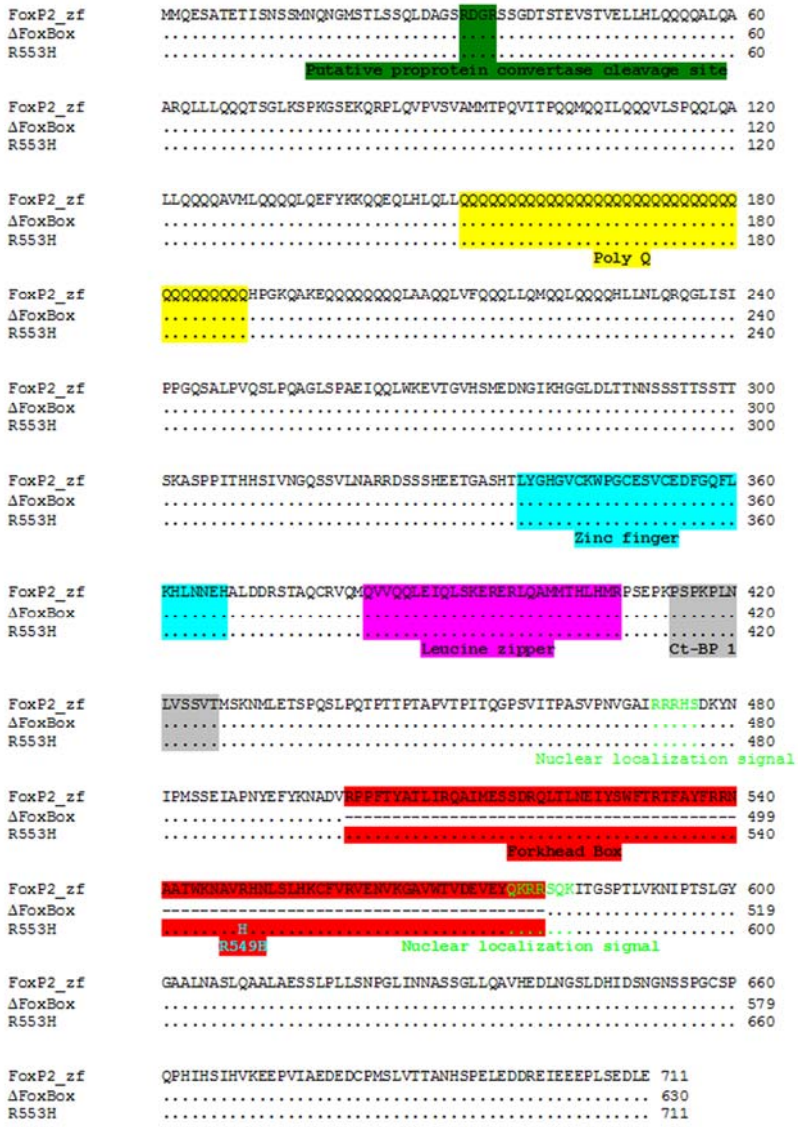



**Figure 3. 20 Sequence alignment of the zebra finch FoxP2 protein to the different mutants.. Starting from the N-terminal to the C-terminal: In green a Pro-protein Convertase cleavage site; in yellow the poly-glutamine tract (Poly-Q); in blue the zinc finger domain; in magenta the leucine finger; in gray the co-repressor C- terminal Binding protein 1; in red the Forkhead Box, absent in the ΔFoxBox version of FoxP2(--); in the Forkhead Box in blue letters the point mutation of amino acid 549, an Arginine to Histidine substitution that mimics the R553H human mutation, refer further on as R553H; in green letters nuclear localization signals.**

## 3.4.1 Interaction of mutant FoxP2 proteins

To test if FoxP2 R553H and ΔFoxBox dimerize, we tagged both with FLAG and with V5. As before, the transient over expression of the R553H and ΔFoxBox versions with the FLAG-tag (Figure 3.21 a) and V5-tag (Figure3.21 b) was tested.


**Figure 3. 21 Co-immunoprecipitation of FoxP2 R553H and ΔFoxBox of zebra finch. HeLa cells were transiently transfected with expression vectors encoding for ΔFoxBox (ΔFox) and R553H tagged with FLAG or V5. In "a" proteins were detected with FLAG and actin; in "b" proteins were detected with V5. In "c" protein extracts were immunoprecipitated with the FLAG antibody and detected with V5 antibody. In panels a and b actin serves as a loading control. In all cases (panels "a", "b" and "c") the order from left to right is ΔFoxBox-FLAG/ΔFoxBox-V5; ΔFoxBox-V5; R553H-FLAG/R553H-V5; R553H-V5. Both mutant versions are able to homo-dimerize (panel "c").**

In all cases and combinations both mutant versions of FoxP2 were successfully over expressed. Bands in the expected size were detected with the FLAG and the V5 antibody (Figure 3.21 a and b, respectively). After Co-IP, a band was detected only in the extracts were the combination with the V5-tag and FLAG-tag were present (Figure 3.21c), and not in the lanes were protein extracts had only one tag. Both mutant versions, the R553H and ΔFoxBox, are able to form a protein complex. In both cases this was expected since the domain involved in dimerization, the leucine zipper, is intact. These results further suggest that the Forkhead box is not involved in dimerization.

### 3.4.2 Interaction of mutant proteins with FoxP1, FoxP2 and FoxP4 proteins

Since both, the R553H and ΔFoxBox variants can dimerize, we tested if they interact with the wild type FoxP subfamily members. We coimunoprecipitated the variant and wild

type FoxP family members in HeLa cells transiently overexpressing these proteins. All of these proteins are similarly over expressed (Figure 3.22 a and b).

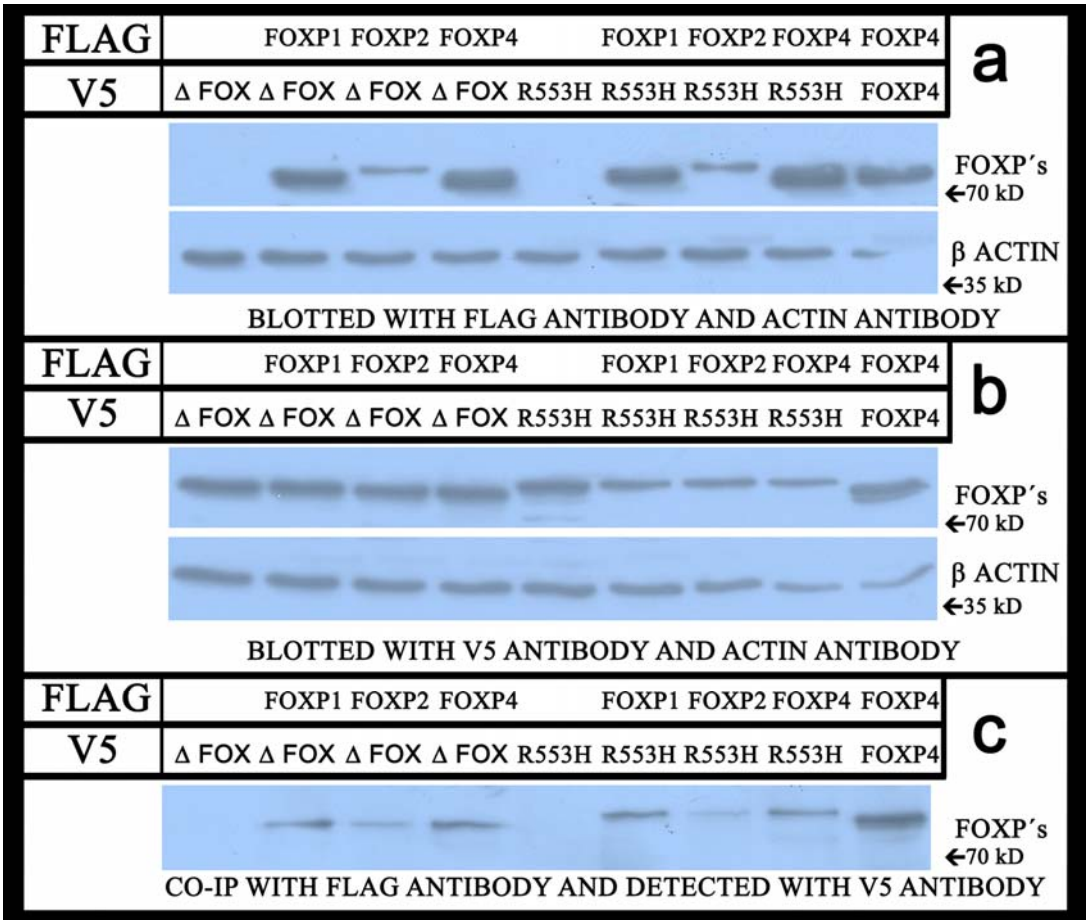



**Figure 3. 22 Co-immunoprecipitation of mutant FoxP2 versions and wild type FoxP subfamily members. In HeLa cells transiently expressing FoxP1, FoxP2 FoxP4, ΔFoxBox (ΔFox) and R553H from zebra finch tagged with FLAG or V5. In "a" proteins were detected with Flag and actin antibody as a loading control; in "b" proteins were detected with V5 and actin antibody. In "c" protein extracts were immunoprecipitated with the Flag antibody and detected with V5 antibody. In all cases (panels "a", "b" and "c") the order from left to right is ΔFoxBox-V5; FoxP1-Flag/ΔFoxBox-V5; FoxP2-Flag/ΔFoxBox-V5; FoxP4-flag/ΔFoxBox-V5; R553H-V5; FoxP1-Flag/R553H-V5; FoxP2-Flag/R553H-V5; FoxP4-Flag/R553H-V5; FoxP4-Flag/FoxP4-V5 as a positive control. Both mutant versions are able to dimerize with all normal FoxP subfamily members (panel "c").**

In all cases we detected the recombinant protein (Figure 3.22 a and b). Also in all cases a band in the Co-IP immunoprecipitate was detected (Figure 3.22 c), which means that mutant FoxP2 proteins bind to the other FoxP subfamily members. This is the first report of a FoxP2 mutant, involved in a human speech phenotype that interacts with the other two FoxP members.

### 3.4.3 Mutant FoxP2 variants change their cellular localization if expressed with wild type FoxP proteins

FoxP subfamily members localize to the nucleus but FoxP2 isoforms that lack the Forkhead box or the R553H and R328X mutants are predominantly citoplasmic (Vernes *et al.*, 2004).. We tested the localization of HeLa cells transiently expressing the V5 tagged version of the mutant FoxP2 alone and in combination with Flag tagged FoxP subfamily members (Figure 3.23).

The ΔFoxBox version was, as expected, predominantly citoplasmic (Figure 3.23 b and d), (Vernes *et al.*, 2006), since a nuclear localization signal (NLS) of FoxP2 is disrupted in this version (Figure 3.20, green letters) (Mizutani *et al.*, 2007).

The zebra finch FoxP2 R553H mutant protein is, surprisingly, localized to the nucleus (Figure 3.23 r, s and t).

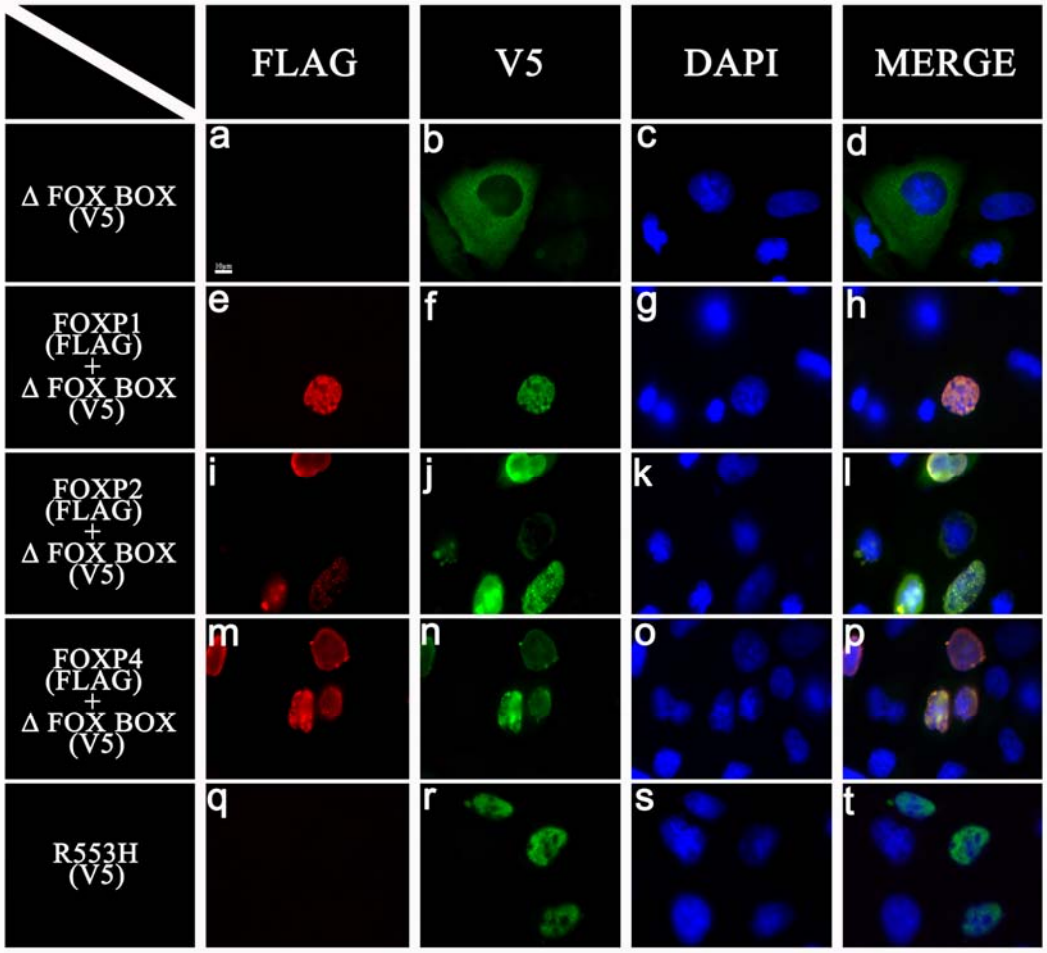



**Figure 3. 23 Immunohistochemistry results showing the intracellular distribution of zebra finch FoxP2 mutant versions R553H and ΔFoxBox in combination with wild type FoxP1, FoxP2 and FoxP4. Photos at 63x magnification of HeLa cells that were transiently transfected with V5 tagged versions of mutant FoxP2 alone (panels "a"-"d" for ΔFoxBox; and panels "q"-"t" for R553H), or in the presence of wild type FoxP1 (panels "e"-"h"), FoxP2 (panels "i"-"l") and FoxP4 (panels "m"-"p") Flag tagged versions. Flag antibody was detected with Alexa 568 (red fluorescence) (panels "a", "e", "i", "m" and "q"); V5 was detected with Alexa 488 (green fluorescence) (Panels "b", "f", "j", "n" and "r"); DAPI staining was used**

**to visualize all nuclei (blue fluorescence in panels "c", "g", "k", "o" and "s"); Merge of all channels (red, green and blue fluorescence in panels "d", "h", "l", "p" and "t"). Scales bar in panel "a" 10µm.**

Interestingly, the citoplasmic localization of FoxP2 ΔFoxBox was nuclear if co-expressed with a wild type FoxP subfamily member (Figure 3.23 b, f, j n, d, h, l and p).

### 3.4.4 Co-expression of mutant FoxP2 variants affect the regulation of wild type FoxP proteins *in vitro*

Since FOXP2 was thought to be the only member of the family expressed in specific cells, mutations in this gene were assumed to only affect, this family member. Our previous data in striatum (Figures 3.10 k-o; Figure 3.11d) and Area X (Figure 3.10 k.o; Figure 3.11c) shows that FoxP2 in zebra finch is expressed with FoxP1 and FoxP4, and they all can interact in the neurons that express them (Figure 3.12). The next question was whether mutations in FoxP2 affect the transcriptional activation of the FoxP family. We approached this problem with the Dual-Glo Luciferase assays in HeLa cells transfected with all combinations of mutant variants and normal FoxP subfamily members (Figure 3.24).

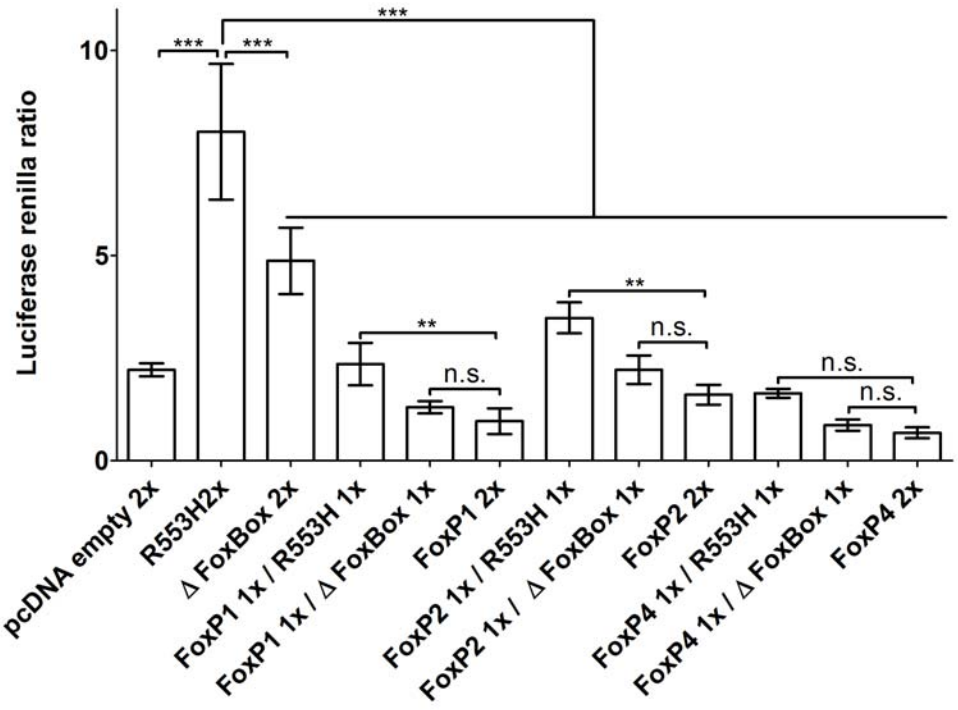



**Figure 3. 24 25 Co-expression of mutant FoxP2 protein affects the repression of the SV40 promoter by wild type FOXP1/2/4 variants. Co-expression of the R553H protein affects the regulation of FoxP1, FoxP2 and FoxP4 (One way ANOVA, Tukey´s Multiple Comparison test; ** P < 0,005) on the repression of the SV40 promoter of the pGL4.17-promoter. The ΔFoxBox version of FoxP2 did not affect the regulatory**

**properties of FoxP1, FoxP2 and FoxP4 (n.s.). Bars show mean of means +/- SEM of 3 independent experiments performed in triplicate presented as luciferase / renilla ratio, corrected for transfection by pGL4.75 Renilla luciferase activity. 1x= 125ng and 2x= 250ng of over expression vector. The control transfection value was obtained with the empty expression vector (pcDNA3.1).**

We found a similar effect to the one described by Vernes *et al*. 2006, on the transcriptional regulation of R553H mutant in zebra finch. All wild type FoxP subfamily proteins repressed transcription (Figure 3.19). The ΔFoxBox deletion repressed transcription more efficiently than the R553H mutant. Cells that express the zebra finch mutant R553H in addition to the wild type FoxP1 or 2 subfamily members are significantly different than expressing the wild type proteins alone (Figure 3.24; P<0,005).

Surprisingly, cells that expressed the ΔFoxBox protein in addition to normal FoxP subfamily members were not affected. This means that either the ΔFoxBox – wild type FoxP heterodimer is able to repress as well as FoxP-FoxP dimer, or that the mutant-wild type dimer does not bind to DNA, and the repression is mediated by wild type - wild type heterodimer.

## 3.5 Knock down of FoxP1 and FoxP4 *in vivo*

FoxP1, FoxP2 and FoxP4 are expressed in neurons in Area X which is important for vocal learning. FoxP2 is known to be important for vocal learning since mutations or deletions in humans (Lai *et al.*, 2001; MacDermont *et al.*, 2005; Sarda *et al.*, 1988; Tyson *et al.*, 2004; Shriberg *et al.*, 2006; Lennon *et al.*, 2007) lead to speech impediments and a reduction of FoxP2 in Area X of zebra finches affects song learning (Haesler *et al.* 2007). FoxP1 was also clearly linked to speech deficits in the last years (Hamdan *et al.*, 2010; Horn *et al*., 2010 and Carr *et al.*, 2010). However, we do not know if FoxP1 is required for song learning in zebra finches. The role of another subfamily member, FoxP4, is not known either in humans or birds. Here, we test the function the role of FoxP1 and FoxP4 in song learning in the zebra finch.

### *3.5.1 In vitro* selection of specific short hairpins to down regulate zebra finch FoxP1 and FoxP4

We tested *in vitro* whether different short hairpins against FoxP1 and FoxP4 cross react in HeLa cells over expressing these proteins. 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 a plasmid vector (Figure 1.6).

Three out of the 12 FoxP1 short hairpins tested (Table 2.19.1; Methods) strongly reduce the expression of this protein (Figure 3.25 a). None of these 3 short hairpins affected the expression of FoxP2 (Figure 3.25 b) or FoxP4 (Figure 3.25 c). Surprisingly, the short hairpin FoxP1-2 has only 2 nucleotide differences if compared to FoxP2 and 5 if compared to FoxP4 (Table 2.18.1), but we did not observe any cross reactivity. The other two short hairpins; FoxP1-1 and FoxP1-3, had similarities from 57% to 63% to the other FoxP members (Table 2.18.1; Methods). The sh-FoxP1-1 was the one that affected the expression of the protein the least of all three short hairpins that is why the remaining two were used for further studies.

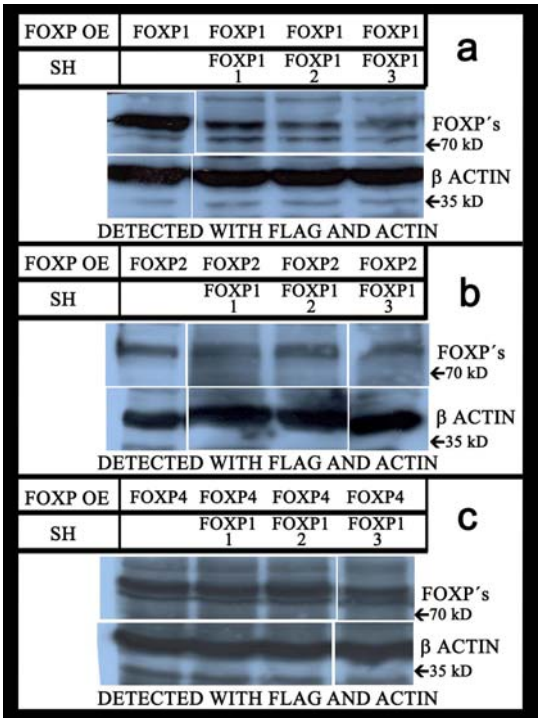



**Figure 3. 26 Functional shRNA targeting of FoxP1 *in vitro*. Hairpin expression constructs were tested for their knockdown efficiency in HeLa cells by simultaneous over expression of zebra finch FoxP1, tagged with the Flag epitope and each of different hairpin constructs. Western Blot analysis using a Flag antibody showed that three hairpins (shown in panel "a"; shFoxP1-1, shFoxP1-2 and shFoxP1-3) reduced FoxP1 levels. Neither, FoxP2 (panel "b") or FoxP4 (panel "c"), were affected, showing specificity against FoxP1. Immunostaining with an actin antibody was used as a loading control (panels "a", "b" and "c"). The ratio of over expression plasmid to sh plasmid was 1:4 respectively, in a total of 4µg DNA transfected.**

We followed a similar approach to identify short hairpins that down regulate FoxP4, specifically. Two (FoxP4-7 and FoxP4-19) of the 11 short hairpins tested (Table 2.21.1; Methods) reduced FoxP4 expression (Figure 3.26 a). Short hairpin FoxP4-5 cross reacted against FoxP2, in spite of a low similarity, and it was not considered further (data not shown). FoxP4-7 and FoxP4-19 have a similarity between 23-71% when compared to the other FoxP subfamily members (Table 2.19.1; Methods).

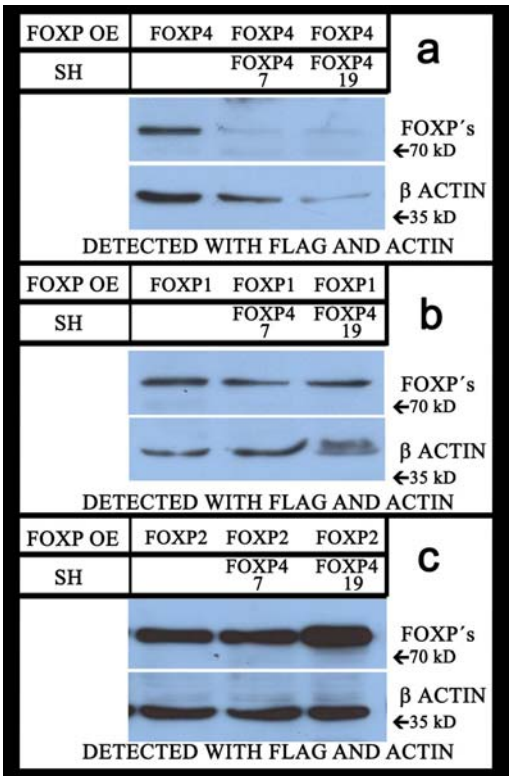



**Figure 3. 27 Functional shRNA targeting FoxP4 *in vitro*. Hairpin expression constructs were tested for their knockdown efficiency in HeLa cells by simultaneous over expression of zebra finch FoxP4, tagged with the Flag epitope and one of different hairpin constructs (shFoxP4-7 or 19). Western Blot analysis using a Flag antibody showed that shFoxP4-7 and shFoxP4-19 (panel "a") that reduced FoxP4 expression. Neither, FoxP1 (panel "b") or FoxP2 (panel "c"), were affected by these short hairpins. Immunoblotting with an actin antibody was used as a loading control (panels "a", "b" and "c"). The ratio of over expression plasmid to sh plasmid was 1:8 respectively, in a total of 4µg DNA transfected.**

In a previous study (Haesler *et al.*, 2007) a non targeting short hairpin control (sh-control) was shown not to target FoxP2 (Table 2.18.1). We used the same sh-control in this study and showed that it did not affect the expression of either FoxP1 or FoxP4 (Figure 3.27).

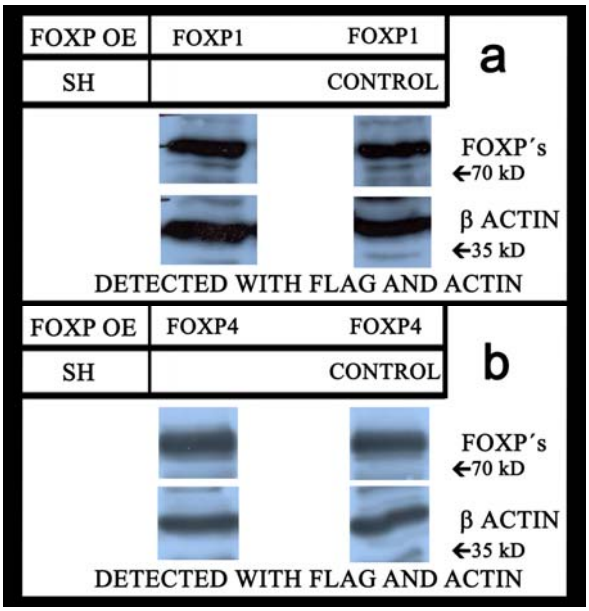



**Figure 3. 28 sh-control does not affect the expression of FoxP1 or FoxP4 *in vitro*. The sh-control hairpin does not affect the expression of FoxP1 or FoxP4 in HeLa cells. Western Blot analysis using a Flag**

**antibody revealed no effect on FoxP1 (shown in panel "a") and FoxP4 (shown in panel "b") protein levels. Immunoblotting with an actin was used as loading control (panels "a" and "b"). The ratio of over expression plasmid to sh plasmid was 1:4 respectively, in a total of 4µg transfected DNA.**

We tested whether the short hairpins selected *in vitro* and expressed in a virus also carrying GFP would co-localize with FoxP1 neurons *in vivo*, targeting the right cells. We injected stereotactically control virus into Area X of a 23 PHD bird and then analyzed the co-localization of FoxP1 by immunohistochemistry with GFP on 40µm slices (Figure 3.28) as described in methods. We were not able to test the co-localization of FoxP4 with GFP because the antibody did not work on perfused tissue, and GFP signal is lost after freezing. The best approximation of FoxP4 cell targeting is the FoxP1 antibody, since the majority of cells that express FoxP1 are also FoxP4 (Figure 3.10 "o"; Figure 3.11 "c") or FoxP2 which was shown in Haesler *et al.*, 2007.

The majority of cells were found to express GFP and also FoxP1 (Figure 3.28 b, c, d and e). About 89% of the GFP cells were FoxP1 positive (Figure 3.28 e). In two different studies in zebra finches that used the same virus, where the short hairpin was under the same promoter (U6 promoter), had similar results. In the first one they did immunohistochemistry against Hu, which is a neuronal marker, and more than 80% of the GFP expressing cells were also Hu positive (Wada *et al.*, 2006). In the second study, Haesler *et al.*, 2007 reported the co-localization of 78.5% ± 3.5% of GFP+/Hu+ neurons in Area X.

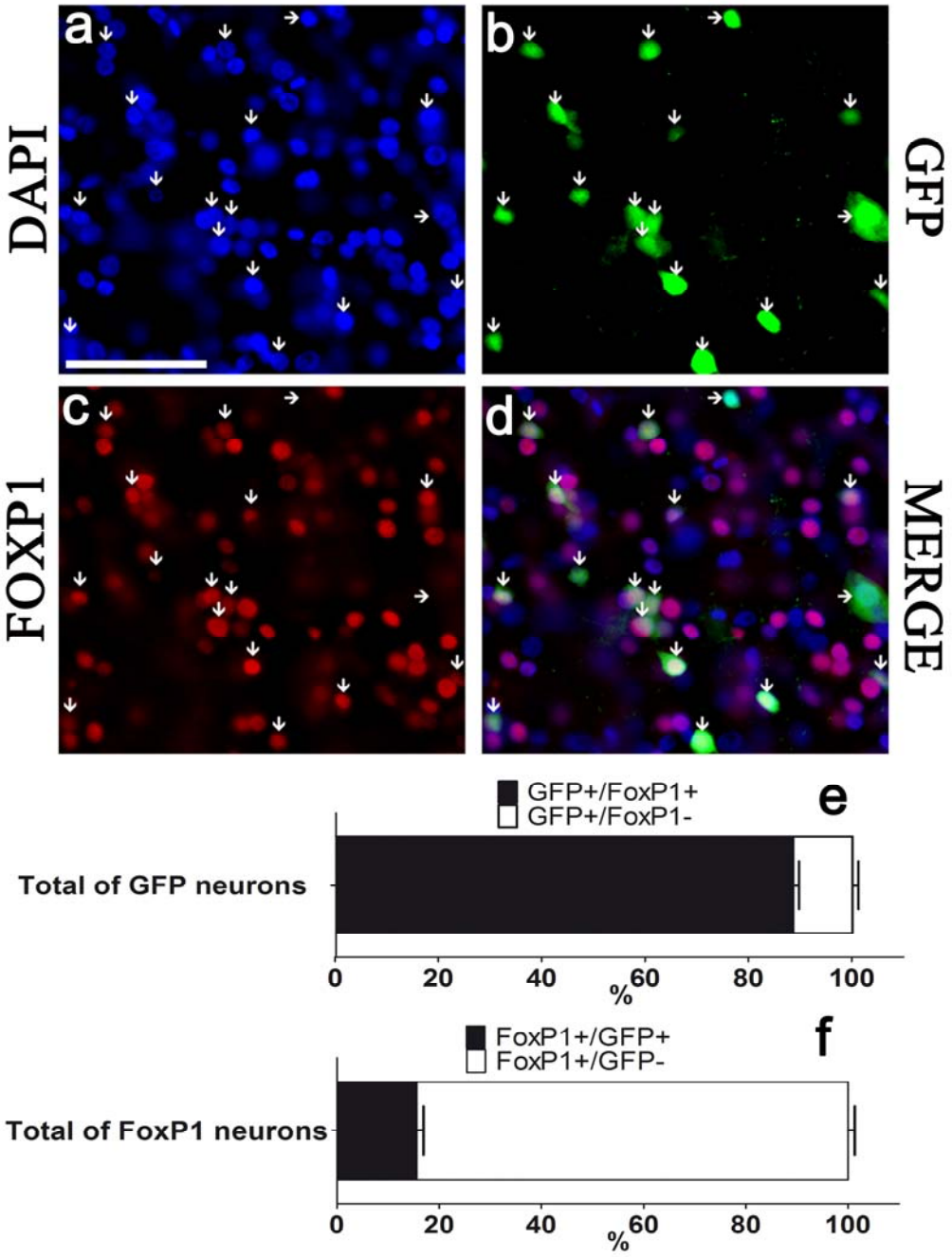



**Figure 3. 29 Quantification of FoxP1 neurons infected by short hairpins not targeting FoxP1. Example of a FoxP1 immunohistochemistry of a 40x magnification Z stack projected photo in Area X of a male zebra finch injected with sh-control virus (in panels "a", "b", "c" and "d"). In panel "a" DAPI staining in blue fluorescence staining all cell nuclei; in "a" scale bar 50µm; in panel "b" virus infected neurons expressing GFP in green fluorescence; in panel "c" immunohistochemistry detecting FoxP1 in red fluorescence. Panel "d" shows the merge DAPI, GFP and FoxP1. Arrows pointing down are GFP+/FoxP1+ and arrows pointing left are GFP+/FoxP1-. In "e" percentage of the total infected neurons expressing GFP and FoxP1. In "f" percentage of the FoxP1 neurons infected by the virus. In "e" and "f" bars refer to mean of means + standard error of the mean [SEM] of 4 photos, in each hemisphere of 3 different animals, 24 in photos total.**

Only around 16% of the total FoxP1 population expressed GFP, indicating a low infection ratio (Figure 3.28 f). Previously we showed that there are more cells expressing

FoxP1 or FoxP4 than FoxP2 positive cells in Area X (Figure 3.11 a). Thus, we could target a higher percentage of FoxP2 cells than for the other FoxPs.

We tested if the virus reduces the amount of mRNA of FoxP1 or FoxP4 *in vivo.* We followed a similar procedure as the one described in Haesler *et al.*, 2007 (Figure 3.29). Birds were injected stereotactically in Area X with the control short hairpin in one hemisphere and a short hairpin against FoxP1 or FoxP4 in the contra lateral hemisphere on 23 PHD. At 50 PHD punches in Area X were cut from 200µm slices and stored at -80°C. Remaining tissue was put in 4% PFA overnight and assessed for a correct Area X punching and GFP. Q-PCR of mRNA extracted of every punch in Area X was used for assessing a reduction in levels of FoxP1 and FoxP4 compared to the controls side.

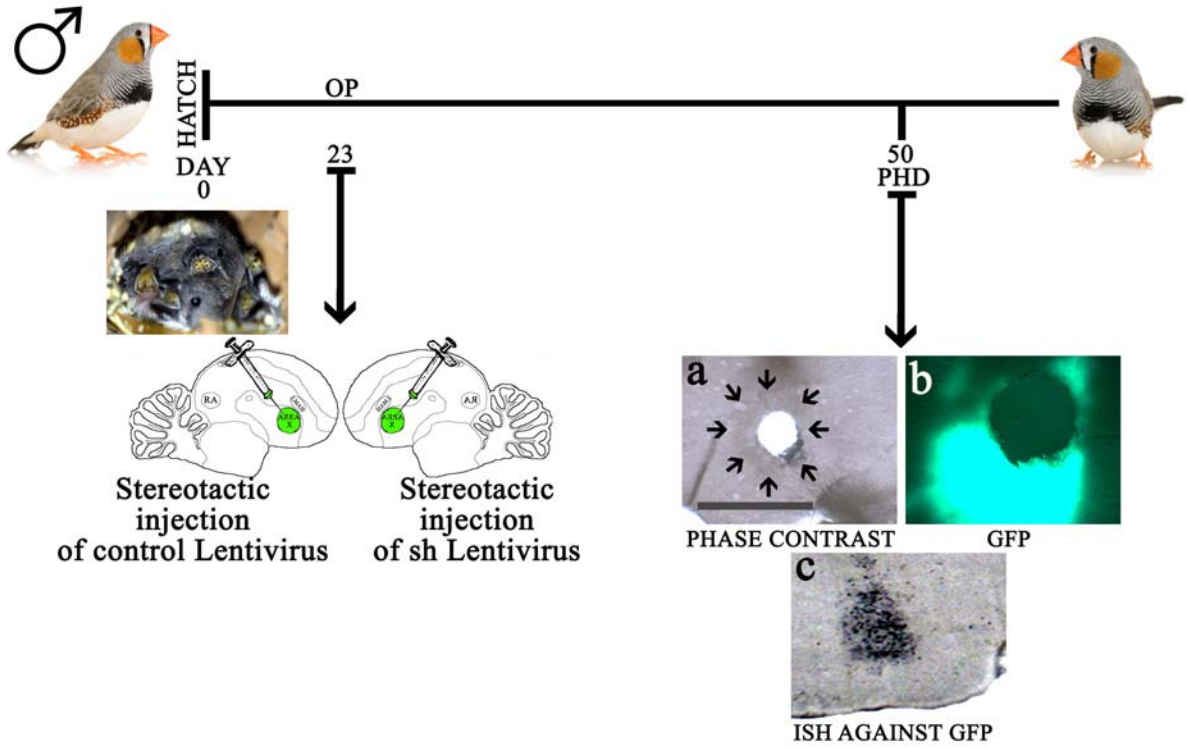



**Figure 3. 30 Timeline of FoxP1 and FoxP4 QPCR experiments using lentiviral mediated RNAi *in vivo*. On day 23 PHD I injected a sh-control virus in one hemisphere and a sh against either FoxP1 or FoxP4 in the contra lateral one of Area X of male zebra finches. After the operation the birds were kept with their parents until day 50 PHD. Then, the brains were extracted, frozen and stored -80°C. Intercalated 12µm and 200µm slices were cut in the cryostat. We punched Area X in the 200µm slices. The punch was stored at -80°C in an eppendorf for mRNA extraction. The remaining 200 µm slices (after punching) were fixed and GFP expression in the surrounding of the slices was assessed (panel "b") and location of the punch in Area X was assessed with normal phase contrast (panel "a") scale bar 2mm.The 12µm slices were used for *in situ* hybridization to detect GFP (panel "c"), to determine the injection size in the adjacent slice of the punch.**

We only used punches that were in Area X, regardless of whether GFP is expressed or not. This allowed us to determine whether there levels of FoxP1 and FoxP4 mRNA were

reduced in Area X. Detection of GFP via ISH reported on whether the injection was in Area X or not. Only birds where GFP was expressed in Area X were included in the study. The MIQE (minimum information for publication of quantitative real-time PCR experiments) information of the Q-PCR results is in Appendix Table 6.1.

We report the mean of the Q-PCR of each punch normalized to HMBS. For FoxP1 and FoxP4, birds were injected on 23 PHD with sh-FoxP1 -2/3 or FoxP4-7/19 respectively into Area X in one hemisphere and the sh-control in the contra lateral one (Figure 3.29).

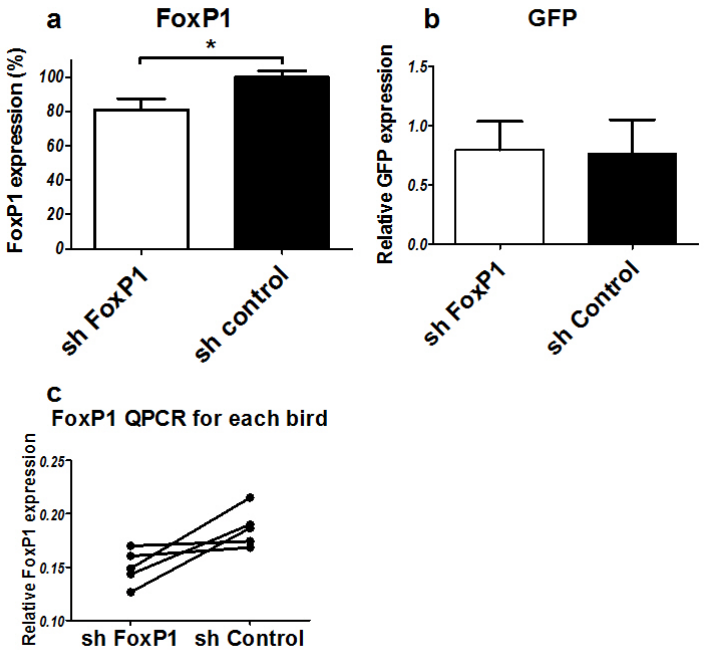



**Figure 3. 31 Quantification of *in vivo* FoxP1 knockdown efficiency. Results of Q-PCR experiments on FoxP1 expression in Area X on PHD50 (graph a and c). Animals were injected with sh-control in one hemisphere and shFoxP1-2/3 virus in the contra lateral one, on PHD23. In "a" bars represent relative gene expression between sh-control and sh-FoxP1-injected hemispheres, normalized to Hmbs [± standard error of the mean (SEM); n=5 animals, two tailed t-test = P< 0,05 ] In "b" Q-PCR of GFP in both hemispheres normalized to Hmbs, no significant differences were seen between the GFP expression of hemispheres injected with sh-control or sh-FoxP1-2/3 (two tailed t-test; n.s. P>0,05). In "c" raw data of the Fluorescence (not normalized to the control side as on "a") of each bird, showing lower expression in 3 of 5 birds in the sh-FoxP1 injected hemisphere compared to the control hemisphere.**

We found a decrease of about 20% of the FoxP1 mRNA in Area X injected with the sh-FoxP1-2/3 virus compared to the sh-control (Figure 3.30 a; two tailed t-test; *P<0,05). The GFP mRNA levels between both hemispheres were not statistically different, indicating that the viral infection in both hemispheres was similar (Figure 3.30 b). Last, the data for each bird (Figure 3.30 c) is shown, 3 of 5 birds injected had a decrease in FoxP1 mRNA in the sh-FoxP1 injected hemisphere if compared to the sh-control one, which shows that virus is not always reducing the mRNA levels as expected.

We reached similar results with the same approach for FoxP4 (Figure 3.31).

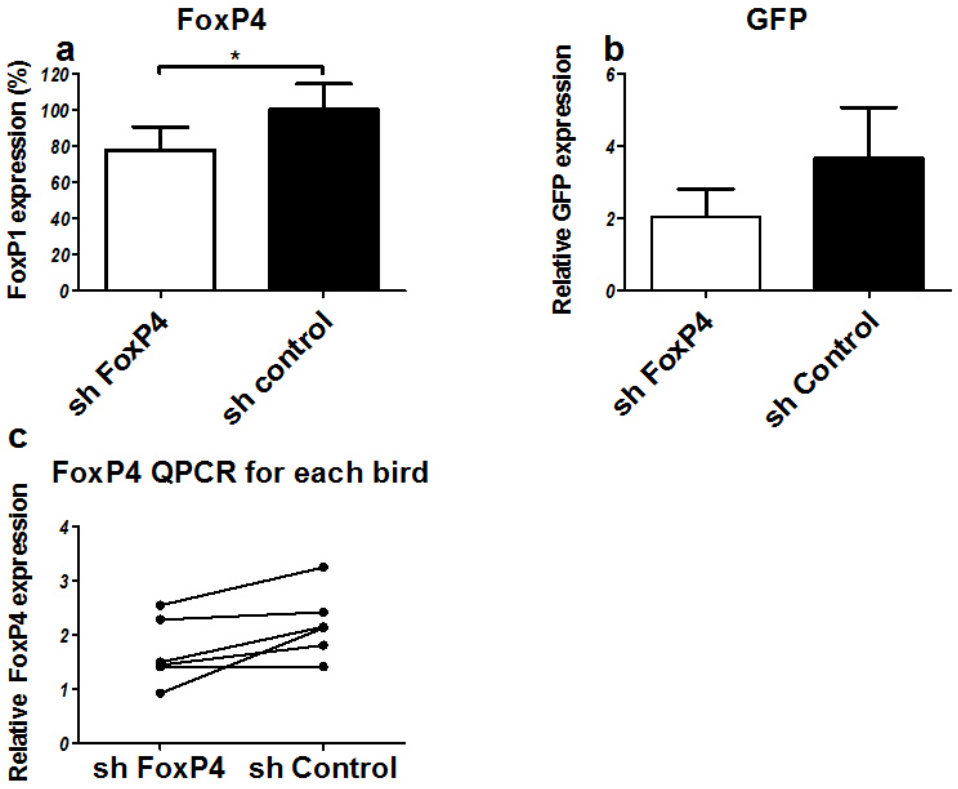



**Figure 3. 32 Quantification of *in vivo* FoxP4 knockdown efficiency. Results of Q-PCR experiments on FoxP4 expression in Area X on PHD50 (graph "a" and "c"). Animals were injected with sh-control in one hemisphere and shFoxP4-7/19 virus in the contra lateral one, on PHD23. In "a" bars represent relative gene expression between sh-control and sh-FoxP4-injected hemispheres, normalized to Hmbs [± standard error of the mean (SEM); n=6 animals, * = P< 0,05 ]. In "b" Q-PCR of GFP in both hemispheres normalized to Hmbs, no significant differences were seen between the GFP expression of hemispheres injected with sh-control or sh-FoxP4-7/19 (two tailed t-test; n.s. P>0,05). In "c" raw data of the Fluorescence (not normalized to the control side as on "a") of each bird, showing lees expression in 4 of 6 birds in the sh-FoxP4 injected hemisphere compared to the control one.**

We observed a reduction of around 20% also for FoxP4 mRNA levels in Area X expressing the sh-FoxP4 (Figure 3.31a; two tailed t-test; *P<0,05). No difference in the GFP expression was observed when comparing both hemispheres (Figure 30.31 b).

Taken together, these data demonstrate that virus-mediated RNAi can induce significant FoxP1 and FoxP4 expression in Area X.

### 3.5.2 FoxP1 and FoxP4 knockdown affects song learning in zebra finches

We analyzed the behaviour of birds where FoxP1 or FoxP4 were knockdown in Area X. Adult zebra finch song is composed of sound elements, called syllables (marked in blue

brackets in Figure 3.32 b), and separated by silent intervals. Syllables are rendered in a stereotyped sequential order, constituting a motif (marked in red brackets in Figure 3.32 “a” and “b”). During a song bout (marked in a black bracket in Figure 3.32 a), a variable number of motifs are sung in short succession.

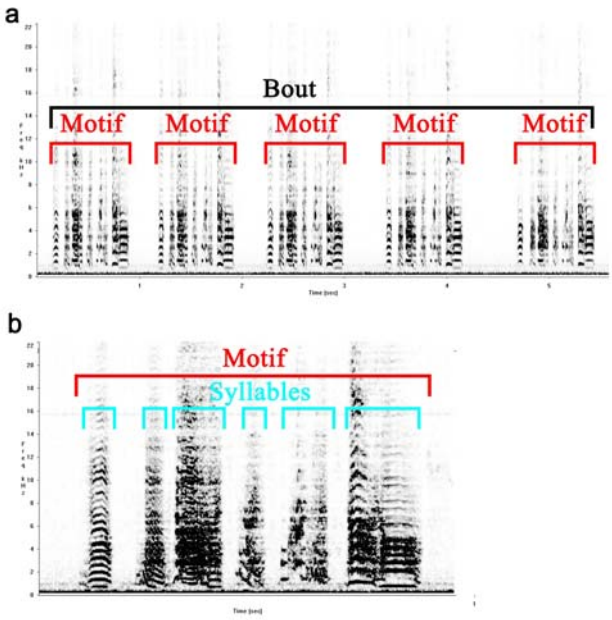



**Figure 3. 33 Sonograms of an adult zebra finch. In “a” an example of a sonogram of a bout of an adult zebra finch tutor bird, marked is the bout in the black brackets and the motifs in red brackets. Tutors that were taken in the behavioural study were selected because they had a very stereotyped song, which means that the same motif is sung all the time with no changes in syllables, as seen in sonogram “a”. In ”b” a motif of the same bird. In red brackets a motif, in blue brackets the different syllables are marked.**

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). Learning success was determined by the degree of acoustic similarity between pupil and tutor motifs. To test whether FoxP1 or FoxP4 contribute to song learning in zebra finches the levels of both genes were reduced separately in Area X *in vivo*, using lentivirus-mediated RNA interference (RNAi). On 23 PHD, the beginning of the sensory learning phase, the virus was injected stereotactically into Area X, afterwards the birds were separated from any male but left in its home cage. Starting on 30 PHD, each pupil was kept in a sound isolation chamber, together with an adult male zebra finch as a tutor and we recoded the song continuously using Sound Analysis Pro (SAP). At the end of the learning phase, at 90 PHD, the tutor was taken out of the isolation chamber and the birds´ vocalization was recorded for another 5 days and used for subsequent song analysis (for timeline of experiments see Figure 3.33).

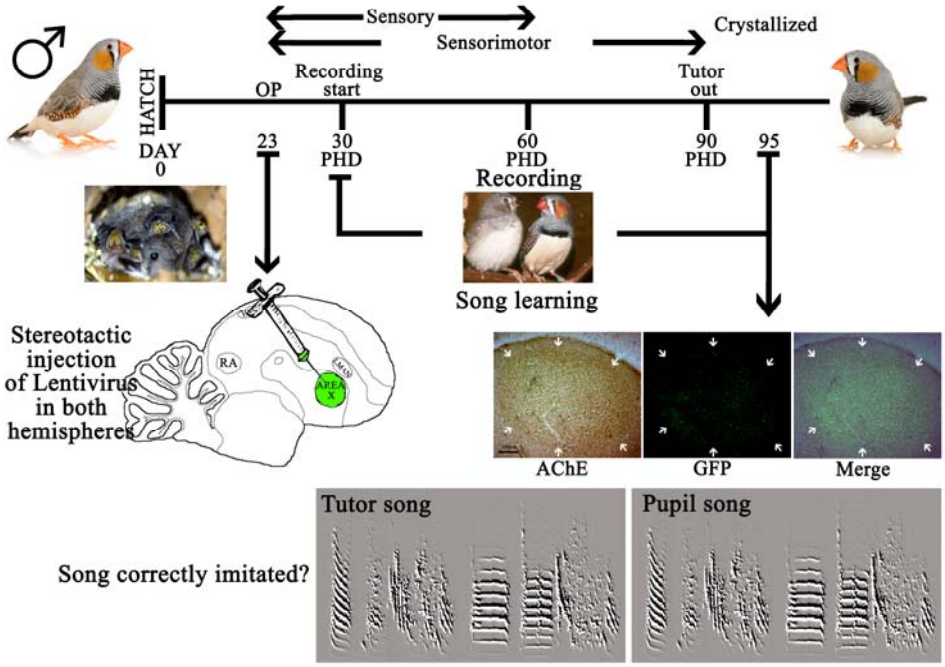



**Figure 3. 34 Timeline of Foxp1 and FoxP4 knockdown in Area X and behavioural experiments. In the first two weeks after hatching the birds were sexed. On day 23 PHD, at the beginning of the sensory learning period, either sh-control, sh-FoxP1-2/3, or sh-FoxP4-7/19 virus was bilaterally injected into Area X of male zebra finches. From 30 PHD on, injected birds were housed in sound-recording chambers together with an adult male zebra finch as tutor. The song was recorded during the learning phase using an automated recording system. I removed the tutor on day 90 PHD from the sound-recording chambers and recorded for another 5 days. After this period I perfused the bird and cut the brain in 40µm slices to assess if virus was targeted to Area X and to quantify the area affected using the GFP marker. I used Acetylcholinesterase (AChE) to identify AreaX. Last I compared the songs of the pupil and the tutor to address if learning was impaired.**

We assessed GFP in Area X in 40µm slices cut on the vibratome. Every fourth slice was stained for AChE to identify Area X. A photo of each of these slides was taken in bright-field and fluorescence (Figure 3.34 a, b and c) and the volume of GFP and Area X was calculated as described in Tramontin *et al.* (1998). We then quantified the percentage of Area X infected and determined the mean of both hemisphere in each bird (Figure3.34 c). The percentage of each hemisphere for each condition is also shown (Figure 3.34 d). The mean of both hemispheres indicates the volume of Area X that was targeted in the bird. The results of FoxP1 and FoxP4 knockdown were compaired with previous results on FoxP2 (Haesler *et al.*, 2007, Figure 3.34 c and d). Birds without detectable GFP or that were not on target were not considered for further analysis. The volume of the infected area was similar across hemispheres, and also similar for all FoxPs and for control birds (One way ANOVA; $P>0.05$; Figure 3.34 d; hemispheres (one way ANOVA; $P>0.05$; Figure 3.34 e). On average, the infected volume of Area X was 35.93% for FoxP1 (SEM 18.3), 22.81% for FoxP2 (SEM 9.6), 28.61% for FoxP4 (SEM 16.27) and 19.64% for the control (SEM 8.9), which is in the same

range but slightly higher than the 20.4% reported before for FoxP2 knockdown birds (Haesler *et al*, 2007).

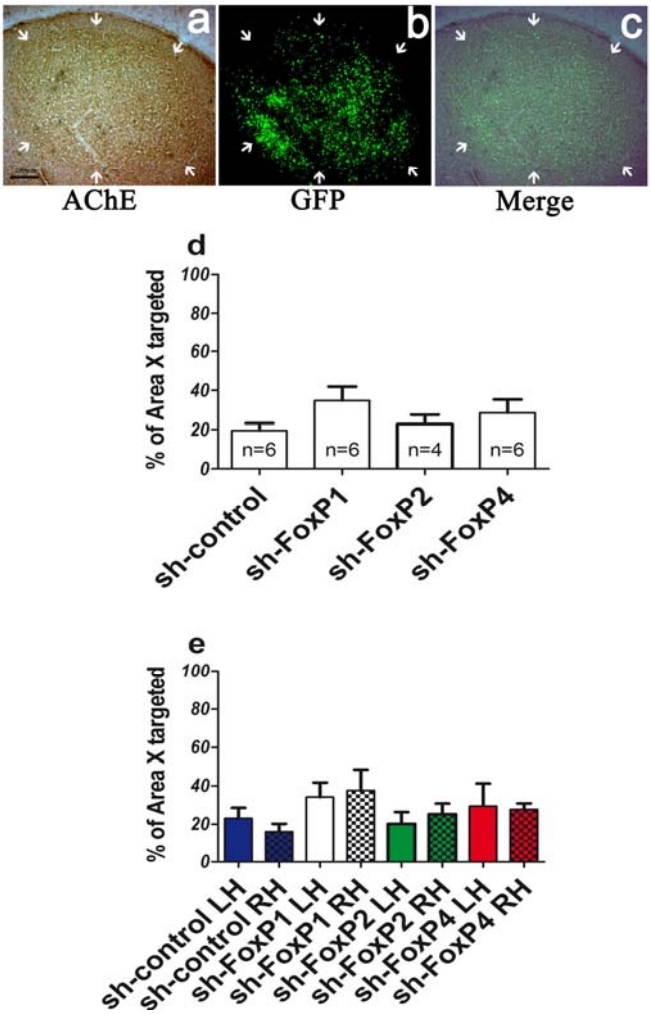
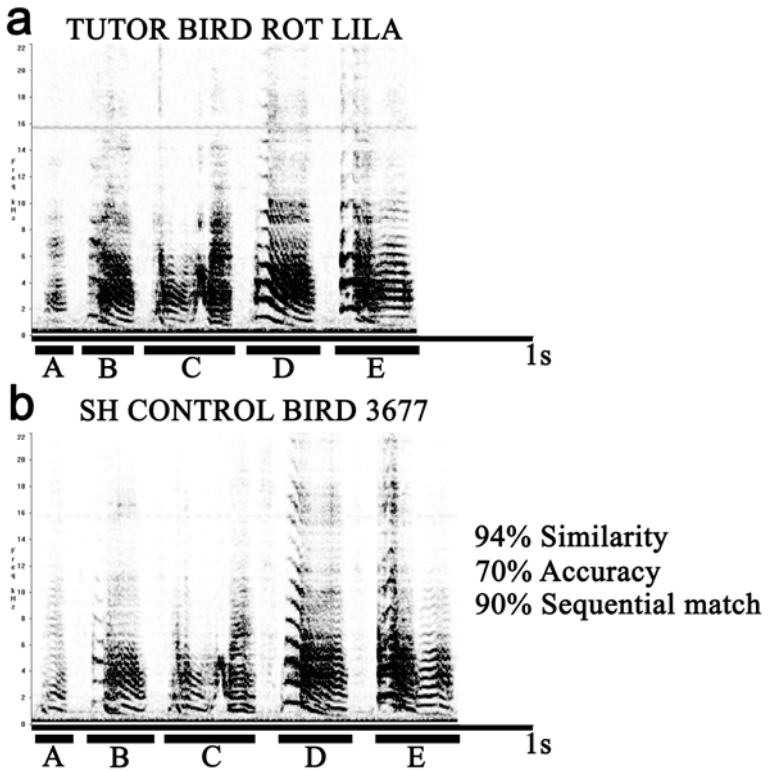



**Figure 3. 35 Quantification of Area X volume targeted by the viral infection. A representative image of a bird expressing GFP in Area X (panel "a", "b" and "c"). In "a" a bright-field photo of a sagittal section stained for AChE showing Area X, delineated by white arrows, scale bar 200µm. In "b" same section showing the expression of GFP. In "c" merged bright-field and GFP channel. In "d" the quantification of the volume of the different birds that expressed GFP in Area X and considered for further analysis (n=6 for sh-FoxP1, sh-FoxP4 and sh-control; n=4 for sh-FoxP2 data from Haesler). Bars represent the percentage of total Area X volume, averaged across hemispheres, expressing the viral reporter GFP (±SEM). In "e" the same birds as in "d" but showing the average for each hemisphere (±SEM).**

Song learning success was quantified with Sound Analysis Pro [SAP2011 (Tchernichovski *et al.*, 2001)] using crystallized song at >95PHD. SAP2011 provides a similarity score that indicates how much of the tutor sound material was copied by the pupil. Accuracy refers to the local average similarity scores across the motif. It is a measure of how precise the different syllables were copied.

An example of a sonogram of a tutor and its respective pupil injected with sh-control is shown in Figure 3.35 a, b and c. The overall similarity of the motif is 94%, accuracy 70% and

sequential match 90% (Figure 3.35 b). All sh-control birds copied all syllables and the sequence from the tutor. Of the tutor's 5 syllables, the pupil sang 4 syllables with a similarity higher than 80% and one with a similarity of 66% (Figure 3.35 c). The sequence of syllables was correct, and the accuracy ranged from a score of 67 to 77 (Figure 3.35 c). Syllable B of the pupil was slightly longer than syllable B of the tutor (Figure 3.35 b).



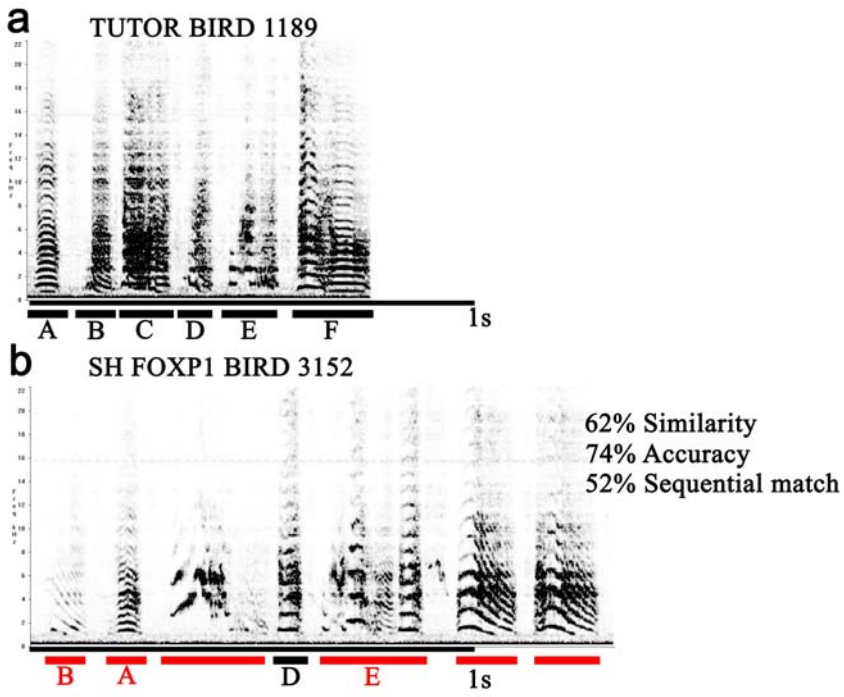

c

| Syllable | Similarity | Accuracy |
|---|---|---|
| A | 86 | 71 |
| B | 93 | 77 |
| C | 98 | 68 |
| D | 80 | 72 |
| E | 66 | 67 |

**Figure 3. 36 Example of song learning in a sh-control injected bird. Sonograms from a tutor and a sh-control pupil are shown in "a" and "b". Each sonogram depicts a representative motif of each animal (scale bars 1s, frequency range 0-22kHz). 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 SAP2011 software. In "a" a sonogram of a motif of the tutor bird. In "b" a sonogram of a motif of a sh-control pupil, overall similarity, accuracy and sequential match scores for this comparison are shown. In "c" table comparing similarity and accuracy scores of each pair of syllables, i.e. syllable A of the tutor with A of the pupil etc.**

Next, the sonogram of a tutor bird and its respective sh-FoxP1 injected pupil is shown (Figure 3.36 "a" and "b"). Typical features of FoxP1 knockdown birds are: omissions of syllables (Syllables C and F were not found in the sh-FoxP1 song; Figure 3.36 b); switching of order (sh-FoxP1 bird sang BA syllables instead of AB sung by the tutor; Figure 3.36 a and b); imprecise copying of syllable duration (Syllable E of the sh-FoxP1 bird; Figure 3.36 b); inaccurate imitation of syllables (Syllable E of the sh-FoxP1 bird; Figure 3.36 b); and addition

or inaccurate copying of syllables (all syllables in the sh-FoxP1 that could not be matched with the tutor song, syllables without a letter; Figure 3.36 b). Overall the motif similarity (62%) and sequential match (52%) are worse than those of the sh-control bird, and the accuracy (74%) was 4% better than that of the sh-control. Of 6 syllables that composed the tutor motif four were copied (syllable A, B, D and E), two of four syllables copied had a good similarity (syllable A and D; about 90%), and the other two were around 48% (syllable B and E). Accuracy of the copied syllables ranged from 61-77%.



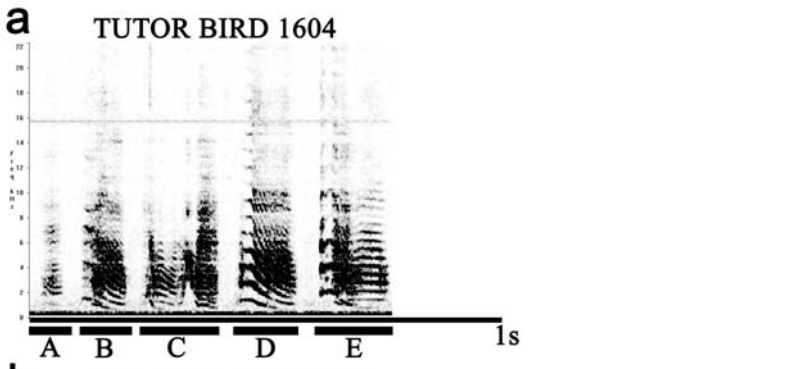

c

| Syllable | Similarity | Accuracy |
|---|---|---|
| A | 90 | 76.61 |
| B | 47 | 77.54 |
| D | 89 | 72.09 |
| E | 49 | 61.63 |

**Figure 3. 37 Example of song learning in a sh-FoxP1 injected bird. Sonograms from a tutor and a sh-FoxP1 pupil are shown in "a" and "b". Each sonogram depicts a representative motif of each animal (scale bars 1s, frequency range 0-22kHz). Tutor syllables are underlined with black bars and identified by letters. For each pupil syllable the letter underneath the bar indicates the syllable that was the best match to the tutor, as determined with SAP2011 software. Red lines and letters identify syllables that were sung in a different order than the pupil and with variable copy fidelity. "a" shows a sonogram of a motif of a tutor bird. "b" shows a sonogram of a motif of a sh-FoxP1 bird pupil. Overall similarity, accuracy and sequential match scores for this comparison are shown. "c" table comparing similarity and accuracy scores of each pair of syllables, i.e. syllable A of the tutor with A of the pupil etc. Syllables without a letter had 0% similarity with any of the tutor motif syllables.**

Another example of a tutor bird and its respective sh-FoxP1 injected pupil bird is shown in the next figure (Figure 3.37 a and b). In addition to the features described for the previous sh-FoxP1 bird syllables repetitions were found (syllable A was sung three times in the sh-FoxP1 bird; Figure 3.37 b). In this example there is no changing of order. Of 5 syllables that compose the tutor motif, the sh-FoxP1 of this example copied only 3, two of them with a

good similarity (more than 90%, syllables A and D; Figure 3.37 c), and one with a bad similarity (68%, syllable C; Figure 3.37 c).

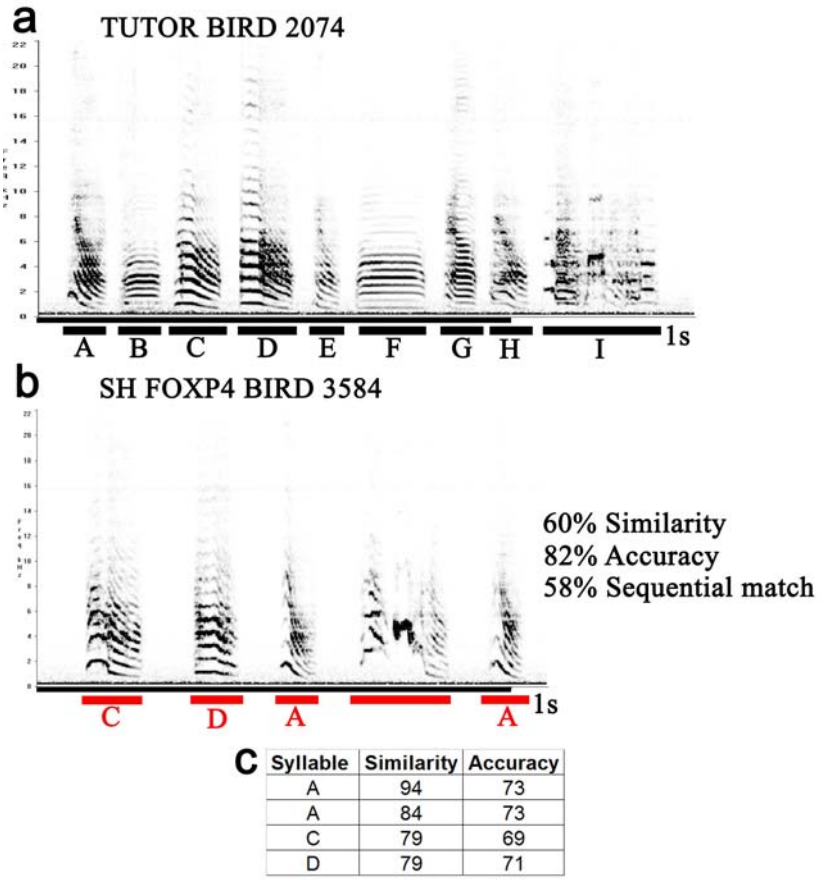



c

| Syllable | Similarity | Accuracy |
|---|---|---|
| A | 91 | 74 |
| A | 91 | 75 |
| A | 91 | 67 |
| C | 68 | 65 |
| D | 96 | 73 |

**Figure 3. 38 Example of song learning in another sh-FoxP1 injected bird. Sonograms from a tutor and a sh-FoxP1 pupil are shown in "a" and "b". ". Each sonogram depicts a representative motif of each animal (scale bars 1s, frequency range 0-22kHz). Tutor syllables are underlined with black bars and identified by letters. For each pupil syllable the letter underneath the bar indicates the syllable that was the best match to the tutor, as determined with SAP2011 software. Red lines and letters identify syllables that were sung in a different order than the pupil and with variable copy fidelity. "a" shows a sonogram of a motif of a tutor bird. "b" shows a sonogram of a motif of a sh-FoxP1 bird pupil. Overall similarity, accuracy and sequential match scores for this comparison are shown. "c" table comparing similarity and accuracy scores of each pair of syllables, i.e. syllable A of the tutor with A of the pupil etc. Syllables without a letter had 0% similarity with any of the tutor motif syllables.**

Next sonograms of a tutor bird and its respective sh-FoxP4 injected pupil bird are shown (Figure 3.38 a and b). The same features as described for FoxP1 knockdown birds are found. In this case of 9 syllables sung by the tutor birds, only 3 were copied (syllables A, C and D). Again that similarity and sequential match were found to be worse than the sh-control bird, but accuracy of the sh-FoxP4 is better than the one of the sh-control bird shown before (Figure 3.35 b).




c

| Syllable | Similarity | Accuracy |
|---|---|---|
| A | 94 | 73 |
| A | 84 | 73 |
| C | 79 | 69 |
| D | 79 | 71 |

**Figure 3. 39 Example of song learning in a sh-FoxP4 injected bird. Sonograms from a tutor and a sh-FoxP4 pupil are shown in "a" and "b". Each sonogram depicts a representative motif of each animal (scale bars 1s, frequency range 0-22kHz). Tutor syllables are underlined with black bars and identified by letters. For each pupil syllable the letter underneath the bar indicates the syllable that was the best match to the tutor, as determined with SAP2011 software. Red lines and letters identify syllables that were sung in a different order than the pupil and with variable copy fidelity. "a" shows a sonogram of a motif of a tutor bird. "b" shows a sonogram of a motif of a sh-FoxP1 bird pupil. Overall similarity, accuracy and sequential match scores for this comparison are shown. "c" table comparing similarity and accuracy scores of each pair of syllables, i.e. syllable A of the tutor with A of the pupil etc. Syllables without a letter had 0% similarity with any of the tutor motif syllables.**

Taken together, these representative sonograms show that sh-control birds copied all syllables of the tutor and sung them in the correct order. Sonograms of FoxP1 and FoxP4 knockdown birds show similar features to the ones described in a previous study about FoxP2 (Haesler *et al.*, 2007). All FoxP knockdown birds did copy de tutor song incomplete.

10 motifs of each of the birds were taken and used compared the songs of sh-control and sh-FoxP-1/4 birds using SAP2011. Again, also song data of FoxP2 down-regulation was taken, and treated in the same way as the data for FoxP1 and FoxP4, to see if our approach would show the same differences and ranges reported by Haesler *et al.* 2007. Results show similarity, accuracy and sequential match scores of an M x N batch similarity analysis (which compares all 10 motifs of the tutor to all the 10 motifs of the pupil, having at the end 100 independent results). The similarity score was significantly lower in all FoxP knockdown birds compared to control animals (Figure 3.39).

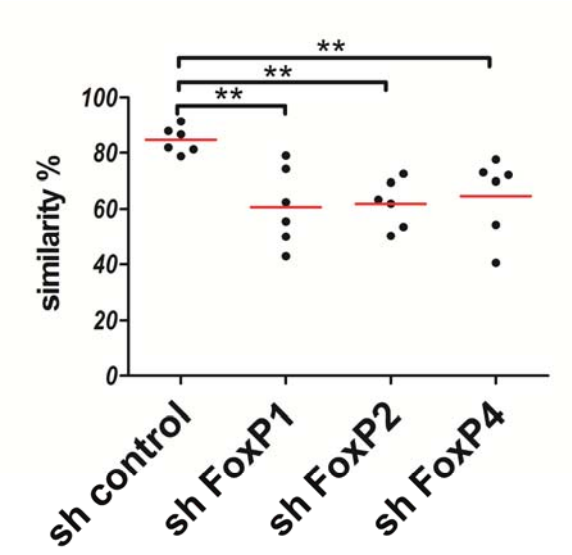



**Figure 3. 40 Knockdown of all FoxP subfamily members reduces motif similarity. The mean similarity between pupil and tutor motifs was significantly lower in all FoxP subfamily members knock-down-injected animals than in sh-control-injected birds, indicating that knockdown animals copied less acoustic material from their tutors (scatter dot plot, each dot represents the mean similarity score of an M x N batch similarity in SAP2011 of an animal, the red line is the mean of means; two-tailed t-test, **P<0.005). FoxP2 song data from Haesler *et al.*, 2007.**

We wondered if the accuracy of the sh-FoxP1/4 birds would be also be significant as it was for FoxP2 in the previous study (Haesler *et al.* 2007). Therefore we obtained motif accuracy values in SAP2011 from M x N batch motif comparisons between pupil and tutor (Figure 3.40). As it was described for FoxP2 (Haesler *et al.*, 2007), tutor song was not precisely copied. FoxP1 and FoxP4 did copy the tutor syllables more precisely than FoxP2 knockdown does, since there were no differences between FoxP1/4 and sh-control. However, there was substantial variability among FoxP1 and FoxP4 knockdown birds, so that some had an accuracy that was as good as sh-control birds, and some were even worse than FoxP2 birds. Another thing that one should consider, is that FoxP2 knockdown birds did copy syllables that could be matched to that of the tutor song in all cases but were not accurate, FoxP1/4 knockdown birds sang syllables that were either so bad or new that could not be matched to the tutor song, and those syllables that had similarity of 0% do not have an accuracy value. The latter syllables were not taken into account by SAP and thus would give and over estimated accuracy value for FoxP1/4.

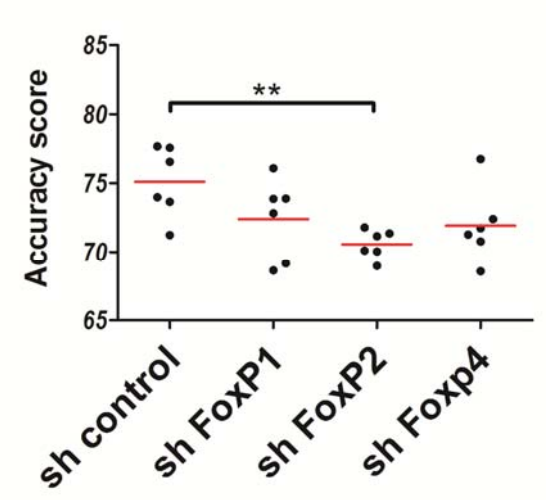



**Figure 3. 41 Knockdown of FoxP1 and FoxP4 did not affect motif accuracy as FoxP2 does. Average motif accuracy was significantly reduced only with sh-FoxP2 knockdown animals, but not sh-FoxP1 or sh-FoxP4, compared to control animals, indicating that they imitated their tutors less exactly (scatter dot plot, each dot represents the mean accuracy score of an M x N batch similarity in SAP2011 of an animal, the red line is the mean of means; two-tailed t-test; **P<0.05). FoxP2 song data from Haesler *et al.*, 2007.**

To get a comprehensive view on how well pupil and tutor motifs matched acoustically, we first plotted similarity and accuracy of all birds (Figure 3.41 a). All control-injected birds had a higher similarity and accuracy scores than the FoxP-knockdown injected birds. We then calculated an overall motif imitation score composed of the mean of the motif similarity and motif accuracy scores, which is a measure commonly used to describe the overall difference between two songs of different birds. FoxP knockdown-injected animals scored significantly lower than control animals (Figure 3.41 b).

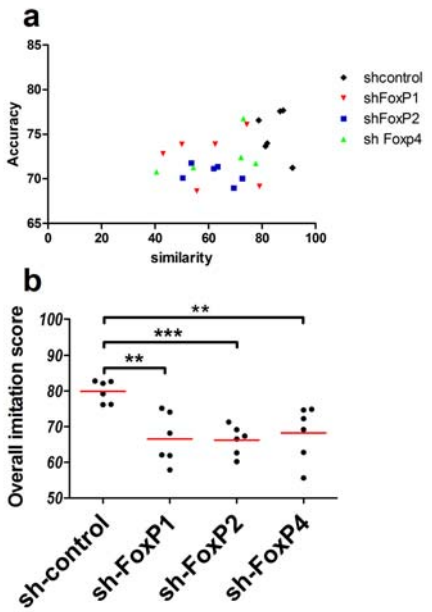


**Figure 3. 42 FoxP subfamily members have reduced overall song imitation scores. In (a) each mean value of similarity and accuracy were plotted. Sh-control birds group together in the higher similarity and accuracy values, while all FoxP subfamily members group in the low similarity and accuracy values. In (b) a reduced overall imitation score in all FoxP knockdown-injected birds (scatter dot plot, each dot represents the mean overall imitation score for each animal, the red line is the mean of means, two-tailed t-test; **P<0.005: ***P<0.0001). FoxP2 song data from Haesler *et al.*, 2007.**

SAP2011 gives also a measure for how well the sequence of the different syllables were copied from the tutor. This is called the sequential match and is calculated by sorting the final sound sections according to their temporal order in reference to the tutor song, and examining their corresponding order in the pupil song (if the order of the syllables of the song of the pupil matches the order of the tutor). Sequential match between pupil and tutor song was measured by an M x N symmetric comparison of pupil and tutor motifs (Figure 3.42). So not only the acoustic features of song were affected, but also the order of syllables was not copied correctly in all FoxP knock-down-injected birds.

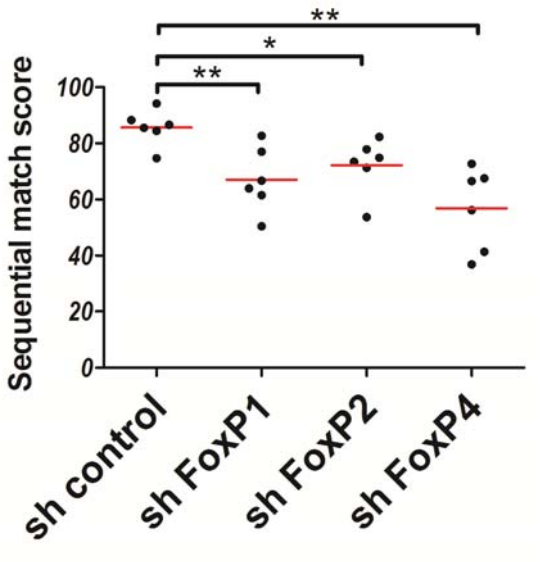



**Figure 3. 43 Knockdown of FoxP subfamily members led to reduced sequential match scores. Average motif sequential match was significantly reduced in all FoxP knockdown-injected birds, compared to control animals (scatter dot plot, each dot represents the mean sequential match of an M x N batch similarity in SAP2011 of an animal, the red line is the mean of means; two-tailed t-test; * P<0.05; **P<0.005). FoxP2 song data from Haesler *et al.*, 2007.**

Last, we compared the effect of both short hairpins for FoxP1 (Figure 3.43 a, b and c) and FoxP4 (Figure 3.43 d, e and f) to rule out different effects with the different hairpins (Figure 3.43). No differences were found in the effect of either short hairpin used for FoxP1 (sh-FoxP1-2 and sh-FoxP1-3) and FoxP4 (sh-FoxP4-7 and sh-FoxP4-19).

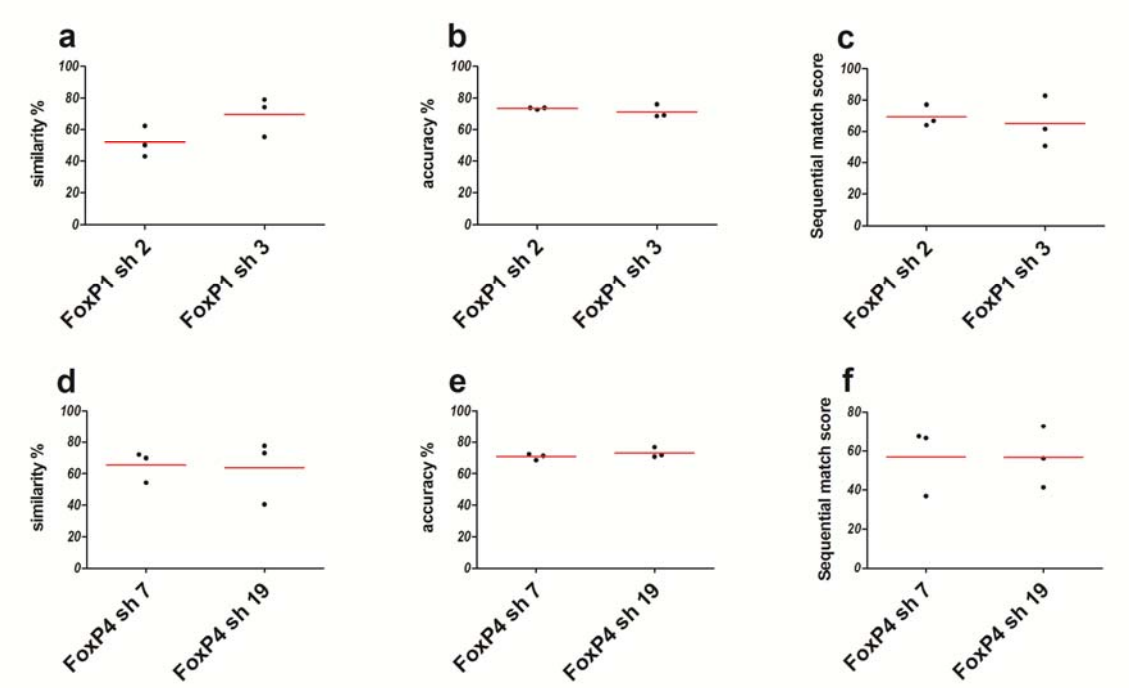



**Figure 3. 44 Both short hairpins constructs, against FoxP1 and FoxP4, impaired song-learning to a similar degree. Neither both FoxP1 short hairpins ("a", "b" and "c") nor both FoxP4 short hairpins ("d", "e" and "f") showed differences in their effect on song learning, none of the different song features showed differences (two tailed t-test; P > 0,05).**

Taken together, FoxP1 and FoxP4 knockdown had a similar effect to the one reported for FoxP2 in similarity, overall imitation score and sequential match. FoxP2 knockdown affected accuracy, something not observed in FoxP1 and FoxP4 knockdown animals, though there was a trend.

# 4. Discussion

## *4.1* FoxP1, FoxP2 and FoxP4 expression in the zebra finch

Although it has been long known that FoxP4 is expressed in the brain (Lu *et al.*, 2002; Teufel *et al.*, 2003) there are almost no studies that have looked at the expression pattern and developmental regulation of this FoxP member. To date there is only one study that looked at the expression pattern of FoxP4 in the brain (Takahashi *et al.*, 2008). In order to study the expression of this gene I successfully cloned the zebra finch *FoxP4* gene. *FoxP1*, *FoxP2* and *FoxP4* zebra finch variants are much conserved, and show a lot of similarities if compared to their human and mice orthologs. A difference between the zebra finch *FoxP4* gene and the human and mice orthologs is that the first one seems to have a full and functional CtBP-1 binding domain. It was reported and tested that due to a change in this domain only the FoxP4 mice and human proteins are not regulated by this co-factor, FoxP1 and FoxP2 are repressed by this protein (Shanru Li *et al.*, 2004). It could be that in zebra finches all three FoxP subfamily members are regulated by this co-factor.

FoxP1 and FoxP2 pattern of expression overlap in the basal ganglia in many species studied so far (Ferland *et al.*, 2003; Haesler *et al.*, 2004; Teramitsu *et al.*, 2004; Takahashi *et al.*, 2003, 2008a, 2008b; Campbell *et al.*, 2009; Tamura *et al.*, 2003, 2004). It is known that the basal ganglia are important for vocal learning, and studies of human FoxP2 mutations show abnormalities in this region. I wanted to know if all three FoxP subfamily members could overlap in their pattern of expression and if this overlapping expression would be in regions important in song learning. For this I did 3 different *in situ* probes of the *FoxP4* zebra finch gene, as well as other 3 for *FoxP2* and one for *FoxP1*. I found a homogenous expression of *FoxP4* in the zebra finch brain. I found an overlapping expression of *FoxP1, FoxP2* and *FoxP4* in striatum or basal ganglia, including in Area X, a region important for vocal learning. This result is interesting because this area is important for vocal learning. Overlapping expression in the basal ganglia of FoxP1/2/4 was also reported in embryonic stages of the rat. A down-regulation of the *Foxp4* expression has been reported in latter stages in rats, and the gene was barely detectable in adult stages (Takahashi *et al.*, 2008). I found a persistent expression of *FoxP4* up to adult stages (>120PHD), so the overlapping of *FoxP4* expression with *FoxP1* and *FoxP2* persists during development. Similarly, striatal expression of FoxP2 persists, in adult stages of mouse, rats and songbirds, but appears to be down-regulated in this region in monkeys of 6 years (Takahashi *et al.*, 2008).

In addition, *FoxP1* and *FoxP4* were also expressed in HVC and RA, two important nuclei of the motor pathway. In HVC, neurons expressing *FoxP1* or *FoxP4* were co-localized with retrograde tracers from Area X and RA, indicating that the projecting neurons express them.
*FoxP1, FoxP2* and *FoxP4* have regions of overlapping expression but all three have specific patterns of expression. In the thalamic region there were nuclei that expressed all three in DLM, as well as regions that express only two of them like OV.

In the cerebellum, I found expression of *FoxP2* and *FoxP4* in Purkinje cells. Another region that had a strong expression of all three FoxP subfamily members studied is IO.
In the pallium there is almost no expression of *FoxP2*, in contrast to mammals were cortical expression in layer VI was reported (Ferland *et al.*, 2003). *FoxP1* and *FoxP4* are expressed in the mesopallium, and *FoxP4* expression is also found in nidopallium and arcopallium.

Next it was known that *FoxP2* expression changes between 35 and 75 PHD, there is an up-regulation in Area X, which is the phase when song learning occurs. After 90 PHD there is a down-regulation of *FoxP2* in Area X if compared with striatum (Haesler *et al.* 2004). Therefore I looked if *FoxP1* and *FoxP4* would also have an up-regulation in different ages. I found that *FoxP1* has a constant higher expression in Area X, if compared to the surrounding striatum in all ages studied. *FoxP4* had a higher expression in Area X at 50PHD, if compared to the surrounding striatum. In other ages I did not found any differences. It might be that expression levels in the different ages and with the different FoxP subfamily members are important for their function in Area X.

One of the most important questions was if more than one FoxP subfamily member could be expressed in the same cell. Although it is long known that FoxP subfamily members are unique in their necessity to homo- and hetero-dimerize for binding to DNA, no one has showed that they could be expressed in the same cell for that to occur. Until now studies in the lung and esophagus tissue show that FoxP1 and FoxP2 are expressed in different populations of cells (Shu *et al.*, 2007). In another study it was shown that Foxp1, Foxp2 and Foxp4 are expressed in the distal epithelium, and FoxP1 and FoxP4 also in the proximal epithelium (Lu *et al.*, 2002). If in these regions they are expressed in the same cells was not addressed. In the brain a lot of studies show overlapping expression of Foxp1 and Foxp2 in the basal ganglia but none has looked if they could be expressed in the same cells. In the cortex a study reported that Foxp1 and Foxp2 are expressed in different projecting neurons, which would suggest that they are not expressed in the same cells, but I did not find expression of FoxP2 in cortical analogous regions in the zebra finch (Hisaoka *et al.*, 2009). In the striatum of mice it was shown that FoxP1 co-localizes with DARPP-32, and was not

expressed in ChAT or PV cells (Tamura *et al.*, 2004). Neurons that were FoxP2 positive in the zebra finch striatum had also the same markers (Haesler *et al.*, 2004), which could suggest that FoxP1 and FoxP2 cells could be express in the same population. To address the question if *FoxP1*, *FoxP2* and *FoxP4* could be expressed with the other members I used double *in situ* hybridization and triple immunohistochemistry. I found that in striatum and Area X there are all types of combinations expressing all three FoxP subfamily members. The majority of cells expressed all three subfamily members and the minority of cells are FoxP2 alone or FoxP1/FoxP2 combination. Given that the striatum is also the site of functional and structural abnormalities in individuals with DVD, it seems possible that all three FoxP subfamily members are involved in the acquisition of motor programs and control over orofacial muscles during speech.

The expression data suggest that FoxP1, FoxP2 and FoxP4 are 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 behaviour (Lieberman, 2001; Marien *et al.*, 2001). In addition, many sites of FoxP2 and FoxP4 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). Of interest is the expression of FoxP subfamily members in IO and Purkinje cells since the topographic connection between this to regions forms the central organization of the cerebellar system (Fujita *et al.*, 2011). Here I found high expression of all three FoxP subfamily members in IO and even saw co-localization in the majority of cells using double *in situ* hybridization (data not shown). In Purkinje cells, all FoxP2 positive cells were also FoxP4 positive. A recent study showed that the FoxP2 pattern of expression in mice, rats and chick is conserved in Purkinje cells and IO. Furthermore they show that almost all Purkinje cells express FoxP2 at P6 in the mice and that at adult stages some Purkinje cells lose their FoxP2 expression resulting in areas that have FoxP2 in all their Purkinje cells, areas that do not have FoxP2 in their Purkinje cells, and areas where there is a mix population. The mean

pattern of FoxP2 expression of these three populations gave a transversely organized pattern conserved in chick and rats. They further divided the FoxP2 positive populations in small neurons and medium size neurons, and weakly stained and strongly stained (Fujita *et al*., 2011). To this data I can add that the FoxP2 positive Purkinje cells are also FoxP4. In IO FoxP2 expression was found almost in all neurons and the pattern of expression did not change in the different ages assessed, or between mice, rats and chicks. Almost all of the IO neurons are excitatory neurons that terminate as climbing fibbers that end in the cerebellum (Fujita *et al*., 2011). To these data I can add that IO highly expresses al 3 FoxP subfamily members and that as far as I could see they all co-localize in double *in situ* hybridization slices I had (data not shown). Last, it is known that mice with knock-out of the FoxP2 gene, knock-in of the affected FoxP2 gene, or point mutation of the FoxP2 gene can generate the cerebellum and Purkinje cells, although the cerebellum is small with foliation deficits and the PCs are less elaborate in dendritic arborisation (Shu *et al.*, 2005; Fujita *et al.*, 2008 and 2010; Gaub *et al.*, 2010). A study on mice Purkinje cells suggest that Foxp4 is dispensable for the early Purkinje cell dendrite outgrowth, but is essential for the maintenance of Purkinje cells dendritic arborisation and subsequent association with Bergmann glial fibers (Tam *et al*., 2011). These reports suggests that FoxP2 and FoxP4 are not a critical molecules for PC differentiation but it may be involved in PC differentiation and consequently in cerebellar development in some modulatory way.

The fact that in striatum cells express all types of combinations gives rise to a lot of possible interactions, which could lead to differential regulation and / or targeting, of the FoxP subfamily members in this region. But for that to occur, I first needed to show that they can actually interact.

In summary, FoxP1, FoxP2 and FoxP4 have characteristic pattern of expression in the zebra finch brain, yet they overlap in a region important for vocal learning, Area X and the striatum. Neurons in Area X can express all different combinations of FoxP1, FoxP2 and FoxP4, so that the combinatorial regulatory possibilities could be used for fine tuning of targets or their regulation. In addition FoxP1 and FoxP4 are expressed in the projecting neurons of HVC, and in neurons in RA. All important nuclei of the motor pathway express FoxP1 and FoxP4, and Area X, important in the anterior forebrain pathway, shows expression of all three FoxP subfamily members, it might be that all of them are important for the development and / or function of this brain pathways.

## 4.2 Analysis of FoxP1, FoxP2 and FoxP4 interaction

Since FoxP1, FoxP2 and FoxP4 can be expressed in the same cells in the basal ganglia and Area X, I wondered if they could interact. The fact that dimerization of the FoxP subfamily members is exemplified by the fact that a deletion of a single Glutamic acid in the leucine zipper of FoxP3, a domain important in dimerization, leads to a human phenotype. It was shown that only the dimerization ability of FoxP3 was affected and was the cause of the malfunction of the protein that lead to a phenotype (Chae *et al.*, 2006). That all other FoxP subfamily member are able to homo- and hetero-dimerize was shown with mice proteins using co-immunoprecipitation assays. Furthermore, a deletion of an analogous glutamic acid as the one mentioned before in the other FoxP subfamily members affected dimerization, as well as regulation and DNA binding (Shanru Li *et al.* 2004). Dimerization is a unique characteristic of the FoxP subfamily member so it is reasonable to think that it has an important function. I showed that zebra finch FoxP proteins are able to homo- and hetero-dimerize as their mice counterparts *in vitro*. I found that native proteins run at about 250kD, which is more than what I would expect for a dimmer, but since native protein run different that denatured and are not separated only by size, but charge and 3D conformation, I cannot conclude anything about this. More studies are needed in order to known if FoxP sub-family members to form only dimmers, or if they are tetramers. Having a crystal structure of the different dimmers would be useful to know how the interactions between FoxP proteins are and which amino acids are important for this, as well as how DNA interacts with the dimmer. Until now there have been reports of the crystal structures of the Forkhead box of FoxP1 (Chu *et al.*, 2011) and FoxP2 (Stroud *et al.*, 2006) where it was shown that they can bind to DNA as monomers or dimers through domain swapping structures. The fact that monomers bind to DNA seems to be contradictory with the fact that the FoxP proteins that cannot dimerize cannot bind to DNA or are affected in their regulatory properties. Things to consider are that the Forkhead box alone was shown to bind DNA (Vernes *et al.*, 2006), but how this binding is in a full protein interacting with another FoxP member is not known.

I was also able to show that hetero-dimerization occurs *in vivo*, but I could not show an interaction for all hetero-dimers. It is possible that in the FoxP1-FoxP4 hetero-dimer there are either other co-factors binding or that the epitope is not available and exposed for the antibody to bind. Since also not from *in vitro* over expressed protein I could pull down this dimmer with the specific antibodies it is possible that it is a conformational problem.

I wanted to see if cells expressing one FoxP subfamily member would differ in their repression propertied to cells that express more than one FoxP protein. To test that idea I quantified luciferase expression of cells expressing all possible combinations of FoxP proteins. I found that targets of cells that express FoxP2 are subject of a weak repression, and if FoxP2 is expressed either with FoxP1 or FoxP4 the repression of the targets would be significantly increased. It is difficult to rule out that the FoxP1 and FoxP4 homo-dimers in those cells were not the ones giving this higher repression, since there was not a significant difference to their single repression. The problem with these assays is that one cannot be sure that only hetero-dimers are regulating the luciferase expression in those cells; for that to work one should be able to manipulate preference of dimerization. Another thing to take into account is the fact that it is not known if there is an interaction preference of the different FoxP proteins or if co-factors could favour a specific dimerization in the cells. The only combination where I saw a clear synergy was with FoxP1-FoxP4, were the cells expressing both had always a stronger repression that cells expressing them separately; this difference was not statistically different but it may biologically important.

What is the function of dimerization? This is a question that should also be addressed in further studies. Are hetero-dimers switching the preference to the DNA binding motif? Do the different dimmers interact with different proteins and therefore change their activity or function?

In summary FoxP protein can dimerize in all possible combinations which could be a way in which switching of targets is achieved or regulation could be fine tune in the cells. Cells that only express FoxP2 have a weaker repression that cells that express FoxP2 and either FoxP1 or FoxP4 which suggest that they could act in a synergic way.

## 4.3 Analysis of the interaction of mutant FoxP2 and wild type FoxP proteins

Until now the DVD phenotype caused by FoxP2 mutations was considered to be a monogenetic disease and caused by the haplo-insufficiency of the protein (Lai *et al.*, 2001). Few studies have tried to address the effects of the FoxP2 mutations and if the mutation does not affect the proper functions of other proteins were not addressed. A first study showed that the R553H mutation leads to an altered citoplasmic protein localization, altered regulation and affected DNA binding (Vernes *et al.*, 2006). This study supported the few of a haplo-insufficiency as the cause of the human DVD phenotype since the mutated protein is not

present in the nucleus and because it cannot bind to DNA. A problem with this study is that it did not show what the scenario would be if a normal FoxP2 would also be there. Mice expressing an analogous mutation (R552H) did not express the mutant protein in the citoplasma, which was not expected, since the cellular localization of the R553H mutant was predominantly in the citoplasma. Even in the homozygous R552H Knock in mice there was nuclear localization of the mutant protein (Fujita *et al.*, 2007). At this point it was not clear why there was this difference in the cellular localization of the mutant FoxP2 protein. A study that gave new insights into this problem showed that the R553H FoxP2 protein was expressed in the nucleus if expressed with a normal, not mutated, FoxP2 protein (Mizutani *et al.*, 2007). But still this did not answer why in the homozygous R552H-KI mice protein was expressed in the nucleus.

Since our results show that in the majority of neurons FoxP2 is not expressed alone, I wanted to see if the FoxP2 R553H mutation would also affect the other FoxP subfamily members. I showed that an analogous mutation to the R553H FoxP2 mutation in the zebra finch did not show strong citoplasmic localization. Therefore I used a FoxP2 version lacking the Forkhead box (ΔFoxBox), which shows a citoplasmic expression if expressed alone. I found that both versions can homo-dimerize and hetero-dimerize with FoxP1/2/4 normal proteins. I found that the expression of the ΔFoxBox is also altered if express with either FoxP subfamily member. This could explain why all mice expressing mutated forms of FoxP2 do not show citoplasmic localization. An example is that in purkinje cells, FoxP2 is expressed with FoxP4, so in the R552H-KI mouse the mutant version could be expressed in the nucleus imported by the FoxP4 protein. Last I showed that regulation of all other subfamily members is affected or altered. Why it seems that there is an up-regulation with R553H zebra finch version and ΔFoxBox is not known. In the Vernes *et al.*, 2006 article they did not discuss why R553H had more signal than the empty vector. A possibility could be that in the empty vector condition there are proteins repressing the SV40 promoter (could be also endogenous FoxP2) and if the mutant is there they cannot repress the SV40 promoter anymore. Analogous to what is shown if normal FoxP1 or FoxP2 are co-expressed with the R553H. Another explanation would be that the R553H acts as an activator, instead of a repressor. It is known that FoxP2 can repress and activate genes (Vernes *et al*., 2007; Spiteri *et al.*, 2007; Konopka *et al*. 2009), and what regulates this change is not known.

In summary our data suggest that what started as a monogenic disease could be in fact a monogenetic based disease that affects the whole FoxP pathway. Discerning if the phenotype is only due to a haplo-insufficiency, or affection of the FoxP pathways is going to

be needed in order to understand the aetiology of the FoxP2 and DVD. This also needs to be considered if trying to identify targets of FoxP2 in R553H mutations, which will not only affect the FoxP2 targets, but also FoxP1 and FoxP4 pathways, if in other species neurons also express more than one FoxP subfamily member.

## 4.4 Analysis of FoxP1 and FoxP4 function *in vivo*

Since FoxP1/2/4 are expressed in the majority of neurons in Area X and striatum, and possible interactions could be a major factor underlying vocal learning. I wanted to address if FoxP1 and FoxP4 lentiviral mediated RNAi would affect song learning as FoxP2 does. I chose these method for the next reasons: i) it allows spatially and temporally restricted genetic manipulations; ii) even if knock- out technology would be available for zebra finches it would not be feasible because knock-out homozygous FoxP1 mice die in embryonic stage E14.5 (Wang *et al.*, 2004) and FoxP4 homozygous mice die at embryonic stage E12.5 (Li *et al.*, 2004), and the possibility that in zebra finches I would have the same problem is high; iii) it was shown that the same phenotype can be achieved with RNAi and knock-out genetic manipulations (Salahpour *et al.*, 2007; Delic *et al.*, 2008); iv) a demonstrated long lasting knock-down effect (Haesler *et al*., 2007; Delic *et al.*, 2008); v) last this method was used for addressing the importance of FoxP2 in song learning in zebra finches (Haesler *et al.*, 2007). A limitation of this approach is the experimental variability in the targeted area and volume achieved by each injection. The best injection yielded a volume of 80% of Area X, which is higher than the one reported in Haesler *et al.*, 2007. Nevertheless, knock down of FoxP1 in an average volume of only 35.93% and FoxP4 of 28.6% was sufficient to cause learning deficits. Another thing that should be taken into account is that 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) and a ~20% volume targeted gave a song learning phenotype in zebra finches with FoxP2 mRNA reduction (Haesler *et al*. 2007). In addition other studies have shown behavioural phenotypes using RNAi in rat (Eren-Kocak *et al*., 2011; Garza *et al.*, 2008; Liu *et al*. 2011) and mice (Rumpel *et al.*, 2005; Inoue *et al.*, 2007; Peters *et al.*, 2009; Salahpour *et al.*, 2007).

Although RNAi is a wide use tool for gene manipulation, non-specific effects were also reported. Among the effects reported are: i) silencing of off-target transcripts (Lew-Tabor *et al.*, 2011; Jackson and Linsley, 2004); ii) induction of interferon response (Jackson and Linsley, 2004); iii) and, fatality and tissue damage (Grimm *et al.*, 2006).

Gene-specific knockdown by RNAi requires careful experimental control. To rule out silencing of off-target genes I use two different shRNA for FoxP1 and FoxP4 that were design to match 100% the targeted gene. I did not see any effects with the sh-control virus. Since I saw the same effects with both sh used for FoxP1 or FoxP4, I would conclude that the phenotype observed in both cases is due to a specific reduction of the mRNA of both genes. I further showed that the shRNA did not reduce the levels of the most homologous proteins, the other FoxP subfamily members *in vitro*. One could argue that the sh-control virus would not compete with the miRNA machinery since it is not targeting any gene, and it is known that only RNAi that have a target compete with the miRNA machinery affecting it through a reduction of miRNA expression (Grimm *et al.*, 2006). But in the previous study a short hairpin against GFP was also used and no differences between the shGFP and the sh-control were reported. In this control there is a short hairpin that is targeting something in the cell (GFP) so a competition would be expected (Haesler *et al.*, 2007). I would not expect to have an interferon response or toxicity with our short hairpins because it is also reported that such effect are induced by hairpins that are longer than 25bp, I used hairpins that were 20bp (Grimm *et al.*, 2006). Furthermore, the FoxP2 short hairpins used in the previous study did not induce apoptosis shown by TUNEL method (Haesler *et al.*, 2007). It could be that sh against FoxP1 and FoxP4 react different that the ones for FoxP2, but I did not see any sign of toxicity or cell damage in GFP cells, though I cannot rule that out. The quantity of virus particles injected in our study was $10^6$ infectious particles/µl, which was higher than those used in some recent publications with behaviour phenotype and/or gene reduction, ranging from $10^5$ particles/µl (Eren-Kocak *et al.*, 2011; Mahairaki *et al.*, 2009; Santamarina *et al.*, 2009) and $10^6$ particles/µl (Garza *et al.*, 2008; Liu *et al.*, 2011; Haesler *et al.*, 2007). The dosage of RNAi was also correlated with toxicity (Grimm *et al.*, 2006), none of the recent reports show toxicity of cells using the same promotor as I did (Di Benedetto *et al.*, 2009; Eren-Kocak *et al.*, 2011; Garza *et al.*, 2008; Mahairaki *et al.*, 2009; Haesler *et al.*, 2007).

The protein reduction of FoxP1 and FoxP4 *in vitro* was evident (Figure 3.25 and 3.26 respectively), but the reduction *in vivo* (Figure 3. 30 and 3.31) was not as strong as the one reported previously for FoxP2 with the same paradigm (Haesler *et al.* 2007). Reason for that could be that I pooled the Q-PCR results for the whole Area X and the mean volume infected

with virus was 30% (Figure 3.34). Adding to that, I have also the problem that many cells express FoxP1 or FoxP4, more than FoxP2 (Figure 3.11), so the percentage of cells affected is not so high (Figure 3.28). The majority of FoxP2 neurons express weakly FoxP2, and FoxP1 and FoxP4 neurons seem to express them in the same levels. Therefore reduction of FoxP2 would be easier to achieve as for FoxP1 and FoxP4. While punching I aimed for Area X and not for GFP injection site, so although in some cases I saw GFP surrounding the punch (Figure 3.29), it can be that there was no GFP in the punch itself. ISH of GFP in adjacent slices did not work in every case, so I are not fully certain to which degree GFP was in the punch, and the only measure I have is Q-PCR. There I found coincidence of GFP Q-PCR signal and punches were I saw GFP in the surrounding tissue of the punch (data not shown). Therefore the difference measured by Q-PCR may actually reflect the scarce volume and cells infected by the virus. Although I reached a weaker reduction measured with Q-PCR and not a high percentage of cells infected, I saw a phenotype with FoxP1 and FoxP4, it might be that a weaker reduction of Foxp1 and FoxP4 affects neurons more than a stronger reduction in FoxP2, because FoxP1 and FoxP4 are stronger repressors than FoxP2 (Figure 3.19) (Vernes *et al.*, 2006; Shanru Li *et al.*, 2004; Chokas *et al*., 2010). The majority of GFP positive neurons were FoxP1 positive and therefore medium spiny neurons. This goes in accordance with previous results using the same promotor where the majority of cells infected are neurons (Haesler *et al*., 2007; Wada *et al.*, 2006; Di Benedetto *et al*., 2009; Garza *et al*., 2008). It was also reported that primers for Q-PCR of RNAi target genes should not flank the shRNA binding site, because this could lead to false positive results (Herbert *et al*., 2011). Primers used for FoxP1 and FoxP4 do not flank either of shRNA used in this study. Other ways to show reduction of the desired gene *in vivo* that could be applied to our settings are: i) *in situ* hybridization of for the gene to be reduced by RNAi (Garza *et al.*, 2008; Di Benedetto *et al*., 2009; Eren-Kocak *et al*., 2011); ii) quantification of the difference of the GFP positive cells that also express the protein of interest detected by immunohistochemistry, comparing control and reduced conditions (Haesler *et al*., 2007; Mahairaki *et al*., 2009; Santamarina *et al.*, 2009); iii) western blot of protein extracts of the tissue affected with RNAi (Salahpour *et* al., 2007; Peters *et al.*, 2009; Delic *et al.*, 2008). In this last study they also did LNA *in situ* hybridization to show expression of the short hairpin in the tissue.

In general we saw a similar phenotype with Foxp1 and FoxP4, and both of which are similar to the one reported for FoxP2 reduction (Haesler *et al*., 2007). There was an incomplete and inaccurate imitation of the tutor song if either of the FoxP subfamily members mRNA levels is reduced by RNAi in Area X. It seems unlikely that FoxP knock-down birds

limited in producing particular sounds, because in all cases birds were able to copy some syllables of the tutor motif with higher similarity scores, up to 96 similarity score (Figure 3.37b and c; syllable D). I saw a trend in the motif accuracy with FoxP1 and FoxP4 knock-down birds, but in general they were able to generate syllables as accurately as control birds. Accuracy at the motif level was significantly low with FoxP2, which was the only difference I saw if compared to FoxP1 and FoxP4 knock-down birds (Figure 3.40). However, FoxP1 and FoxP4 knockdown birds did sang syllables that were not possible to match to the tutor song, which were not counted in the accuracy measure, and such a feature was not seen with FoxP2 knock-down birds. The overall imitation score of all FoxP knock-down birds (Figure 3.41b) was significantly lower if compared to the control birds. And last, the Sequential match score was also affected in al FoxP knock-down birds (Figure 3.42). Since FoxP1 and FoxP4 knock-down birds were able to produce syllables with a high similarity and accuracy, it is improbable that they were not able to produce particular syllable types. Since I did not assess song stereotypy within FoxP1 and FoxP4 knock-down birds I cannot rule out that birds did not reach the end of the sensory-motor period, but song data is available to further analyse such questions and also look at other features at the syllable level where FoxP2 knock-down birds did also show impairment. But given that crystallization was reached with the FoxP2 knock-down birds, I would think that FoxP1 and FoxP4 knock-down birds would have reached crystallization as well.

As proposed for FoxP2, the limited learning success of FoxP1 and FoxP4 knockdown birds could result from an imprecise neural representation of the tutor model or that animals failed to reconcile their own vocalization with the memorized tutor model (Haesler *et al.*, 2007). Since FoxP2 is up-regulated during the sensory-motor phase, it was proposed that it would be involved in motor integration (Haesler *et al*., 2004). Since expression of FoxP1 is high in Area X during all ages examined, I would conclude that there does not appear to be developmental regulation of this gene. On the other hand, FoxP4 was up-regulated at 50 PHD, thus it could be involved in motor integration and/or template matching. Looking at all FoxPs during the different ages it is clear that at different time points there are different and specific expression patterns of the three FoxP subfamily members that may be important for motor integration. It was shown that FoxP2 is regulated by singing in different social contexts (Teramitsu *et al*. 2006; Miller *et al*., 2008; Teramitsu *et al*., 2010). To date such regulation was not been observed for FoxP1 and FoxP4, although preliminary experiments indicate some regulation (data not shown). It was also proposed that the FoxP2 knock-down birds failed to reconcile their own vocalization with the memorized tutor model (Haesler *et al*., 2007) which

could be extended to FoxP1 and FoxP4. This is supported by the phenotypic similarities of song deficits observed in FoxP1/2/4 knock-down and birds that were prevented to match their vocal output with their memorized tutor song through online auditory feedback of altered song. Features they reported by such manipulation were repetition of syllables or stuttering, addition and deletion of syllables and modify syllable sequence (Leonardo and Konishi), all features that were seen with FoxP knock-down. Comparing our knock-down approach with a study where Area X of juvenile male birds was lesioned (Scharff and Nottebohm, 1991), I see different phenotypes. It was reported that lesioned birds had unusual longer syllables and reduced stereotypy. This also supports that the song features observed in our approach cannot be explained by the procedure of injecting and perhaps damaging Area X.

It is known that cortico-basal ganglia circuits promote learning of action sequences through trial-and-error learning and that basal ganglia drives the variability necessary for this reinforcement-based learning. This learning could be driven by the reward-related dopamine signalling that projects to the basal ganglia from the VTA and SNpc (Graybiel, 2005). In the striatum of the zebra finch there is expression of D1A, D1B and D2 receptors, and even higher expression in Area X (Kubikova *et al.*, 2009). In Area X, pallial auditory and song motor efference information converges with nigral dopaminergic reinforcement signals in the medium spiny neurons (Reiner et al., 2004). Our preliminary results show that FoxP1/2/4 positive cells co-localize with dopamine receptors, so it is possible that dopamine may regulate the plasticity of those neurons and that the regulation of the FoxP subfamily members during times of vocal plasticity could be functionally related to this process. In a mouse model in which FoxP2 was manipulated to resemble the human FoxP2, a decrease in dopamine levels was reported (Enard *et al.*, 2009 and 2011), further suggesting that a link between FoxP2 and dopamine exists. That FoxP2 is involved in plasticity of neurons was shown in the zebra finch using the same FoxP2 short hairpins. In this work it was reported that neurons expressing the shFoxP2 virus had fewer spine density (Schulz *et al.*, 2010). Furthermore, studies on targets of FoxP2 reported genes involved in neurite outgrowth, synaptic plasticity and axon guidance (Spiteri *et al.*, 2007; Vernes *et al*., 2007 and 2011). In addition, results of mouse FoxP2 manipulations support this showing alterations in dendrite length and synaptic plasticity (Reimers-Kipping *et al*., 2011; Enard *et al*., 2009; Groszer *et al.*, 2008; French *et al.*, 2011). FoxP2 manipulations in mouse also resulted in abnormal vocalizations (Fischer *et al.*, 2011; Fujita *et al.*, 2008; Gaub *et al.*, 2010; Shu *et al.*, 2005).

The fact that all FoxP subfamily members show a song learning phenotype can be explained in different ways. For FoxP2 and FoxP1, there are human phenotypes that support

their role in vocal learning. But for FoxP4 there are no mutations known. Possible explanations would be that hetero-dimers of the FoxP subfamily are important for regulating pathways important for vocal learning, so the absence of one affects the whole machinery. This would suggest that either hetero-dimers have a different binding site, which is not known, or bind to a specific co-factor that is needed for controlling targets important in vocal learning, which is also not known. FoxP subfamily members regulate different targets all needed for vocal learning, and the absence of one affects vocal learning. Area X equilibrium is affected no matter which gene is down-regulated and thereby affecting song learning. Since I used the same virus and short hairpins against the same conserved subfamily members it could be that the effect is due to the induction of the same off-target effect were the short hairpin affects the miRNA machinery and this affects vocal learning. This last hypothesis is not probable because: i) I used different short hairpins for each FoxP subfamily members; ii) I demonstrated that down-regulation a possible cross-reaction to the closest homologs, and short hairpins are specific even if compared in the same subfamily, therefore it is not probable that this short hairpins would have off-targets; iii) I used small short hairpins proofed not to be toxic or induce other side effects.

Although there are many studies about FoxP2, there is only a paltry number of experiments that focus on FOXP2 protein. It is not known what regulates FoxP2 or FoxP1/4. It is not known what other interactions partners (co-factors) interact with them, that may be important for their function. It is not known if the protein is phosphorylated, glycosylated, cleaved or sumoylated. It is not known which isoforms are doing which function. It is not known what the role of zinc-finger is. What is the role of hetero-dimerization, which is a unique characteristic of the FoxP subfamily that must have a specific and important function?
Taking all the data together, I have shown that FoxP1/2/4 are expressed in the basal ganglia and Area X, and the majority of neurons expressed all three members. FoxP subfamily members have the unique characteristic to homo- and hetero-dimerize, which was shown in mice and now in zebra finches. Cells expressing FoxP2 and another FoxP sub-family member show a stronger repression of target genes. FoxP2 mutations that do not affect the leucine zipper can lead to alterations in the whole FoxP machinery through interaction with the other FoxP subfamily members that are also expressed in the neurons. Last we showed that all FoxP subfamily members affect song learning in a similar way. Altogether, this body of work suggests that the FoxP subfamily, FoxP1/2/4, act in the basal ganglia in concert and regulate pathways important in song learning in the zebra finch.

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# 6. Appendix

## 6.1 Abbreviations

***A***

AChE: Acetylcholinesterase
AFP: Anterior Forebrain Pathway
ALV: Avian Leukosis Virus
AMPA: alfa-3-amino-5-hydroxy-methyl-4-isoxazole proprionic acid
ANOVA: Analysis of variance
AOS: Apraxia Of Speech
AR: Androgen receptor
Av: Avalanche

***B***

BCIP: 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
BLAST: Basic local alignment search tool
BOS: Birds Own Song
bp: Base Pairs
BPES: Blepharophimosis/ptosis/epicanthus inversus syndrome

***C***

CAS: Childhood Apraxia of speech
CC10: Clara cell 10 protein
CDH: Chromobox Helicase DNA binding Gene
cDNA: copy DNA, complementary DNA
ChAT: Choline acetyltransferase
CLM: Caudal Lateral Mesopallium, 4; Lateral portion of the caudal mesopallium
CMM: Medial portion of the caudal mesopallium
CMV: Cytomegalovirus
CN: Cochlear nucleus
CNTNAP2: Contactin-associated protein like 2
Co-IP: Co-immunoprecipitation assay
CP: cortical plate
CS: FoxP2 affected patient
CSt: Caudal striatum
CtBP-1: C -terminal binding protein 1

***D***

D1: Dopamine receptor type 1
D2: Dopamine receptor type 2
DARPP-32: Dopamine- and cyclic AMP-regulated phosphoprotein with molecular weight 32kDa
DIG: Digoxigenin label
DLM: Nucleus dorsolateralis anterior thalami, pars medialis
DM: Dorsal Medial Nucleus of the Thalamus
DMP: Nucleus dorsomedialis posterior thalami
dsRNA: double stranded RNA
DT: Dorsal thalamus
DVD: Developmental Verbal Dyspraxia

***E***

E: Entopallium
eGFP: Enhanced Gree Fluorescence Protein
EH1: Engrailed Homology Protein 1
ER: Estrogen receptor
ES: Embrio stem
ESCs: Embrionic stem cells
ET: Epithalamus

***F***

*FACS*: Flow cytometry
Field L: Field L complex
Fitc: Fluorescein
FnTm2: Novel Fibronectin Type III protein
FOX: Forkhead box
FoxC1: Forhead box protein, Subfamily C, member 1
FoxC2: Forkhead box protein, subfamily C, member 2
FoxE1: Forkhead box protein, subfamily E, member 1
FoxE3: Forkhead box protein, subfamily E, member 3
FoxL2: Forkhead box protein, subfamily L, member 2
FoxN1: Forkhead box protein, subfamily N, member 1
FoxP: Forkhead box protein subfamily P
FOXP1: Forkheadbox protein, subfamily P, member 1
FOXP2: Forkheadbox protein, subfamily P, member 2
FOXP3: Forkhead box protein, subfamily P, member 3
Foxp4: Forkhead box protein, subfamily P, member 4

***G***

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase gene
GFP: Green Fluorescence protein
GluR1: Glutamate Receptor 1
GluR2: Glutamate Receptor 2
GluR3: Glutamate Receptor 3
GluR4: Glutamate Receptor 4

***H***

HAT-2: n Chimaerin
HIV-1: Human imminodeficiency virus Type 1
*Hox*: Homeobox protein
HVC: used as a proper name

***I***

IB: Immunoblotting
IC: Inferior colliculus
IEG: Imediate early genes
IF: Immunofluorescence
IO: Inferior olive
IP: Immunoprecipitation
IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ISH: In situ hybridization
IVZ: intermediate ventricular zone

***K***

KA: Kainate
kD: Kilo-Dalton
KE: Family in which the FoxP2 human mutation was found
kHz: Kilo-Hertz
KI: Knock In

***L***

L1: L1 subdivision of Field L
L2: subdivision of Field L
L3: Subdivision of Field L
LEF1: Lymphoid enhancer-binding factor 1
LGE: Lateral ganglionic eminence
LLD: Lateral Lemniscus dorsal Nucleus
LLI: Lateral lemniscus intermediate nucleus
LLV: Lateral lemniscus ventral nucleus
LMAN: Lateral Magnocelular nucleus of the anterior nidopallium
LMO: Lateral oval nucleus of the mesopallium
LNA: Locked Nucleic acid
LTD: Long Term Depresion
LTP: Long Term Potentiation

***M***

MBP: Myelin Basic Protein
MCS: Multiple Cloning Site
MEK-1: signal transduction kinase
MGE: Medial Ganglionic eminence
mGluR2: metabotropic Glutamate Receptor 2
MIQE: minimum information for publication of quantitative real-time PCR experiments
miRNA: micro RNA
MLd: dorsal lateral nucleus of the mesencephalon, 4; mesencephalicus lateralis dorsalis
mM: mili Molar
MoMLV: Moloney murine leukemia virus
MOR: mu-opoid receptor
MPI: Max Planck Institute
mRNA: message RNA
MSCV: Murine stem cell virus
MTA: Metastasis associated protein 1

***N***

NBT: Nitro Blue tetrazolium chloride
NCM: Caudomedial nidopallium
NEUM: Neuromodulin
NFm: Neurofilament
ng: nano gram
NIf: Intrafacial nucleus of the nidopallium
NLS: Nuclear localizing signal
NMDA: N-methyl-D-aspartate
nNOS: Nitric oxide synthase 1 (neuronal)

NPY: Neuropeptide Y
NRGR: Neurogranin
nXIIts: Tracheosyringeal portion of the nucleus hypoglossus nucleus XII

***O***

ORF: Open Reading Frame
Ov: Nucleus Ovoidalis, 3; Ovoidalis
OV: Nucleus ovoidalis

***P***

P215A: FoxP1 mutation proline to alanin mutation in amino acid 215
PAm: Para-ambioguus
*Pax6*: Paired Box Gene 6
PB: Phosphate Buffer
PBS: Phosphate Buffer saline
PBST: Phosphate Buffer Saline Tween
PBS-Tx: Phosphate Buffer Saline Triton X
PCR: Polymerase Chain Reaction
PGCs: Primordial germ cells
PHD: Post hatching days
PKC: Protein Kinase C
PV: Parvalbumin

***Q***

Q17L: FoxP2 mutation Glutamine to Leucine substitution in amino acid 17
Q40-Q44: FoxP2 mutation elongacion of the Poly Q by four amino acids
QPCR: Quantitative Polymerase Chain Reaction

***R***

R328X: FoxP2 mutation leading to a premature stop codon in amino acid 328
R525X: FoxP1 premature stop in amino acid 525
R553H: FoxP2 ariginine to histidine substitution in amino acid 553
RA: Nucleus robustus acropallii, 76; Robustus nucleusof the arcopallium
RAm: Retroambiguus
REV: Reticuloendotheliosis virus
RISC: RNA inducing silencing complex
RNAi: RNA interference
RT: Nucleus rotundus
RT-PCR: Real Time Polymerase Chain reaction

***S***

S_DYS: Spastic Flaccid dysarthria disorders
SAP: Sound Analysis Pro programm
SEM: Standart error of the mean
shRNA: short hairpin RNA
siRNA: short interference RNA
SLI: Specific Language Impairment
SMP: Song Motor Pathway
SNc: substantia nigra pars compacta
SO: Superior olive
SP: Subplate
SPL: Nucleus spiriformis lateralis

SSCs: Spermatogonial stem cells
*ssDNA*: Single stranded DNA
SV40: Simian virus 40
SVZ: subventricular zone

***T***

TH: Tyrosin Hydroxylase
TLE: Transducin like Enhancer
TUNEL: Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling

***U***

UV: Ultra violet
Uva: nucleus uvaeformis

***V***

VSV-G: Vesicular stomatitis virus G protein
VTA: ventral tegmental area
VZ: Ventricular Zone

***Z***

Zenk: Avian homolog of the mammalian zif268/EGR-1/ NGFI-A/krox24 gene

***Δ***

ΔFoxBox: FoxP2 protein without a Forkhead box

## 6.2 MIQE Q-PCR

**Table 6. 1 "MIQE" guidelines proposes a minimum standard for the provision of information for publications utilising RT-qPCR experiments for FoxP1 and FoxP4.**

| **Sample / Template (in General)** | | | | |
|---|---|---|---|---|
| Source | Punches of Area X of different zebra finches | | | |
| Method of preservation | Frozen in Liquid N2 and stored at -80°C until cut in the cryotom, punches stored again at –80°C until mRNA extraction. | | | |
| Storage time | Less than 1 month | | | |
| Extraction method | TRIZOL (Invitrogen) following manufacturer's protocol | | | |
| RNA : DNA-free | Turbo DNAse from AMBION and –RT of all samples run in QPCR with HMBS primers only | | | |
| Concentration | Quant-iT™ RNA assays kit (Invitrogen, Q32855) following manufacturer's protocol and determined the yield by fluorescence using the Qubit ® fluorometer (Invitrogen) | | | |
| RNA integrity | Was not assessed | | | |
| **Assay optimization** | | | | |
| | **FoxP1** | **FoxP4** | **GFP** | **HMBS** |
| Accession numbers | JN800727 | JN800728 | JN800726 | NM_013551 |
| Amplicon details | 150bp, from 1602-1751 bp of the ORF | 135 bp, from 1622-1756 bp of the ORF | 135bp, from 476-610 bp of the ORF | 107bp published in Haesler *et al.* 2007 |
| Primer sequence | qRT_FoxP1_3_for CGTTAAAGGGGCAGTATGGA<br><br>qRT_FoxP1_1_rev GCCATTGAAGCCTGTAAAGC | EM_qRTPCR_FoxP4_7f TGACAGGGAGTCCCACCTTA<br><br>EM_qRTPCR_FoxP4_7r AGCTGGTGTTGATCATGGTG | EM_GFP_RT_1f AGAACGGCATCAAGGTGAAC<br><br>EM_GFP_RT_1r TGCTCAGGTAGTGGTTGTCG | Hmbs-SH2-for GCAGCATGTTGGCATCACAG<br><br>Hmbs-SH2-rev TGCTTTGCTCCCTTGCTCAG |
| *In silico* | Blat (UCSC) "for" primer into the zebra finch genome gives only FoxP1 as a result. For the "rev" primer the last 29bp are needed to get as the only result FoxP1, less than 29bp was not recognize. | Blat (UCSC) needs the "for" primer plus 4 bp more to get a result that is FoxP4. The "rev" primer is fully recognize if blat in the UCSC browser, the only result is FoxP4. | No results if both primers are blat (UCSC) with the zebra finch genome. | Blat (UCSC) both primers, "for" and "rev", gives HMBS as the only result. |
| Primer concentration | 450nM both<br>Not optimized | 450nM both<br>Not optimized | 900nM both<br>Optimized concentration | 450nM both<br>Not optimized |

| Annealing Temperature | 60°C | 65°C | 65°C | 64°C |
|---|---|---|---|---|
| Priming conditions | Random Hexamers | Random Hexamers | Random Hexamers | Random Hexamers |
| PCR efficiency | Efficiency 90,3% | 115,6% | 96,9% (FoxP1 Q-PCR)<br>107,1% (FoxP4 Q-PCR) | 111,8% (FoxP1 Q-PCR)<br>111% (FoxP4 Q-PCR) |
| Slope | -3,58 | -2,997 | -3,399 (FoxP1 Q-PCR)<br>-3,162 (FoxP4 Q-PCR) | -3,069 (FoxP1 Q-PCR)<br>-3,083 (FoxP4 Q-PCR) |
| **RT/PCR** | | | | |
| | **FoxP1** | **FoxP4** | **GFP** | **HMBS** |
| Protocols | 1.- 95°C for 10 min<br>2.- 95°C for 30 s<br>3.- 60°C for 30 s<br>4.- 70°C for 1 min<br>2-4 40 cycles | 1.- 95°C for 10 min<br>2.- 95°C for 30 s<br>3.- 65°C for 30 s<br>4.- 70°C for 1 min<br>2-4 40 cycles | 1.- 95°C for 10 min<br>2.- 95°C for 30 s<br>3.- 65°C for 30 s<br>4.- 70°C for 1 min<br>2-4 40 cycles | 1.- 95°C for 10 min<br>2.- 95°C for 30 s<br>3.- 64°C for 30 s<br>4.- 70°C for 1 min<br>2-4 40 cycles |
| Master Mix | For 1x 25µl<br>Water 4,5µl<br>Buffer 1x SYBR 12,5µl<br>450nM sense primer 1,25µl<br>450nM anti primer 1,25µl<br>Rox Low 50nM 0,5µl<br>20µl Mix in each well<br>+ 5µl cDNA of each probe | For 1x 25µl<br>Water 4,5µl<br>Buffer 1x SYBR 12,5µl<br>450nM sense primer 1,25µl<br>450nM anti primer 1,25µl<br>Rox Low 50nM 0,5µl<br>20µl Mix in each well<br>+ 5µl cDNA of each probe | For 1x 25µl<br>Water 2,5µl<br>Buffer 1x SYBR 12,5µl<br>900nM sense primer 2,25µl<br>900nM anti primer 2,25µl<br>Rox Low 50nM 0,5µl<br>20µl Mix in each well<br>+ 5µl cDNA of each probe | For 1x 25µl<br>Water 4,5µl<br>Buffer 1x SYBR 12,5µl<br>450nM sense primer 1,25µl<br>450nM anti primer 1,25µl<br>Rox Low 50nM 0,5µl<br>20µl Mix in each well<br>+ 5µl cDNA of each probe |
| Reagents | Stratagene Mx3005P QPCR system<br>Kapa SYBR Fast Universal mix Cat. No 07-KK4600-01 Code KM4100<br>Kapa SYBR Fast Rox Low (50x) Code KD 4601 | | | |
| Duplicate RT | ΔCq = 0,71 | ΔCq = 0,21 | ΔCq = 0,49 for FoxP1<br>ΔCq = 0,45 for FoxP4 | ΔCq = 0,8 for FoxP1<br>ΔCq = 0,22 for FoxP4 |
| NTC | Cq=35,42-35,73 | Cq=30,23-30,72 | Cq=31,04-31,14 (For FoxP1)<br>Cq=30,88-32,40 (For FoxP4) | Cq= 30,51-30,78 (For FoxP1 –RT)<br>Cq=31,17-32,31 (For FoxP1 +RT)<br>Cq=29,41-29,98 (For FoxP4 |

| | | | | |
|---|---|---|---|---|
| | | | | –RT)<br>Cq=31,90-33,15 (For FoxP4 +RT) |
| Positive control (inter-run calibrator) | 10^4 of the pGEM t easy plasmid having the fragment amplified by the primers used | 10^4 of the pGEM t easy plasmid having the fragment amplified by the primers used | 10^4 of the pGEM t easy plasmid having the fragment amplified by the primers used | 10^4 of the pGEM t easy plasmid having the fragment amplified by the primers used |
| **Data analysis** | | | | |
| Software used | MxPro-Mx3005P | MxPro-Mx3005P | MxPro-Mx3005P | MxPro-Mx3005P |
| Statistical justification | 5 individuals, each injected in one hemisphere sh-control and in the contra lateral sh-FoxP1 | 6 individuals, each injected in one hemisphere sh-control and in the contra lateral sh-FoxP4 | 5 animals for FoxP1<br>6 animals for FoxP4 | 5 animals for FoxP1<br>6 animals for FoxP4 |

## 6.3 Abstract

The Forkhead transcription factor FoxP2 is important both for human speech and for bird song learning. *In vitro*, transcriptional activity of FoxP2 requires dimerization, either with itself of with other members of the Forkhead P family, FoxP1 and FoxP4. *In vivo*, the brain expression patterns of FoxP1, FoxP2 and FoxP4 have not been systematically compared for regional or cellular co-localization. To provide the means for future functional studies I cloned FoxP4 from zebra finches and compared both the mRNA and protein expression patterns of FoxP1, FoxP2 and FoxP4 at different ages. I found overlapping expression of FoxP1, FoxP2 and FoxP4 in striatum and there, in Area X, a nuclei important for song learning. HVC and RA, two important nuclei of the motor pathway of the song system, express FoxP1 and FoxP4. All FoxP subfamily members studied had a specific pattern of expression, shown in regions of overlapping expression and regions were there are either expressed alone or with another FoxP member. FoxP2 and FoxP4 expression increase in Area X during time when song learning occurs. A similar developmental regulation was not evident with FoxP1, were I saw a similar expression during all ages assessed. I further characterize HVC neurons expressing FoxP1 and FoxP4 as Area X and RA projecting neurons. To address whether specific combinations of FoxP expression existed I analyzed co-expression in the most important song nuclei using double *in situ* hybridization and triple immunohistochemistry. I provide the first evidence that FoxP subfamily members can be co-express in the same neurons. In Area X and striatum, I found neurons expressing all combinations of FoxP expression consistently during the different ages assessed. Surprisingly I found few cells that expressed only FoxP2, the majority of cells were FoxP1+/FoxP2+/FoxP4+, FoxP1+/FoxP4+ or FoxP1 alone. In addition all Purkinje cells express FoxP2 and FoxP4, HVC projecting neurons express FoxP1 and FoxP4 as well as RA neurons.

A second part of my work focus on the interaction of zebra finch FoxP1, FoxP2 and FoxP4 proteins and what the functional implication of this interaction would be. I first show *in vitro* that zebra finch FoxP1/2/4 proteins are able to homo- and hetero-dimerize as shown for their mouse counterparts. I further shown that *in vivo* hetero-dimerization occurs, and that the only hetero-dimer that I were not able to pull down (FoxP1-FoxP4) may be because the epitope was maybe not accessible. Last I show that cells expressing only FoxP2 are not as repressed as cells expressing FoxP2 and FoxP1 or FoxP4. These results imply that a variety of regulatory possibilities exist via dimerization of the FoxP members in cells expressing them.

Human FoxP2 mutations lead to a speech deficit known as developmental verbal Dyspraxia (DVD) and is considered to be a monogenetic disease, were haploinsufficiency is the etiological cause. Since mutant FoxP2 may be expressed in the same neurons as the normal FoxP1, FoxP2 and FoxP4, a third part of my work focuses on the interaction between mutant FoxP2 variant R553H and ΔFoxBox and normal FoxP proteins and what the outcome of such interaction could be. First I provide the first evidence that mutant FoxP2 proteins can homo- and hetero-dimerize with normal FoxP subfamily members, and that the Forkhead box in not essential for such interaction. Furthermore, I show that the cellular localization of the ΔFoxBox protein is altered if normal FoxP subfamily members are present, leading to a nuclear localization of the mutant version which would be otherwise localize in the citoplasma and isolated from the other FoxP members. Such interaction can have a huge effect on the regulation of all FoxP subfamily members express in the same neurons were a FoxP2 mutant is expressed. I provide the first evidence that mutation of FoxP2 that do not affect the leucine zipper can affect the whole FoxP machinery in the cell and that the R553H, Q17L and Q40-44 mutations should not be considered to affect only FoxP2.

In the last part of my work I assessed the functional consequence of a reduction of FoxP1 and FoxP4 in Area X using lentiviral mediated RNAi. I observed a similar phenotype with FoxP1 and FoxP4 to the one observed with FoxP2. Birds did not copy completely the tutor song and had a lower similarity and sequential match if compared to control birds. In contrast to FoxP2, FoxP1 and FoxP4 knock-down birds had a non significant accuracy score. The overall imitation score of all FoxP subfamily members was significantly lower that control birds. This data corroborates recent data on FoxP1 involvement in speech deficits in humans and opens the question if FoxP4 might mutations in humans might lead to a similar phenotype as the one seen with FoxP1 and FoxP2. This is the first evidence that the auditory-guided vocal learning in the basal ganglia requires all FoxP subfamily members, together as a family.

## 6.5 Zusammenfassung

Der Transkriptionsfaktor FOXP2, dessen Mutationen mit einer erblichen Sprachstörung, Childhood Apraxia of Speech, CAS, (auch Developmental Verbal Dyspraxia, DVD genannt) assoziiert sind, spielt auch beim Erwerb des Vogelgesangs eine wichtige Rolle. Es ist bereits durch *in vitro* Studien bekannt, dass FoxP2 um an DNA binden zu können, als Dimer vorliegen muss, entweder als Homodimer (FoxP2/FoxP2) oder in Kombination mit FoxP1 oder FoxP4. *In vivo*, im Gehirn des Zebrafinken, wurde bisher nicht systematisch untersucht ob FoxP1, FoxP2 und FoxP4 regional und zellulär koexpremiert werden. Um die Basis für zukünftige funktionelle Studien zu schaffen, klonierten wir das FoxP4 Gen des Zebrafinken und untersuchten die Expressionsmuster von FoxP1, FoxP2 und FoxP4 mRNA und Protein in Zebrafinken unterschiedlichen Alters. Regionale Koexpression von FoxP1, Foxp2 und FoxP4 konnte im Striatum und dort in Area X, einer für das Gesangslernen essentiellen Struktur nachgewiesen werden. HVC und RA, zwei Kerne der motorischen Bahn des Gesangssystems zeigten eine Koexpression von FoxP1 und FoxP4. Alle FoxP Mitglieder die wir analysiert haben zeigten regional spezifische Expressionsmuster mit teilweiser Überlappung. Während der Gesangslernphase wurden FoxP2 und FoxP4 in Area X verstärkt expremiert. FoxP1 dagegen wurde immer stärker in Area X als im Striatum expremiert und zeigte keine entwicklungsabhängen Änderungen des Expressionsniveaus. Weiterhin konnten wir zeigen, dass FoxP1 und FoxP4 koexpremierende Neurone des HVC entweder zu Area X oder RA projizieren.

Um der Frage nachzugehen, in welche Kombinationen FoxP1, FoxP2 und FoxP4 in Neuronen von Area X koexpremiert werden, führten wir dreifach-fluoreszente Immunhistochemie und duale *in situ* Hybridizierungen durch, wobei simultan zwei verschiedene Sonden unterschiedlich fluoreszensmarkiert nachgewiesen werden. Wir konnten so zeigen, dass alle Kombinationen von FoxP1, FoxP2 und FoxP4 in Neuronen koexpremiert werden können. In Area X sind die meisten Neurone FoxP1+/FoxP2+/FoxP4+, FoxP1+/FoxP4+ oder FoxP1+ positiv. Es gab sehr wenige Neurone die nur FoxP2 expremieren. Purkinjezellen zeigten sehr starke Expression von FoxP2 und FoxP4, während Projektionsneurone in HVC und Neurone des RA FoxP1 und FoxP4 expremierten.

Im zweiten Teil meiner Dissertation habe ich die Interaktionen zwischen FoxP1, FoxP2 und FoxP4 funktionell untersucht. Anhand von *in vitro* Studien konnten wir belegen, dass FoxP1, FoxP2 und FoxP4 des Zebrafinken, wie auch schon für die Mausorthologen gezeigt wurde, als Homodimere und Heterodimere vorliegen können. *In vivo*, im Gehirn von

Zebrafinken, konnten wir Heterodimere von FoxP1/FoxP2 und FoxP2/FoxP4 nachweisen. Während Heterodimere von FoxP1 und FoxP4, die mit V5 und/oder Flag-Tags versehen waren, *in vitro* über die entsprechenden Tags koimmunoprezipitiert werden konnten, gelang dies nicht mit endogenem FoxP1und FoxP4, was an der Maskierung der entsprechenden Epitope im FoxP1 und FoxP4 Heterodimer-komplex liegen könnte. Zuletzt konnten wir zeigen, dass FoxP2 Zielgene in vitro stärker reprimiert werden, wenn FoxP1 und FoxP4 koexpremiert wurden. Diese Ergebnisse weisen darauf hin, dass differentielle Genregulation durch FoxP´s in verschiedenen Zelltypen durch die Zusammensetzung von FoxP Dimeren beeinflusst werden kann.

DVD in Menschen wird als monogenetische Krankheit mit ursächlicher FoxP2 Haploinsuffizienz angesehen. Unsere Ergebnisse werfen die Frage auf, ob FoxP2 Mutationen auch die Funktion von anderen zellulär koexpemierten FoxP Mitglieder beeinflussen könnten, die somit an der Etiologie von DVD beteiligt sein könnten.

Im dritten Teil meiner Dissertation untersuchte ich daher ob durch DVD erzeugende Mutationen verändertes FoxP2, mit unmodifiziertem FoxP1, FoxP2 und FoxP4 dimerisieren können und inwiefern sich dies auf die FoxP-abhängige Genregulation auswirkt. Es wurden zwei FoxP2 Varianten erzeugt: eine die der menschliche FoxP2 Mutation R553H ähnlich ist, und eine andere, die keine Forkhead Box besitzt (ΔFoxBox). Wir konnten zeigen, dass diese beiden FoxP2 Varianten Homodimere bilden können. Darüber hinaus waren beide FoxP2 Varianten fähig mit allen anderen „Normalen“ FoxP Mitgliedern Heterodimere zu bilden, was impliziert, dass die Forkhead-Box nicht für die Ausbildung von Dimeren notwendig ist. Während nicht mutierte FoxP´s im Kern lokalisiert sind, zeigte die ΔFoxBox FoxP2 Mutante eine cytoplasmatische Lokalisation. Interessanterweise gelangte diese Mutante durch Koexpression mit anderen (normalen) FoxP Proteinen in den Zellkern. Weiterhin konnten wir zeigen, dass die Anwesenheit von mutiertem FoxP2 (ΔFoxBox oder R553H) in FoxP-Komplexen deren genregulatorische Aktivität beeinflusst.

Somit konnten wir erstmalig zeigen, dass FoxP2 Mutationen, die ausserhalb des für die Dimerisierung notwendigen Leucine Zippers liegen, die genregulatorische Aktivität aller anderen koexpremierten FoxP Proteine beeinflussen können.

Abschliessend zeigte die experimentelle Reduktion von FoxP1 und FoxP4 eine Beeinträchtigung des Gesangslernens, ähnlich derer, die schon für FoxP2 beschrieben wurde. Im Gegensatz zu Kontrollvögeln konnten Vögel mit RNAi-vermittelter Verminderung der FoxP1 oder FoxP4 in Area X verschiedene Gesangsmerkmale des Tutorgesanges nicht

akkurat kopieren. Diese Ergebnisse deuten darauf hin, dass FoxP1, FoxP2 und FoxP4 notwendig für auditorisch geleitetes, vokales Lernen sind.

# Lebenslauf

Der Lebenslauf ist in der Online-Version
aus Gründen des Datenschutzes nicht enthalten

## *6.7* Affidavit

I declare that my Ph.D. thesis entitled “FoxP1, FoxP2 and FoxP4 in the song control system of zebra finches: molecular interactions and relevance for vocal learning” has been written independently and with no other sources and aids than quoted.

_____

Ezequiel Mendoza, Nov. 2011

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