Genetic manipulation of the FoxP locus and expression analysis of FoxP-isoformB in *Drosophila melanogaster*

Bachelor Thesis

Faculty for Biology and Preclinical Medicine

University of Regensburg

Institute of Zoology

Department of Neurogenetics

Supervisor: Prof. Dr. Björn Brembs

Submitted by

Julia Dobbert

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Table of contents

Zusammenfassung

FoxP gehört zur Unterfamilie der forkhead box (Fox) Transkriptionsfaktoren, die sich durch eine evolutiv konservierte DNA-Bindedomäne auszeichnen. FOXP1 und FOXP2 sind erforderlich für kognitive Entwicklungsprozesse beim Menschen. In Sprachstörungen wie Autismus-Spektrum-Störung und Sprachfehlern wurden Mutationen in den *FOXP1* und *FOXP2* Genen entdeckt, was auf eine wichtige Rolle dieser Proteine hindeutet. In *Drosophila melanogaster* zeigten bisherige Entdeckungen die Bedeutung von dFoxP für normales Balz-, Lauf- und Flugverhalten, sowie bestimmte Formen des Bewegungslernens (motor learning).

Der Fokus der Arbeit lag auf der weiterführenden Charakterisierung des *dFoxP* Gens. Mit der modernen CRISPR/Cas9 Methode konnte die neue transgene FoxP-K.O. *Drosophila* Fliegenlinie in und für unser Labor generiert werden. Zusätzlich wurde die Expression des FoxP-isoformB Proteins, mit Hilfe der bereits etablierten FoxP-isoformB-Gal4 Linie, im adulten und L3 larvalen zentralen Nervensystem erstmalig untersucht. Die FoxP-isoformB Expression konnte ausschließlich in Neuronen, nicht in Gliazellen in adulten und Larven Gehirnen beobachtet werden. Außerdem wurde die FoxP-isoformB im Gehirn und im ventralen Nervenstrang während der larvalen Entwicklung exprimiert. Bei adulten Fliegen fand man ebenfalls IsoformB-exprimierende Zellen im Gehirn und im ventralen Nervenstrang. Jedoch enthüllte das FoxP-isoformB Expressionsmuster gruppierte Neuronen in mehreren Bereichen des adulten Gehirns. Die FoxP-isoformB Neuronen innervieren verschiedene Regionen des Neuropil im zentralen Nervensystem, insbesondere die protozerebrale Brücke, die bereits früher in anderen Studien mit motorischer Steuerung assoziiert wurde.

Abstract

The FoxP is a subfamiliy of the forkhead box (Fox) transcription factors characterized by an evolutionary conserved DNA-binding domain. FOXP1 and FOXP2 are essential for cognitive developmental processes in humans. In speech and language disorders like autism spectrum disorders and language impairments mutations in the *FOXP1* and *FOXP2* genes were discovered, that indicates the important role of these proteins. In *Drosophila melanogaster* recent discoveries demonstrated the importance of dFoxP for normal courtship, walking and flight behaviour and certain types of motor learning.

The focus of the thesis was further characterization of the *dFoxP* gene. With the modern technique CRISPR/Cas9 the new transgenic dFoxP-K.O. *Drosophila* fly line was generated in our laboratory. In addition, the dFoxP-isoformB protein expression of the already established FoxP-isoformB-Gal4 line was analysed in the adult and L3 larval central nervous system (CNS) for the first time. The dFoxP-isoformB expression was observed only in neuron cells, not in glia cells in adult and larvae brains. Furthermore, the dFoxP-isoformB was expressed during larval development in the central brain and in the ventral nerve cord. In adult flies, isoformB expressing cells were also found in the brain and the ventral nerve cord. But additionally, the dFoxP-isoformB expression pattern revealed clustered neurons in several adult brain areas. dFoxP-isoformB neurons innervate various neuropil regions in the CNS, in particular the protocerebral bridge, that was previously in other studies associated with motor control.

1. Introduction

Drosophila melanogaster became an important model organism especially for developmental, genetic and behavioural studies for eukaryotes through the decades (Rubin and Lewis, 2000). The flies are easy and inexpensive to handle in the laboratory, they have a short life cycle (10 days at 25°C) and a wide range of tools for genic manipulations was introduced (Jennings B.H., 2011). Interestingly, the *forkhead* (*fkh*) gene was originally discovered in *Drosophila melanogaster* by a random mutagenesis screen (Weigel et al., 1989). The related forkhead box (*Fox*) genes are evolutionary conserved transcription factors that are relevant for many biological functions. The proteins contain a highly conserved DNA-binding domain among various species (Hannenhalli and Kaestner, 2009; Golson and Kaestner, 2016). In the subfamily *dFoxP*, the *Drosophila melanogaster* ortholog dFoxP has a 110 amino acids long DNA-binding motif (Santos et al., 2011). Alternative splicing results in different transcripts of the *dFoxP* gene, the Isoform A, Isoform B and the intron-retention form (**Figure 1**) (Mendoza et al., 2014; Santos et al., 2011).

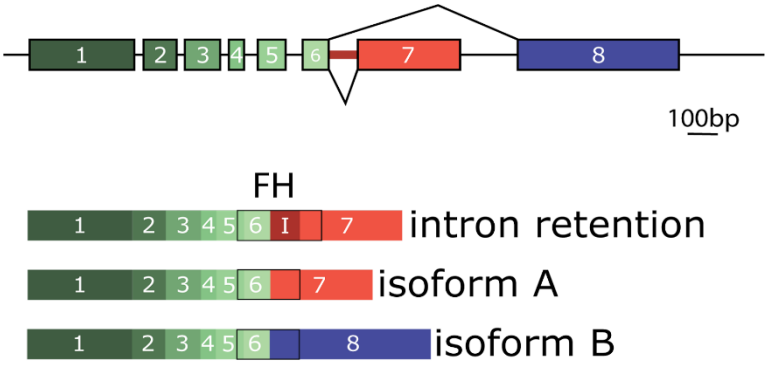

Figure 1. The dFoxP locus and its isoforms. The genomic structure of the dFoxP gene (above) and the differently transcribed isoforms (below). FH: Forkhead-Box Domain, I: Intron. (Mendoza et al., 2014).

In humans, scientists were investigating the functions of *FOXP* genes in neurodevelopmental diseases (Bowers and Konopka, 2012; Golson and Kaestner, 2016). Especially FOXP1 and FOXP2 are involved in developmental processes affecting neuronal tissues. They are linked to autism spectrum disorders and language impairments (Hamdan et al., 2010; Bowers and Konopka, 2012). The *FOXP2* gene was the first gene of this subfamily discovered to be involved in severe speech and language disorders (Lai et al., 2001). The expression of FOXP2 in developing brains in mammalian species reveals amongst other things expression in basal ganglia, cerebellum and thalamus striatum that have a role in development of motor-related circuits (Lai et al., 2003; Takahashi et al., 2003). Similar FoxP2 protein expression regions were found in zebra finches (Haesler et al., 2004; Teramitsu et al., 2004). The neural parallels to humans led to experiments where *FoxP2* mutations in vertebrates were used to characterize the phenotype of mice and songbirds concentrated on vocal and song learning (White et al., 2006). Vocal and song

learning is a type of motor learning, analogue to operant conditioning. In birds it is the vocalization from imitating a ‘subsong’ until a correct song crystallizates (Marler P, 1991; Fee MS, 2014). An inaccurate or incomplete vocal imitation of tutor song for instance, resulted by reduction of FoxP2 levels (via RNAi) in the Area X in zebra finch, that is necessary for song learning (Haesler et al., 2007). It was discovered that analogous features to vocal learning are found in operant learning in *Drosophila melanogaster*. The *dFoxP* locus mutants revealed the disruption of operant self-learning in adult *Drosophila* flies. (Mendoza et al., 2014). In addition, reduced dFoxP expression (via RNAi) affected courtship behavior and song structure and it influenced walking and flight of *Drosophila* (Lawton et al., 2014).

2. Aim of the study

The *FoxP* gene and the FoxP-isoformB transcript are hardly studied in *Drosophila melanogaster*. The study attempted further characterization of the dFoxP and the dFoxP-isoformB. Especially the dFoxP-isoformB protein is hypothesized to play a role in motor learning, because of the gained data in operant self-learning experiments (Mendoza et al., 2014) and was chosen for analysis in this thesis. The expression pattern of this specific isoform had to be characterized for a better understanding of molecular and functional pathways. We wanted to figure out in which cell type and where dFoxP-isoformB is expressed in the adult and larval fly brain. In addition, in this thesis a dFoxP-isoformB-K.O. fly line, without a functional dFoxP-isoformB protein and a dFoxP-K.O. line, without a functional dFoxP protein should be generated. The lines could be utilized for behavioural experiments (especially operant self-learning experiments) in the laboratory to analyse and compare functional aspects of these phenotypes with the knocked-out genes.

3. Materials

3.1 Fly stocks

Table 1. Drosophila stocks used for experiments

Genotype	Explanation	Origin
w; UAS-6xGFP/CyO; D3/TM3	Effector line for GFP expression	Chair stock collection
w; D3/TM3; Sb	Balancer flies for crossing	Chair stock collection
w; +/+; FoxP-isoformB-Gal4 /TM3	Driver line for Gal4 expression in FoxP-isoB neurons	Chair stock collection
+/+; VasCas9/CyO; +/+	Fly line for injection	Chair stock collection
+/+; UAS-Stinger; +/+	Effector line for GFP expression	Chair stock collection
w; FoxP-K.O/TM3; w	Knock-out (K.O) of the FoxP locus	This work

3.2 *E.coli* Strain

Competent DH5α *Escherichia coli* cells (Genotype: F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) from Thermo Fisher Scientific Inc. were used for heatshock transformation.

3.3 Chemicals

Table 2. Chemicals

Name	Origin	Application
Acetic acid	Sigma-Aldrich	TAE buffer
Agarose	Carl Roth GmbH	Agarose gel electrophoresis
Ammonium sulfate $(NH_4)_2SO_4$	Merck KGaA	LSB buffer
Ampicillin	Carl Roth GmbH	LB_{Amp} medium
Bacto™ Tryptone	Becton, Dickinson and Company	LB_0 medium

Bacto™ Yeast Extract	Becton, Dickinson and Company	LB_0 medium
Bovine Serum Albumin (BSA)	Thermo Fisher Scientific Inc.	LSB buffer
DanKlorix Hygiene-Reiniger (Chloride)	DanKlorix	Injection
DPX	Sigma-Aldrich	Slices mounting
Ethylendiamintetraacetic acid (EDTA)	Merck KGaA	TAE buffer
Ethanol	Merck KGaA	Apple agar plates
Hydrogen chloride (HCl)	Merck KGaA	LSB buffer, PFA
Magnesium sulfate ($MgSO_4$)	Merck KGaA	LSB buffer
Nipagin	Merck KGaA	*Drosophila* food
Normal-goat-serum (NGS)	PAN™-Biotech	Immunohistochemistry
Paraformaldehyde (PFA)	Merck KGaA	PFA
Potassium chloride (KCl)	Merck KGaA	Injection buffer, LSB buffer
Sodium chloride (NaCl)	Carl Roth GmbH	LB_0 medium
Sodium dihydrogen phosphate monohydrate (NaH_2PO_4)	Merck KGaA	PBS buffer
di-Sodium hydrogen phosphate dihydrate (Na_2HPO_4)	Merck KGaA	PBS buffer
Sodium hydroxide (NaOH)	Merck KGaA	LB_0 medium, PFA
Silica Gel Orange	Carl Roth GmbH	Injection
D(+)-Saccharose	VWR Chemicals BDH Prolabo®	Apple agar plates
Trizma® base	Sigma-Aldrich	LSB buffer, TAE buffer
Triton X-100	Carl Roth GmbH	PFA, PBST buffers
VECTASHIELD®	Vector Laboratories Inc.	Coverslip mounting
VOLTALEF® Oil	PROLABO	Injection

3.4 Medium

Table 3. Medium

Name	Composition	Application
Apple agar plates	• 25 g sucrose • 250 ml apple juice (from EDEKA) • Heated up to 60°C in waterbath until sucrose dissolved • 20 g Agarose • 750 ml H_2O • Heat up in microwave until agarose dissolved • Mix both solutions • 15 ml of 10% nipagine dissolved in Ethanol added • Poured in plates	Deposition of fly eggs
LB_0 medium	• 10 g/l Bacto™ Tryptone • 5 g/l Bacto™ Yeast Extract • 10 g/l NaCl • 3 ml/l 1M NaOH • autoclaved	Full medium for *E.coli* growth
LB_{Amp} medium	LB_0 medium with ampicillin (100 ng/µl)	Selection medium for *E.coli*
LB_{Amp} plates	LB_{Amp} medium with Bacto agar (15 g/l)	Selection plates for *E.coli*

3.5 Buffer and solutions

Table 4. Buffer and solutions

Description	Composition/ Origin	Application
Agarose gel	1% agarose in TAE buffer	Agarose gel electrophoresis
AMPUWA® injection water	(Commercial) AMPUWA®	Injection
Antarctic Phosphatase reaction buffer	New England BioLabs® Inc.	Dephosphorylation reaction
CutSmart® buffer	New England BioLabs® Inc.	Restriction digestion
dNTP mix (10 mM)	PeqLab	PCR
Gel Loading Dye Blue (6X)	New England BioLabs® Inc.	Agarose gel electrophoresis
GeneRuler^{TM} 1kb DNA Ladder	Thermo Fisher Scientific Inc.	Agarose gel electrophoresis
Injection buffer (10X)	• 5 M KCl • 0,1 mM $NaPO_4$ • pH 6,8	Injection
Low Salt Buffer (LSB)	• 200 mM Tris/HCl, pH 8,75 • 100 mM KCl • 100 mM $(NH_4)_2SO_4$ • 20 mM $MgSO_4$ • 1% Triton X-100 • 1 mg/ml BSA	PCR with *Taq* polymerase
PBS (10X)	• 1.3 M NaCl • 0.07 M Na_2HPO_4 • 0.03 M NaH_2PO_4	Brain dissection, immunohistochemistry
PBST (0,1%)	PBS with 0,1% Triton X-100	Brain dissection, immunohistochemistry
PFA (8%)	• 0,8 g PFA • 8 ml H_2O • 100 µl NaOH (1 M) • Heated up to 60°C in waterbath until PFA dissolved • 80 µl HCl (1 M) • 1 ml PBS (10X) • 100 µl Triton X-100	Brain fixation

Phusion® HF Reaction Buffer (5X)	New England BioLabs® Inc.	PCR with phusion polymerase
T4 DNA Ligase Buffer (10X)	New England BioLabs® Inc.	Ligation reaction
Tris-acetate-EDTA (TAE) buffer (10X)	• 40 mM Tris-acetate • 1 mM EDTA • pH 8,0	Agarose gel electrophoresis

3.6 Kits

Table 5. Kits

Description	**Origin**	**Application**
E.Z.N.A.® Gel Extraction Kit	Omega Bio-tek Inc.	Gel extraction
QIAfilter™ Plasmid Midi Kit	QIAGEN	Midi preparation
QIAprep® Spin Miniprep Kit	QIAGEN	Mini preparation
QIAquick® PCR Purification Kit	QIAGEN	Purification

3.7 Enzymes

Table 6. Enzymes

Name	**Origin**	**Application**
Antarctic Phosphatase	New England BioLabs® Inc.	Dephosphorylation reaction
Calf Intestinal Alkaline Phosphatase (CIP)	New England BioLabs® Inc.	Dephosphorylation reaction
Nhel-HF®	New England BioLabs® Inc.	Restriction digestion
Sacll-HF®	New England BioLabs® Inc.	Restriction digestion
Spel	New England BioLabs® Inc.	Restriction digestion
Pstl-HF®	New England BioLabs® Inc.	Restriction digestion
Phusion® HF DNA Polymerase	New England BioLabs® Inc.	PCR with Phusion polymerase
T4 DNA Ligase	New England BioLabs® Inc.	Ligation reaction
Taq polymerase	Made in the laboratory	PCR

3.8 Antibodies

Primary Antibodies

Table 7. primary antibodies

Antibody	Antigen	Host animal	Origin	Dilution
7E8A10	ELAV	Rat, monoclonal	DSHB	1:50
8D12	REPO	Mouse, monoclonal	DSHB	1:1000
nc82	Bruchpilot	Mouse, monoclonal	AH	1:500

Secondary Antibodies

Table 8. secondary antibodies

Antibody	Antigen	Host animal	Marker	Origin	Dilution
Anti-Mouse IgG	Mouse	Goat, polyclonal	Cy3	Jackson (Dianova)	1:200
Anti-mouse IgG	Mouse	Goat, polyclonal	DyLight 633	Thermo Fisher Scientific Inc.	1:200
Anti-rat IgG	Rat	Goat, polyclonal	Alexa Fluor 555	Invitrogen	1:400

3.10 DNA/RNA templates, primers and vectors

DNA/RNA templates

Extracted genomic DNA (courtesy of Ottavia Palazzo) was used for PCR amlification of the homology inserts (HomI and HomII). The Inserts comprised fragments of exon 6 (HomI) and exon 8 (HomII).

Table 9. Homology inserts

Name	Size
Homology insert (HomI)	1 Kb
Homology insert (HomII)	1 Kb

The two guide RNAs (gRNA) and the dFoxP-K.O.-construct (both courtesy of Ottavia Palazzo) for the injection via CRISPR/Cas9 was already prepared. The dFoxP-K.O.-construct contained homologous regions from exon 1 to 3 (HomI) and exon 3 to 6 (HomII).

Primers

Table 10. Primers

Name	Sequence 5`→ 3`	Origin
dFoxPB knockout Homl fw (10X)	GGG GGC TAG CGA CAA GCA GTT AAC CCT AAA CGA	Invitrogen
dFoxPB knockout Homl rv (10X)	GGG GCC GCG GAA GAG TCA GAA ACA GGA AAT TGC	Invitrogen
dFoxPB knockout Homll fw (10X)	GGG GAC TAG TAG CAC AAT CGA TAA ATA ATA TAT CAT	Invitrogen
dFoxPB knockout Homll rv (10X)	GGG GCT GCA GTA ATT TTT CAA CTA TTC ATG TAC ACA G	Invitrogen

Vectors

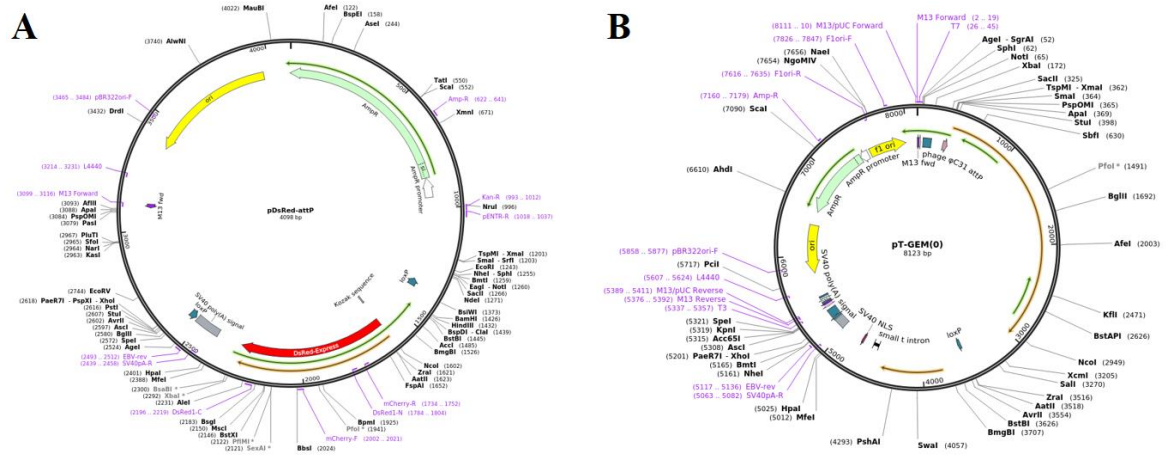

Figure 2. Vectors used for cloning experiments. Both contain origin of replication, resistance against ampicillin for selection, cut sites for restriction enzymes and marker regions for insertion. A: Vector pHD-Dsred-attP. B: pT-GEM(0).

The vector pHD-Dsred-attP was used to produce a plasmid with the homology regions of the dFoxP protein for the CRISPR/Cas9 system (Harris et al., 2016). The Dsred is a marker that drives expression

of red fluorescents in the fly eyes. The vector pT-GEM(0) was used to produce a plasmid with the homology regions of the dFoxP protein and to additionally introduce a Gal4 into the *dFoxP-isoformB* gene for the CRISPR/Cas9 system.

4. Methods

4.1 Fly handling

All fly strains of *Drosophila melanogaster* were maintained in glass vials with standard *Drosophila* food consisting of agar, cornmeal, malt extract, soymeal, sugar beet molasses, yeast and 0,3% nipagine (fungicide) with dried yeast powder. The vials were kept on 25°C and/or 18°C in an incubator room with 60% relative humidity and a simulation of a 12 h light/ 12 h dark cycle. The flies were transferred into fresh vials every week. All flies were kept on 25°C except for *w ; D3/TM3 ; Sb* flies, which were instead kept at 18°C. For determination of virgins and sex of the flies the Stemi 305 (Zeiss) was used.

Fly virgins

Virgin female flies of *w ; D3/TM3 ; Sb* were seperated from the stocks daily and kept in vials without dried yeast powder on 18°C until they were crossed.

Fly crossing

1-4 virgin female flies and 1-2 male flies were placed together in a new vial (as described above) for each cross. This parental generation deposited eggs for 4-7 days until they were removed. After 10 days the enclosed offspring flies were selected against their marker phenotype and placed in new vials or used directly for further experiments.

4.2 Construct generation

4.2.1 DNA Preparation

To propagate the amount of the vector, heatshock transformation with competent DH5α *E.coli* cells (see **3.2**) and subsequently Plasmid mini preparation were used.

The Inserts were propagated via PCR amplification. Afterwards the products were analysed with agarose gel electrophoresis. The appropriate bands of 1 Kb were cut out of the gel and extracted with the gel extraction kit (see **table 5**). Then the concentration of DNA was measured at the NanoDrop. The Inserts were stored at -20°C or used directly for further experiments.

Gradient PCR Amplification of the Homology-Inserts

The DNA of Homl and Homll for the Vector were amplified with already extracted genomic DNA (gDNA) (see **3.10**) from wild type flies. The gradient PCR was done to figure out which annealing temperature fit best to the primers. The following two mastermixes were prepared in 1,5 ml Eppendorf tubes:

Table 11. Composition of the mastermixes for amplification

Composition	Volume (Homl)	Volume (Homll)
dNTP-Mix	10 µl	10 µl
gDNA	10 µl	10µl
Phusion buffer	100 µl	100 µl
Phusion polymerase	5 µl	5 µl
Primer Homl fw (1X)	6,25 µl	-
Primer Homl rv (1X)	6,25 µl	-
Primer Homll fw (1X)	-	6,25 µl
Primer Homll rv (1X)	-	6,25 µl
H_2O_{MilliQ}	Ad 500 µl	Ad 500 µl

20 µl each of the mastermixes were pipetted into five 0,5 ml PCR reaction tubes. The cups were placed into the Thermocycler (Biometra TOne) and the following programme was used to perform the amplification.

Table 12. Programme for gradPCR

Step	Temperature	Lenght	Cycles
Activation (Denaturation)	98°C	30 sec	
Denaturation	98°C	10 sec	38X
Annealing	55°C + 2°C per line	20 sec	
Amplification	72°C	40 sec	
Termination (Amplification)	72°C	2 min	
Storage	16°C	∞	

4.2.2 DNA Extraktion

Plasmid mini preparation

The Vector (pHD-Dsred-attP) was extracted with the QIAprep® Spin Miniprep Kit (see **table 5**). Initially the plasmid was isolated from LB_{Amp} plates (see **table 3**). One single cell colony was picked with a tip and transferred into an autoclaved glass vial with 3 ml LB_{Amp} medium (see **table 3**). Then the cells were shaked at 220 rpm for 14-16h in the 37°C room. The next day the protocol of the manufacturer was followed. The DNA was eluted with 26 µl of Buffer EB. The concentration of the DNA was measured at the NanoDrop. After that the Vector was used immediately or stored at -20°C for future experiments.

Plasmid midi preparation

The midi preparations with the QIAfilter™ Plasmid Midi Kit (see **table 5**) were performed for CRISPR constructs (Vectors and guideRNAs) to receive purer and higher concentrations of DNA and RNA. The manufacturers instructions were followed. The air-dry pellet was redissolved in 75 µl of AMPUWA® injection water (see **table 4**). To determine the concentration of DNA or RNA the NanoDrop was used. After that the CRISPR constucts were stored at -20°C until the injection.

Agarose gel electrophoresis

Agarose gel electrophoresis is a widespread technique for fractionation of DNA molecules within an electric field. 5 µl of blue loading dye (5X) (see **table 4**) were added to all DNA probes to visualize and to weight down the DNA during the electrophoresis. An 1% agarose gel was prepared and loaded with DNA ladder as a marker (see **table 4**) and the DNA probes. The electrophoresis was run in TAE buffer (see **table 4**) at 120 V for 30-40 minutes.

Gel extraction

Specific DNA molecules can be isolated by gel extraction. For this the E.Z.N.A.® Gel Extraction Kit (see **table 5**) was used. After agarose gel electrophoresis the gel was analysed under UV light (Transilluminator by INTAS) and the appropriate bands were cut out with razorblades. The gel slices were transferred into 1,5 ml Eppendorf microcentrifuge tubes and their volume was determined by weight. Further steps were carried out as decribed in the protocol. To elute the DNA 26 µl of Elution Buffer were used. Afterwards the concentration of the DNA was measured and it was frozen at -20°C for further experiments.

Determination of DNA and RNA concentrations

The concentration of DNA and RNA was measured at the NanoDrop1000 Spectrophotometer from peqLab. The blank was done against the respective Elution Buffer or water.

4.2.3 Restriction digestion of DNA

For the injection via CRISPR/Cas9 suitable constructs had to be generated. Therefor the vector and the Inserts were digested with the same correct enzymes to create appropriate overhangs for ligating them together. The following approaches were prepared in 1,5 µl Eppendorf microcentrifuge tubes on ice:

Table 13. Composition of a restriction digestion of DNA

Composition	**Quantity**
CutSmart® Buffer (10X)	5 µl
DNA	500 ng/µl
Restriction enzymes	1 µl each
H_2O_{MilliQ}	Ad 50 µl

A construct that should contain HomI was digested with NheI-HF® and SacII-HF® while a consruct contained HomII was digested with PstI-HF® and SpeI (see **table 6**). The reaction was incubated at 37°C over night. After that the Vectors were dephosphorylated.

3.2.4 Dephosphorylation of pHD-Dsred-attP

After the vector was cut with the restriction enzymes, it was possible that it ligated intramolecular. To minimize the occurence of such religation of the ends, they were incubated with a Phosphatase. The Phosphatase removes the 5` and 3` terminal phosphates. For this, 1 µl of CIP or 1 µl of Antarctic Phosphatase (see **table 6**) with 5 µl Antarctic Phosphatase reaction buffer (see **table 4**) were added to the restriction digested Vectors. The reaction was incubated at 37°C for 1-2 h until further experiments.

Purification of DNA

The Inserts and dephosphorylated Vectors were either purified with the QIAquick® PCR Purification Kit (see **table 5**) following the manufacturer instructions. Instead of the PCR samples the restriction digested reaction were used and the DNA was eluted with 26 µl Buffer EB. Or they were analysed on

an agarose gel and the appropriate bands for Vector and Inserts were cut out under UV light and extracted with the E.Z.N.A.® Gel Extraction Kit. After that the DNA concentration was determined and they were used directly for ligation and/or stored at -20°C.

4.2.5 Ligation of pHD-Dsred-attP and Homology-Inserts

To create CRISPR constucts that can be injected into flies it was necessary to ligate the Homology-Inserts into the Vector with the help of a ligase. For Ligation the following approach was pipetted into 1,5 ml Eppendorf microcentrifuge cups on ice:

Table 14. Compostition of a ligation

Compostition	**Quantity**
Homology-Insert	37,5 ng
T4 DNA Ligase	1 µl
T4 DNA Ligase Buffer (10X)	1 µl
Vector (pHD-Dsred-attP)	50 ng
H_2O_{MilliQ}	Ad 10 µl

The T4 DNA Ligase was added at least. Only Vector and Inserts, that were cut with the same restriction enzyme were ligated together. In order to control if the ligation worked, control approaches without the Homology-Insert (with water instead of the Insert) were prepared, too. Afterwards, the ligation reactions were incubated at 18°C for 4 h or over night.

4.2.6 Heatshock transformation of the construct into competent *E.coli* cells

The constructs and the two guideRNA for injection were transformed via heatshock into competend *E.coli* cells to ensure that their amount could be increased by mini or midi preparation. The competent DH5α-*E.coli* cells in an 1,5 ml Eppendorf microcentrifuge cup were thawed 10 minutes on ice until the ligation reaction was added and mixed by pipetting up and down. In order to retransformate already created constructs or empty vectors 1 µl was added to the cells. After that the Eppendorf cup was put on ice for 10 minutes again. The construct got into the bacteria cells while the cell mixture was incubated in the 42°C waterbath for 45 seconds. Then it was incubated on ice for 2 minutes and 800 µl of sterile LB_0-Medium (see **table 3.4**) were added. After shaking 45 minutes at 37°C in the Thermomixer compact (Eppendorf) the *E.coli* bacteria were centrifuged at 6000 rpm for 30 seconds. The pellet was resuspended

in 100 µl supernatant, the rest of the supernatant was discarded. Then the bacteria suspension was plated on LB_{Amp} plates under sterile conditions and the plates were incubated at 37°C over night.

4.2.7 Colony polymerase chain reaction (colonyPCR)

With polymerase chain reaction (PCR) specific DNA fragments can be amplified by using suitable primers. In order to figure out if the heatshock transformated bacteria cells contain the desired construct, a colony PCR was used. The two following mastermixes were prepared in 1,5 ml Eppendorf microcentrifuge tubes on ice for 17 PCR reactions each:

Table 15. Composition of the mastermixes for colonyPCR

Composition	Volume (construct HomI)	Volume (contsruct HomII)
dNTP-Mix	20 µl	20 µl
LSB Buffer (10X)	10 µl	10 µl
Polymerase (*Taq* or Phusion)	20 µl	20 µl
Primer Homl fw (1X)	20 µl	
Primer Homl rv (1X)	20 µl	
Primer HomII fw (1X)		20 µl
Primer HomlI rv (1X)		20 µl
H_2O_{MilliQ}	280 µl	280 µl

The polymerase wad added at least. 20 µl each were places into 0,5 ml PCR reaction tubes on ice. One randomly chosen single colony from the LB_{Amp} plates of the heatshock transformated cells was picked with a small tip and mixed into the PCR reaction mix. After that the tip was inoculated into a sterile glass vial containing 2 ml sterile LB_{Amp} and incubated at room temperature over night. This was repeated 15 times and the last PCR reaction tube was transfected with a colony of the control plates. The PCR reaction tubes were placed into the Thermocycler (Biometra TOne) and the following programme was used for amplification.

Table 16. Programme for colonyPCR

Step	Temperature	Lenght	Cycles
Activation (Denaturation)	98°C	30 sec	
Denaturation	98°C	10 sec	
Annealing	55°C	30 sec	35X

Amplification	72°C	1 min	
Termination (Amplification)	72°C	3 min	
Storage	10°C	∞	

After that the amplification products were analysed with agarose gel electrophoresis.

4.3 CRISPR/Cas9

Genome engineering is a powerful tool to edit the genome of many organisms and analyse structural and functional aspects of the genome (Gratz et al., 2013; Bassett A.R., Liu J.-L., 2014; Gratz et al., 2014). The CRISPR (clustered regularly interspaced short palindromic repeats) /Cas9 system is a simple and efficient technique to generate mutations in a desired target position in the genome in several model organisms (Cong et al., 2013; Mali et al., 2013; Basset A.R., Liu J.-L., 2014) including *Drosophila* flies (Liu et al., 2012; Gratz et al. 2013). An optimized system comprising the crRNA and tracrRNA to single chimeric RNA (gRNA) was introduced (Jinek et al., 2012, Bassett et al., 2013) and used in this thesis, too. With CRISPR/Cas9 it was possible to induce site-specific double strand breaks (DSB) with the help of the Cas9/gRNA complex (Gratz et al., 2013). The gRNA contained an average of 20 nucleotide (nt) long sequence that was complementary to the genomic target sequence to guide the Cas9 endonuclease to the desired target site in the DNA. For the cleavage it was also necessary that the target sequence contained a protospacer adjacent motif (PAM), NGG, next to the homologue 20 nt sequence (Jinek et al., 2012). The endonuclease Cas9 was expressed in the germline (Kondo and Ueda 2013; Ren et al. 2013; Sebo et al. 2013; Gratz et al., 2014) by the *VasCas9* fly line (see table 1). After DSB the cell could repaired the break with either non-homologous end joining (NHEJ) or homology-directed repair (HDR). The HDR required a homologous DNA sequence as a template (donor template) and could be triggered (Bibikova et al., 2002; Liu et al., 2012). This was exploided by using CRISPR constructs containing two homologus regions to the target sequence. The HomI region was equal upstream to one DSB and Hom II sequencewas equal downstream of the other cleavage. After hybridization the region between the homologous inserts of the Vector functioned as a donor template and gets then inserted into the genome of the fly (Gratz et al., 2013; Gratz et al., 2014).

4.4 Injection

A popular and safe way to create *Drosophila melanogaster* transformants of interest is to inject CRISPR constructs with a micro capillary into fly embryos (see **4.3**). The injection-mix was prepared in 1,5 ml Eppendorf microcentrifuge tubes:

Table 17. Composition of an injection-mix

Composition	Quantity
Injection buffer	2 µl
Vector with HomI and HomII	500 ng/µl
Guide RNA	200 ng/µl
AMPUWA® water	Ad 20 µl

The mix was centrifuged at 13,2 rpm for 5 minutes and refilled into a new 1,5 ml Eppendorf microcentrifuge tube. The injection flies deposited eggs on agar plates (see **table 3**) with some yeast for 30 min. After that the embryos were collected carefully with a brush. The chorion was removed by washing the embryos in chloride (see **table 2**) for 2-3 minutes and then washed with water. Afterwards the embryos were lined up on microscope slides (20-60 eggs each) and dried in a box of Silica Gel Orange (see **table 2**). The eggs were coated with VOLTALEF® oil (see **table 2**) before the injection to prevent them to dry out meanwhile. The amount of mix that was injected into each egg varied respectively but 20 µl was enough for 700 eggs. With the micro capillary the injection-mix was injected into the pole cells and the glass slides were put afterwards onto apple agar plates on 18°C for 2 days. On the third and the fourth day after injection, hatched larvae were counted and transferred into glass vials containing standard *Drosophila* food without yeast. Hatched flies were crossed with balancer flies (see **table 1**). The descentant flies were checked at the fluorescent microscope Leica MZ FLlll for transformant flies, these could be identified by having red fluorescent eyes (Dsred). Transformants were crossed with balancer flies again and their progeny were checked for shortened hairs on the back and no green fluorescent eyes (from the injection line) additionally. These flies were crossed with each other to create a stock.

4.5 Brain dissection

After crossing the driver line (*dFoxP-isoformB-Gal4* mutant) flies with flies of an effector line *(UAS-6xGFP* or *UAS-Stinger*) (see **table 1**), the offspring expressed the GFP protein within the *dFoxP-isoformB* locus of the genome. To analyse this expression, the brains and/or ventral nerve cords of the GFP expressing flies were dissected at the Leica M60 microscope (Leica) and after that analysed at the

fluorescent and/or confocal microscope. The whole flies were fixated in an Eppendorf microcentrifuge tube in 1 ml 4% PFA (see **table 4**) (500 µl of 8% PFA and 500 µl 0,1% PBST) at room temperature for 2 h. Afterwards the PFA was removed and replaced by 1 ml of 0,1% PBST (see **table 4**). The brains were dissected in 0,1% PBST in a glass reservoir with forcers. Then the brains were mounted with VECTASHIELD® (see **table 2**) on prepared slices. The mounting slices were prepared with DPX (see **table 2**) to arrange some space in the middle of the glasses for the fly brains. The brains were prevented from dehydration by the VECTASHIELD® medium (see **table 2**) and the borders were coated with clear nail polish (from different companies).

4.6 Immunohistochemistry

To further analyse the expression of dFoxP-isoformB in *Drosophila* flies stainings with specific antibodies were used. The brains and/or ventral nerve cords of GFP expressing flies were fixated and dissected as described above (**4.4**). After that they were washed with 0,1% PBST 4-5 times within 1 h. The fly brains were blocked in 7% normal-goat-serum (see **table 2**) for 1 h. Then the primary antibody was added to the brains and incubated over night at 4°C. The next day the brains were washed again 4-5 times within 1 h in 0,1% PBST and the secondary antibody was pipetted on the brains and incubated 3-4 h at room temperature. Afterwards the washing steps were repeated (4-5 times within 1 h) and the brains were mounted (see **4.4**).

4.7 Imaging

Fluorescent microscopy

The *Drosophila* brains were analysed at the Leica MZ FLlll (Leica) fluorescent microscope. The magnification was 20X and/or 40X.

Confocal laser scanning microscopy

The fly brains were analysed at the TCS SP8 (Leica) confocal microscope to get high-resolution and detailed images of the brains. The magnification was 40X and/or 20X. The images were edited with the ImageJ (version 1.5) software.

5. Results

5.1 no creation of the dFoxP-isoformB-K.O. *Drosophila* strain via CRISPR/Cas9

The creation of the dFoxP-isoformB-K.O. *Drosophila* stock requires a construct for injection into fly embryos to knock-out the dFoxP-isoformB protein. For this construct the homology regions HomI and HomII had to be amplified with gradient PCR first and after that analyzed via agarose gel electrophoresis. The highest annealing temperature led to the highest amount of insert DNA. The bands at 1 Kb indicate the appropriate DNA fragments and were extracted from the gel with a razorblade (**Figure 3**). After that, the homology inserts and the vector pHD-Dsred-attP were digested with the appropriate restriction enzymes and the vectors were dephosphorylated and analyzed on agarose gel. The dephosphorylation prevents the religation of the vector. Digested vectors are smaller when the restriction enzymes cut the vector correctly because the enzymes cut parts of the vector away to create similar cut sites for insertion of HomI and HomII (**Figure 4**). The vector (digested with NheI-HF® and SacII-HF®) and HomI were ligated together and the vector (digested with PstI-HF® and SpeI) and HomII were ligated together. After heatshock transformation the amount of grown DH5α *E.coli* bacterial colonies of the controls were lower than the dFoxP-isoformB-K.O. construct colonies. Less bacterial colonies. To test the bacteria for containing the correct construct a colony PCR was performed and the products were analysed on an agarose gel. The vector with HomI or HomII have a size of 1 Kb. You don`t see a band at this size in any of the tested bacterial cells, that indicate that there is no construct that contains neither HomI nor HomII (**Figure 5**). The use of different concentrations of Vectors and Inserts to digest with restriction enzymes and different polymerases (*Taq* and Phusion) for the colony PCR also did not lead to any positive clone. It was not possible to create a dFoxP-isoformB-K.O. construct to inject into *Drosophila* embryos.

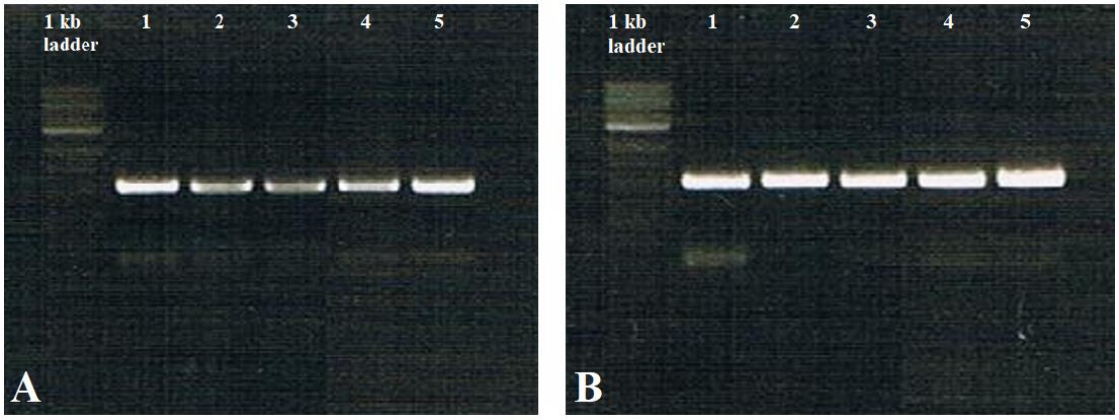

Figure 3. Amplification of the homology inserts HomI and HomII. Increasing temperatures of 2°C each from line 1 (55°C) to line 5 (65°C). The highest temperature results in the highest amount of DNA. Bands at 1Kb. 1kb ladder. **A**: HomI; **B**: HomII.

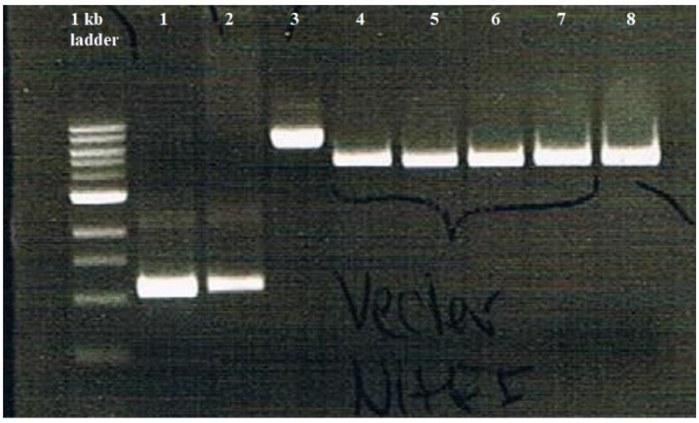

Figure 4. Digestion of homology inserts HomI and HomII and the vector pHD-Dsred-attP with different restriction enzymes. The undigested vector is bigger than the digested ones. 1 kb ladder. Band 1: HomI; Band 2: HomII; Band 3: undigested vector; Band 4-7: Vector digested with NheI-HF® and SacII-HF®; Band 8: Vector digested with PstI-HF® and SpeI.

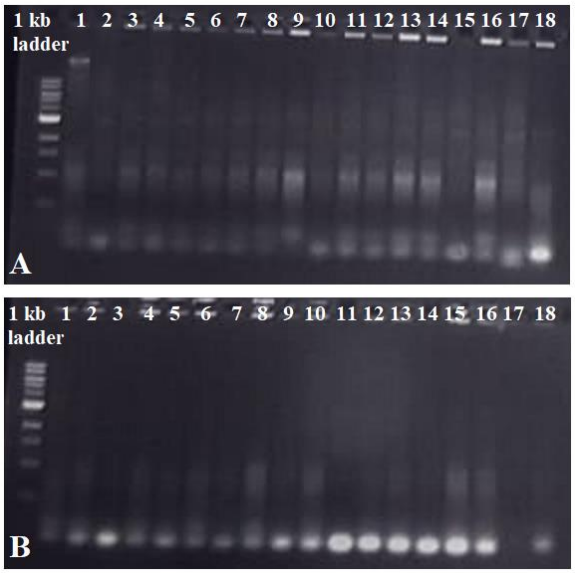

Figure 5. Colony PCR of DH5α cells after transformation with pHD-Dsred-attP-HomI/II constructs. No positive cell colonies. 1kb ladder. **A**: Band 1-17: pHD-Dsred-attP-HomI construct; Band 18: negative control. **B**: Band 1-17: pHD-Dsred-attP-HomII construct; Band 18: negative control.

5.2 Creation of the dFoxP-K.O. *Drosophila* strain via CRISPR/Cas9

The dFoxP-K.O. construct for injection was produced with the pHD-Dsred-attP vector and two homology domains as described in **5.1**. The dFoxP-K.O. construct and the two gRNAs (see 3.10) were injected into *VasCas9* flies. Here, the homology inserts recognized specific regions within the *dFoxP* gene and so stimulate the HDR mechanism of the cell (Bibikova et al., 2002; Liu et al., 2012). HomI targeted the region from exon 1 to exon 3 and HomII the sequence from exon 3 to 6. After the DSB a region from exon 1 to 3 were knocked out (**Figure 6**). The DsRed marker of the donor vector was inserted in between. The injection mix was injected into 700 *Drosophila* embryos. 197 (28%) larvae survived the injection and 75 (10,7%) hatched and were crossed with *w; D3/TM3; Sb* flies. The F1 generation of these flies were checked for transformants under the fluorescent microscope, positive

transformants contained the dFoxP-K.O. construct and had red fluorescent eyes (**Figure 7**). There were two positive transformants that were recrossed to w; D3/TM3; Sb flies in order to obtain a transformant with the appropriate balancer chromosome (TM3) and the progeny of this cross was again crossed to itself to create a stock of dFoxP-K.O. flies.

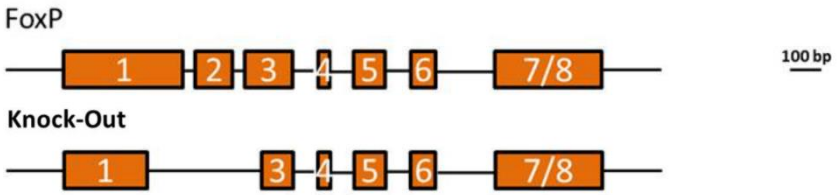

Figure 6. The dFoxP locus before and after the knock-out. The structure of the dFoxP gene before (above) and after the knock out (bottom). A region including a part of exon 1 to a part of exon 3 is removed.

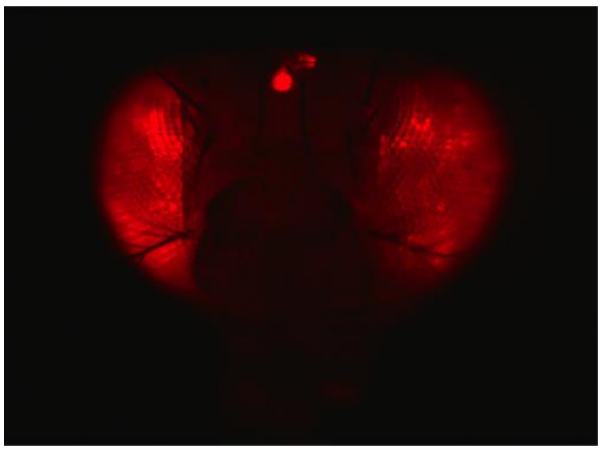

Figure 7. Red fluorescent eyes in Drosophila melanogaster. Red fluorescence caused by gene editing, in this case by the Vector pHD-ScarlessDsRed. (http://flycrispr.molbio.wisc.edu/scarless)

5.3 Expression analysis of dFoxP-isoformB in *Drosophila* flies

For the analysis of the FoxP-isoformB protein expression, the driver line *w; +/+; FoxP-isoformB-Gal4 /TM3* was used. This fly line was indroduced to the laboratory in a similar way as described above (**5.2**). The pT-GEM Vector was used to create the FoxP-isoformB-Gal4 construct that was injected into *VasCas9* embryos. The crossing scheme is the same described in 5.2. This driver line was crossed with an effector line to induce GFP expression. The cross with *UAS-Stinger* effector flies led to nuclear expression of Stinger-GFP and *UAS-6xGFP* crosses expressed cell membrane localized CD8-GFP, visualized the projections of the neurons. The nervous system of the offspring generation was dissected and stained with different antibodies (Immunohistochemistry) for analysis. Immunostainings with anti-ELAV visualize neurons (ELAV is a neuron-specific protein) and stainings with anti-REPO makes glial cells visible (REPO is expressed specifically in glia cells). Immunohistochemistry with BruchPilot is useful for understanding the anatomical structure of the nervous system, BruchPilot marks the synapses. All analysed brains and ventral nerve cords were from heterozygous flies (larvae and adult). The dissected larvae brains were L3 larvae and the adult nervous systems was from 1-5 old day flies.

5.3.1 dFoxP-isoformB expression in adult flies

Immunohistochemistry revealed dFoxP-isoformB protein expression in neurons. There was a co-localization of neurons and dFoxP-isoformB cells in anti-ELAV stainings (**Figure 8 A, C**). On the contrary, in labelled anti-REPO cells we found no co-localization with dFoxP-IsoformB positive cells (**Figure 8 B, D**). All of the dFoxP-isoformB cells nucleai were found in the cortex. The cortex is the outer layer of gliacells and neuron, that encase the inner neuropil part of the brain. We identified several dFoxP-isoformB densely packed cells, called clusters. Most of the clusters were bilateral in both left and right part of the fly brain, but we also found central ones. Clustered positive cells were especially in the central brain and between the optic lobes and the central brain. There were dFoxP-isoformB cell populations close to the region of the central complex and close to the saddle and the vest. Furthermore dFoxP-isoformB cells were localized close to the region of the gnathal ganglion and the superior medial protocerebrum. Some more distributed dFoxP-isoformB positive neurons showed GFP signals in other regions and also in the optic lobes (**Figure 8**).

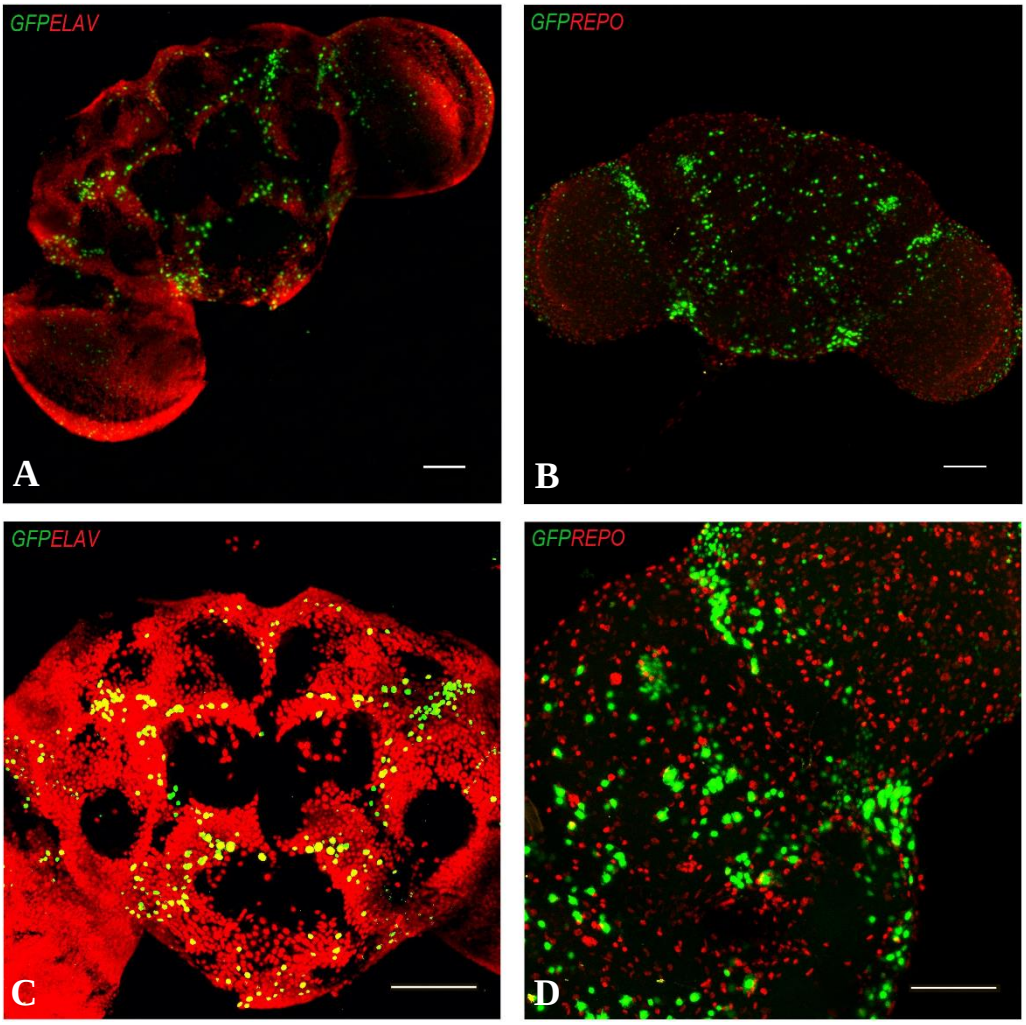

Figure 8. Confocal images of the dFoxP-isoformB expression pattern of adult *Drosophila* brains stained with different antibodies. The FoxP-isoformB-Gal4 strains drive nuclear UAS-GFP (green). The GFP signals are distributed in the whole brain ans clustered cells are in several regions of the brain. The dFoxP-isoformB is located in two clusters between the optic lobes and the central brain (above and below). GFP cell populations are close to the gnathal ganglion and the superior medial protocerebrum. Labelled neurons are also close to the saddle close to the central complex. **A**, **B**: brain at 20X. **C**, **D**: closer look at the brain at 40X. **A**, **C**: Staining with anti-elav show neurons (red). Co-localisation of neurons and FoxP-isoformB neurons (yellow). **B**, **D**: Staining with anti-repo show gliacells (red). No co-localisation of gliacells and FoxP-isoformB neurons.

For a better understanding of the dFoxP-isoformB protein it was useful to analyse which part of the nervous system was innervated by the dFoxp-isoformB neurons. The neuropil regions that seemed to be more marked by the CD8-GFP were: the protocerebral bridge (PB), the region of the saddle, vest and superior medial protocerebrum (**Figure 9 A**, **B).** There were also neuron populations visible, that show labelled cells in similar regions, that were identified with Stinger-GFP stainings. Furthermore, with this kind of staining, we were able to see projections of neurons between different parts of the brains, for instance the axons protruding from one of the most visible clusters that were linking the two hemispheres of the brains (**Figure 9 A**, **B**). The dFoxP-isoformB neurons prolongated also along the linkage of the brain and the ventral nerve cord (**Figure 9 D**). The ventral nerve cord was highly marked, too. The ventral nerve cord contained dFoxP-isoformB neurons prolongating especially in outer part of the nerve cord close to other parts of the fly body (**Figure 9 C**).

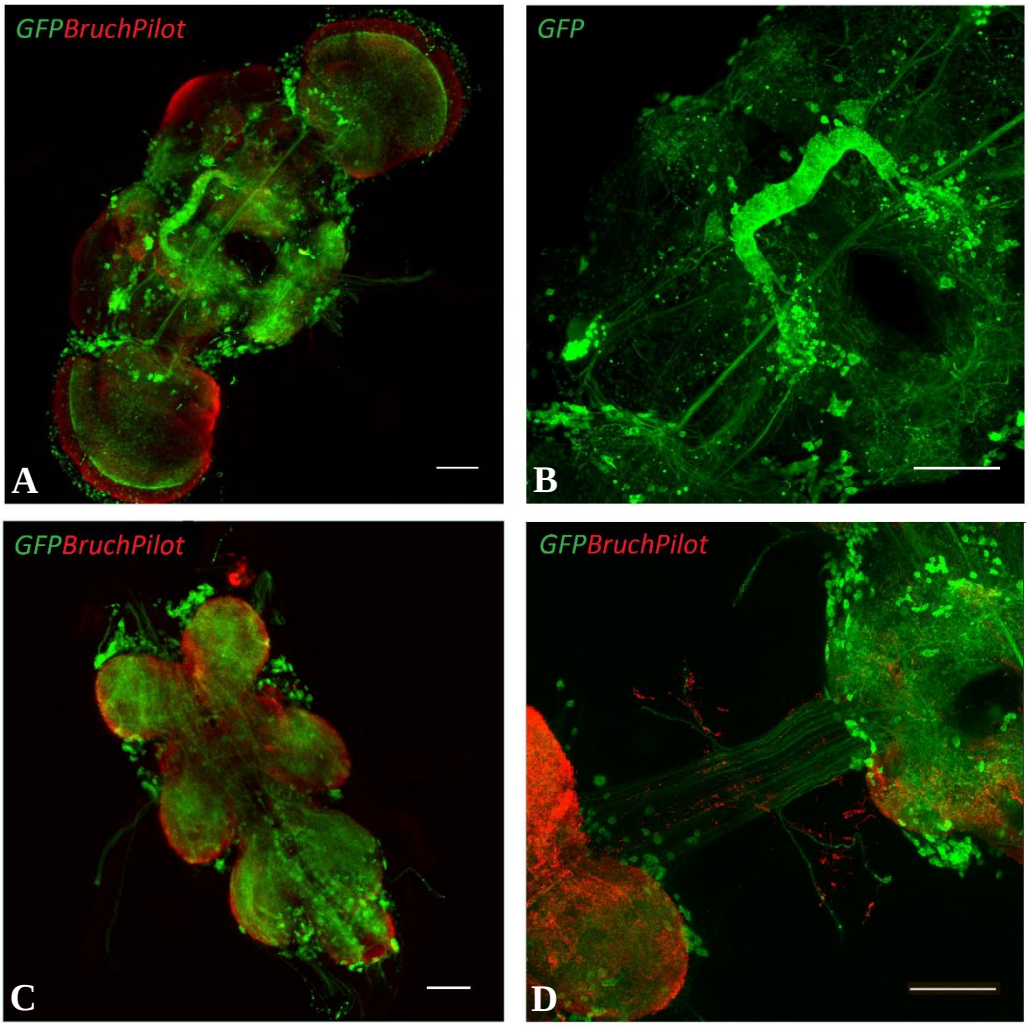

Figure 9. Confocal images of adult brains with the projections of dFoxP-isoformB neurons. The dFoxP-isoformB-Gal4 strains drive cell membrane bound UAS-GFP (green). Immunostaining with Bruchpilot (red). There are dFoxP-isoformB marked regions in the brains and the ventral nerve cord (**A**-**D**). The dFoxP-isoformB neurons project especially into the protocerebral bridge, saddle, vest and superior medial protocerebrum. There is a marked axon linking the two hemispheres (**A**, **B**). **A**: the brain at 40X. **B**: central brain at 20X. **C**: the ventral nerve cord at 20X. **D**: marked connection between brain and ventral nerve cord.

5.3.2 dFoxP-isoformB expression in larvae

dFoxP-isoformB cells were found in the developing brain (L3 larvae) of *Drosophila*. It looks like a lower expression of dFoxP-isoformB cells in the larval brain. There are distributed dFoxP-isoformB positive cells in the brain and in the nerve cord. The anterior part will become the central brain, whereas the posterior part will become the ventral nerve cord in adult flies (**Figure 10**). The immunohistochemistry with anti-ELAV and anti-REPO replicate the results seen in the adult: we can see co-localized staining of GFP and ELAV cells (**Figure 10 A**, **C**) and there is no co-labelling of GFP and REPO in the brain glial cells (**Figure 10 B**, **D**).

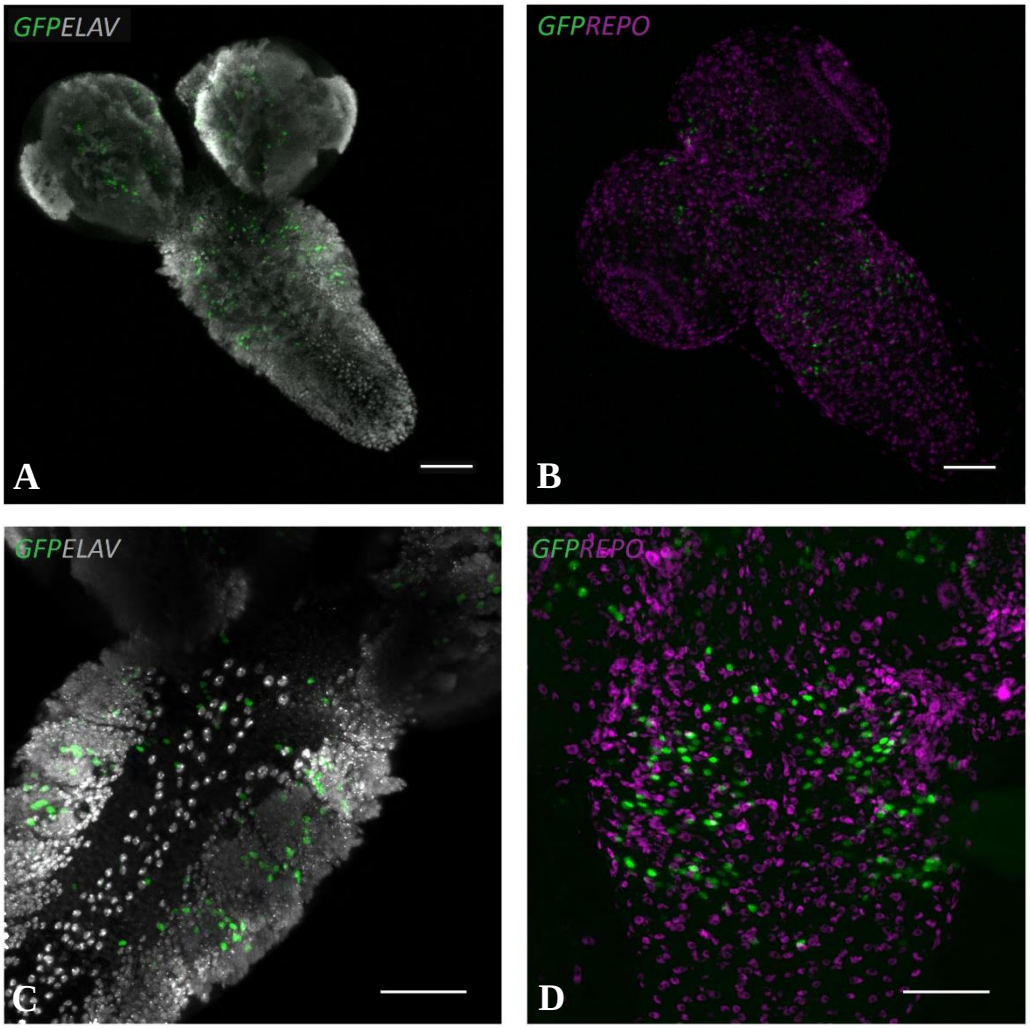

Figure 10. Confocal images of the dFoxP-isoformB expression pattern of larval *Drosophila* brains stained with different antibodies. The FoxP-isoformB-Gal4 strains drive nuclear UAS-GFP (green). There are FoxP-isoformB neurons distributed in the brain and in the nerve cord. **A**, **B**: larval brain at 20X. **C**, **D**: closer look at the brain at 40X. **A**, **C**: Staining with anti-elav show neurons (grey). Co-localisation of neurons and FoxP-isoformB neurons (light green). **B**, **D**: Staining with anti-repo show gliacells (violet). No co-localisation of gliacells and FoxP-isoformB neurons.

The analysis of dFoxP-isoformB positive neurons innervating other parts of the larval brain replicated the results of the adult brain. We can see prolongation of positive neurons in the brain and in the nerve cord. More marked CD8-GFP regions are in the center of the brain, that will become the central brain in the adult fly and in the ventral nerve cord. Furthermore, we can see more GFP at a structure between the two brain hemispheres that looks like a precursor of the PB (**Figure 11**).

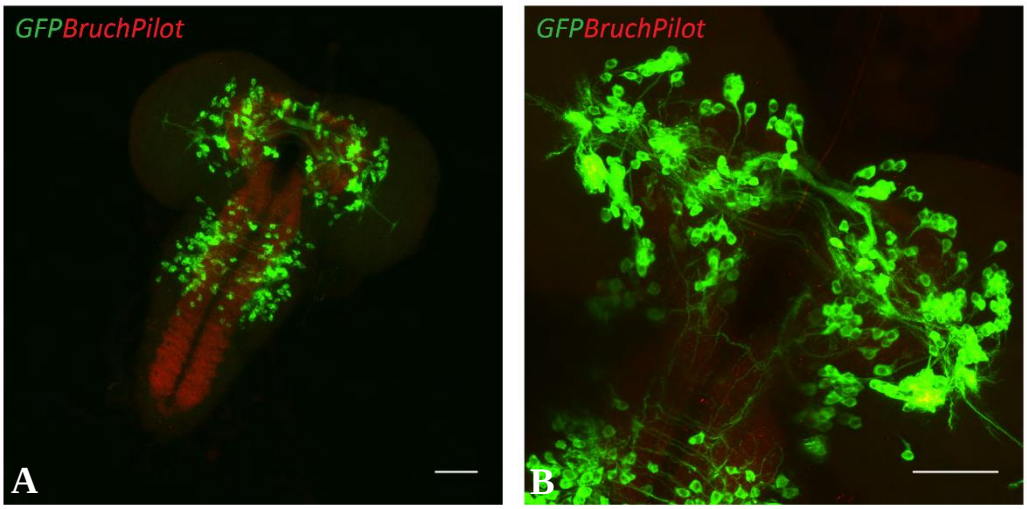

Figure 11. Confocal images of the larval brain with the projections of the FoxP-isoformB neurons. The FoxP-isoformB-Gal4 strains drive cell membrane bound UAS-GFP (green). Immunostaing with Bruchpilot (red). The FoxP-isoformB neurons innervert neurons in the brain and in the ventral nerve cord. Linking both hemispheres there is a structure that looks like the protocerebral bridge that is marked. **A**: larval brain at 20X, **B**: closer look at the larval brain at 40X.

6. Discussion

6.1 no creation of the dFoxP-isoformB-K.O. construct

Little is known about the *dFoxP* locus and its differently transcribed isoforms in *Drosophila*. To study and analyze more of this gene, a fly line, with an inoperable dFoxP and dFoxP-isoformB protein is indispensable. One aim of this study was to create a dFoxP-isoformB-K.O. *Drosophila* line, that can be used to further characterize the dFoxP-isoformB in flies. This requires a dFoxP-isoformB-K.O. construct, that can be used for injection and establish the *Drosophila* line. We thus decided to knock out the dFoxP-isoformB protein expression with the CRISPR/Cas9 technique. We expected to create the dFoxP-isoformB-K.O. with these methods, because they performed well for the creation of the dFoxP-K.O. construct (**5.2**) and the FoxP-isoformB-Gal4 construct by Ottavia Palazzo. The vector pHD-Dsred-attP was used as a donor template for the construct. This vector was already utilized successfully for genome editing via CRISPR/Cas9 in Drosophila in previous studies (Gratz et al., 2014).

We attempted to clone the homology regions HomI and HomII after another into the pHD-Dsred-attP Vector. Therefor we decided to do both in parallel, to get one of the homology regions into the plasmid. However, there were no positive *E.coli* colonies detectable after the colony PCR. After heatshock transformation there were less colonies grown on the control plates. This is due to the expectation that without an insert no complete vector occurs. The colonies on the control plate might grew with religated vectors without the inserts, but they still contained the ampicillin resistance. The examination of the restriction digestion homology revealed that the homology inserts and the vector had different sizes after the digestion (**Figure 4**). This indicates correct cut of the digestion enzymes. The next step was to vary the polymerases during the colony PCR to exclude that there are replication problems, like to low efficiency, with the *Taq* polymerase. But the colony PCR with Phusion polymerase, that has a higher efficiency during the PCR led to similar results (no positive clone after colony PCR). This suggests that the problem why this experiment did not work could be the ligation step. We tried different incubation times (4h or over night) but could not find any colony with the correct construct.

6.2 Creation of the dFoxP-K.O. *Drosophila* fly strain via CRISPR/Cas9

For further analysis of the *dFoxP* gene and it`s function in *Drosophila* a fly line without a no longer operational *dFoxP* gene and so protein is necessary. Therefor the dFoxP-K.O. construct, that was already generated, was used for our injection. The dFoxP-K.O. fly line was produced via CRISPR/Cas9. The vas-Cas9 flies that express the Cas9 protein in the germline were found out to lead to the maximized efficiency in the injection compared to other systems (Gratz et al., 2014). That is why we expected to increase the probability for finding transformant flies with this technique. The dFoxP-isoformB-Gal4 fly line was produced similarly and there were found 3 positive transformant flies after injection.

6.3 Expression analysis of the dFoxP-isoformB in *Drosophila* flies

The dFoxP-isoformB protein expression pattern revealed on one hand expression in neurons, not in glial cells and five clusters of GFP positive neuron populations. In the report from Lawton et al. (2014) in Drosophila, expression of dFoxP was also found only in neurons and not in glial cells. This fits to our data, that at least dFoxP-isoformB is expressed exclusively in neurons. Considering all of our dFoxP-isoformB stainings, several clusters of densely packed cells could be identified. We can see three major bilateral clusters (3-5), and two central major clusters (1,2) (**Figure 12**). Expression analysis of other insects of hymenoptera showed expression in some similar regions as in *Drosophila*. Specific clusters and groups of AmFoxP and AmFoxP^{IsoB} (IsoformB transcript of AmFoxP) were identified in honeybees and compared to two other bee species. Among other, AmFoxP and AmFoxP^{IsoB} cells appeared in a region of the gnathal ganglia (vGNG) that corresponded to our labeled cells in the region of the gnathal ganglion cluster 2 (Schatton et al., 2018). This begs the question of the function of these *FoxP* neurons close to the gnathal ganglion. They also found expression in the region between the optic lobes and the central brain (mvLO) in stainings for all isoforms of *FoxP* in honeybees and bumblebees and further characterized it. The cluster 5 in the similar part of the brain was found in our labellings, too. Nevertheless, there were differences within the isoforms. They hymenopteran FoxP-isoformB was not expressed in mvLO specific neurons, whereas the Drosophila isoformB was expressed in this neuron population (Schatton et al., 2018). This indicated differences within the *FoxP* splicing variants within the studied species (honeybee, bumblebee, Drosophila). A further study for comparison of these animals is supported in order of these results. The clusters 1, 3 and 4 are more difficult to compare with the honeybee stainings. The cluster 4 dFoxP-isoformB neurons might be in a comparable clsuteres region to alLCA in bees they found in all-isoform stainings (Schatton et al., 2018). Furthermore, we identified several structures the dFoxP-isoformB neurons projected into. Previous studies in Drosophila found dFoxP expression only in Kenyon cells of the mushroom bodies. The $\alpha\beta_{core}$ and γ (weak) were labelled in the stainings by their *FoxP-Gal4* system (DasGupta et al., 2014). The expression in special regions in the Kenyon cells of the mushroom bodies was also observed in other insect species in honeybees and

bumblebees (Kiya et al., 2008; Schatton & Scharff, 2017). With our dFoxP-isoformB stainings we could not confirm this expression in *Drosophila*. However, the expression of dFoxP in the mushroom bodies could not be excluded with the results of this thesis. Further analysis of all isoforms of *dFoxP* is required. However, according to our data the isoformB is not expressed in the mushroom bodies, this expression is likely due to other isoforms of *dFoxP*. However, dFoxP expressing neuron populations in the central complex were found in several studies (Kiya et al., 2008; Lawton et al., 2014; Schatton et al., 2018). In honeybees the central complex contains single neuron populations, but the PB as a subregion of the central complex was not labelled. Our dFoxP-isoformB stainings revealed also two populations of neurons close to the PB. Interestingly, dFoxP neurons projected into the PB in *Drosophila* brains in one study. (Lawton et al, 2014). We could identify among other things the PB as a marked structure in our stainings. The central complex is expected to play a role in locomotion control and is involved in visual learning (Strauss and Heisenberg, 1993; Strauss R., 2002). The dFoxP-isoformB (and also dFoxP) proteins likely have a role in motor control. PB mutants like *no-bridge* and *tay bridge* has been associated with affecting the fly legs that leads reduction of walking speed and activity (Strauss et al., 1992; Poeck et al., 2008). The results of Strauss et al. (1992) in flight experiments, where *no bridge* mutants showing a certain phenotype support more the hypothesis of dFoxP mutations in the operant self-learning experiment as in Mendoza et al. (2014). In addition, dFoxP in *Drosophila* was also found out to be involved in other motor coordination circuits like courtship and song and motor related impairments in walking and flying behavior (Lawton et al., 2014). Corresponding to our data (especially PB) dFoxP may have a role in several motor control involving behaviors. For the other regions we found dFoxP-isoformB neurons projected into (saddle, vest and superior medial protocerebrum) further studies are necessary to define their exact localization and function in *Drosophila*.

Lawton et al. (2014) found distributed dFoxP expression in the whole CNS. This corresponds to our larval GFP stainings with dFoxP-isoformB cells spread in the brain and the ventral nerve cord. But further experiments are necessary to identify their exact localisation and function.

The first impression of the expression pattern of dFoxP-isoformB in adult and larval brains illustrates that further analysis like co-stainings for transmitter (GABA, Glutamate, Dopamine etc.) or expression patters of embryos are required to extend functional aspects of these proteins.

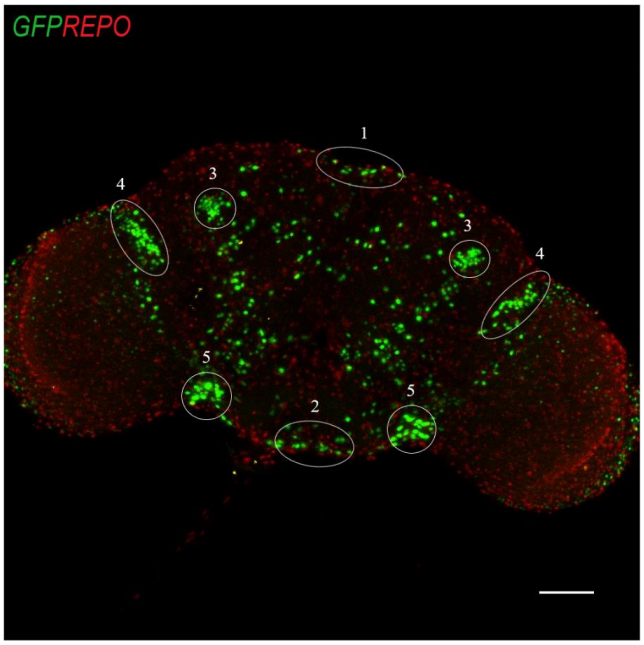

Figure 12. Confocal image of adult Drosophila brain dFoxP-isoformB expression clusters. GFP (green) and anti-elav (red) staining. The clustered regions from 1-5 (white circles) are marked in the brain.

6. Appendix

6.1. References

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Website:

(http://flycrispr.molbio.wisc.edu/scarless)

6.2 List of figures

6.3 List of tables

6.4 Index of abbreviations

%	Percent
µ	Mikro
Amp	Ampicillin
bp	Base pair
C	Celsius
CIP	Calf Intestinal Alkaline Phosphatase
CNS	Central nervous system
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DsRed	*Dicosoma sp.* red fluorescence protein
DSB	Double strand break
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
F	First filial generation
fw	forward
g	Gram
GFP	Green fluorescence protein
grad	Gradient
gDNA	genomic DNA
gRNA	guide RNA
h	Hour
H_2O	Water
H_2O_{MiliQ}	MiliQ water
Hom	Homologous region
K	Kilo
Kb	Kilo bases
K.O.	Knock-out
l	Liter
LB	Luria Bertani medium
LSB	Low salt buffer
m	Meter
M	Molar
min	Minutes
ml	Mililiter

n	Nano
NGS	Normal goat serum
PAM	Protospacer adjacent motiv
PB	Protocerebral bridge
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	“power of hydrogen”, negative decimal cologarithm
rev	reverse
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
SDS	Sodium dodecyl sulphate
sec	Seconds
TAE	Tris-acetate-EDTA
Taq	Polymerase from *thermus aquaticus*
Tris	Tris (hydroxyl methyl) aminomethane
UV	Ultra Violette
V	Volt

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Declaration of Authorship

The bachelor thesis was written independently, I used no other sources and tools than those indicated and have not yet submitted it to any other examining authority.

Date Signature