universität wien

MASTERARBEIT

Titel der Masterarbeit

„Functional Analyses of Inner Brain Opsins in Zebrafish and Medaka“

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2014

Studienkennzahl lt. Studienblatt:	A 066 877
Studienrichtung lt. Studienblatt:	Masterstudium Genetik und Entwicklungsbiologie
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1. Introduction

When we think of sensation and sensory cells we usually think of the five senses: hearing, sight, touch, smell, and taste. However, evidence has accumulated that the brain itself is sensory as well, in particular light sensory. This was suggested by different approaches: functional analyses and histologically. KARL VON FRISCH observed that blinded minnows (*Phoxinus laevis*) are still able to change the intensity of their body color (FRISCH, 1911). SCHARRER demonstrated that even ophtalectomized fish react to light when shone on their head (SCHARRER, 1927). In the beginning, both authors postulated that the pineal organs might be responsible for their findings. Removing the pineal falsified their hypotheses; the fish were still able to detect light. Hence, FRISCH and SCHARRER considered deep brain regions to be light sensitive (FRISCH, 1911; SCHARRER, 1927). YOUNG discovered that even blinded and pinealectomized lamprey larvae react to light (YOUNG, 1935). BENOIT and OTT found out that the hypothalamus of ducks is light-sensitive; exposure to light leads to testicular enlargement (BENOIT and OTT, 1944). Experiments with the Japanese quail (*Coturnix japonica*), which adapts strongly and robustly to changes in light conditions (NAKANE *et al.*, 2010), revealed that not the assumed pineal organs react to light but inner brain regions like the mediobasal hypothalamus or septal region of the telencephalon (MENAKER *et al.*, 1970; WILSON, 1974; SIOPES and HOMMA, 1979; OLIVER and BAYLE, 1982; FOLLET *et al.*, 1998; YOSHIMURA *et al.*, 2003).

A recent publication by FERNANDES *et al.* reported that zebrafish larvae lacking eyes and the pineal show still light-seeking behavior. However, *orthopedia*-deficient fish (*otpa*) have reduced dark photokinesis and and a small reduction of the melanopsin *opn4a*. The authors were able to narrow the light-sensitive neurons to the preoptic area. This provides evidence that deep brain photoreceptors mediate light-depending behavior (FERNANDES *et al.*, 2012). KOKEL and co-workers removed surgically eyes, pineal organs, fore-, and midbrain in zebrafish larvae. These larvae show still robust photomotor response driven by the hindbrain (KOKEL *et al.*, 2013).

1.1 Opsins

Opsins are G-protein coupled photoreceptors, which interact with a co-factor; in most cases 11-*cis* retinal (*Figure* 1 (b); TERAKITA, 2005).

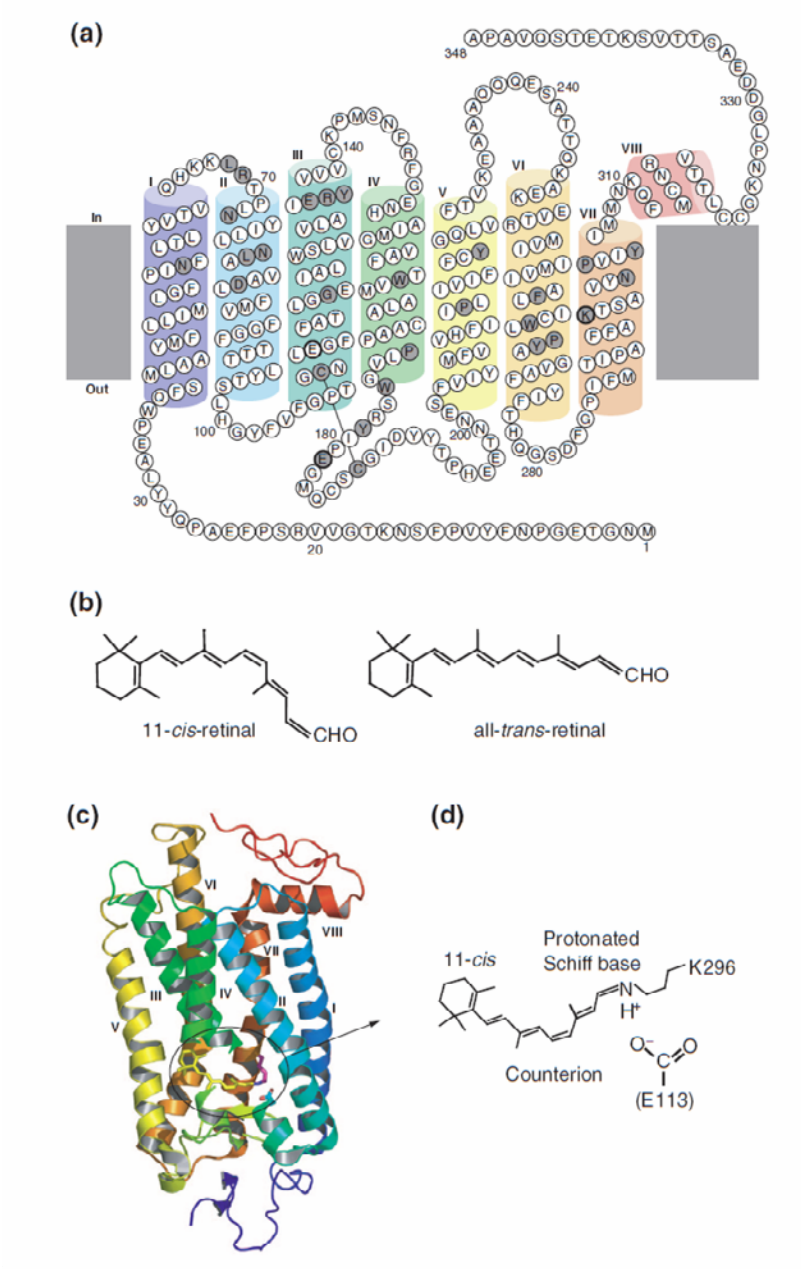


Figure 1: *Characteristic opsin structure and chromophore retinal (TERAKITA, 2005). (a) Bovine rhodopsin. Conserved amino acids are highlighted in gray. K296 and E113 are depicted as bold circles. The disulfide bond is formed between C110 and C187. (b) shows the structures of chromophore retinal. (c) The crystal structure of bovine rhodopsin. (d) Schiff base linkage between K296 and 11-cis-retinal stabilized by E113.*

Opsins are divided into seven subfamilies: the vertebrate visual and non-visual opsin subfamily, the encephalopsin/tmt-opsin subfamily, the G_q-coupled opsin/melanopsin subfamily, the G_o-coupled opsin subfamily, the neuropsin subfamily, the peropsin subfamily, and the retinal photoisomerase subfamily (*Figure* 2; TERAKITA, 2005).

The G_q-coupled opsin/melanopsin subfamily (rhabdomeric opsin) and the encephalopsin/tmt-opsin subfamily together with the vertebrate visual and non-visual opsin subfamily (ciliary opsin) evolved before deuterostomes separated from protostomes. However, the phylogenetic tree of the other four subfamilies (the G_o-coupled opsin subfamily, the neuropsin subfamily, the peropsin subfamily, and the retinal photoisomerase subfamily) is not resolved well. The depicted opsins of the lophotrochozoa Patinopecten and Todarodes are closer related than it is shown in the phylogenetic tree (*Figure* 2). In addition, support values like maximum likelihood and neighbor joining at critical branches are missing. The protostome *Platynereis dumerilii* possesses opsins of the neuropsin and peropsin subfamily each (unpublished data, personal communication). To sum up, the putative bilaterian ancestor had most likely more than three different opsins. (TERAKITA, 2005).

Opsins like bovine rhodopsin (*Figure* 1 (a)) possess specific characteristics, which are prerequisites for their function: Seven transmembrane α-helices domains are anchored within the membrane (*Figure* 1 (a)). K296, located in transmembrane domain VII, interacts with the chromophore 11-*cis*-retinal by forming a Schiff-base linkage, whereas E113 acts in the vertebrate visual and non-visual opsin subfamily as counterion (*Figure* 1 (d)). However, in other opsins tyrosine, phenylalanine, methionine, or histidine are located at position 113 and E181 serves as counterion (TERAKITA, 2005).

Another functional feature of bovine rhodopsin are two cysteins at the positions 110 and 187 in the second and third extracellular loop, whose disulfide bond formation is crucial for conformational stability (*Figure* 1 (a); MOUTSAKI *et al.,* 2003).

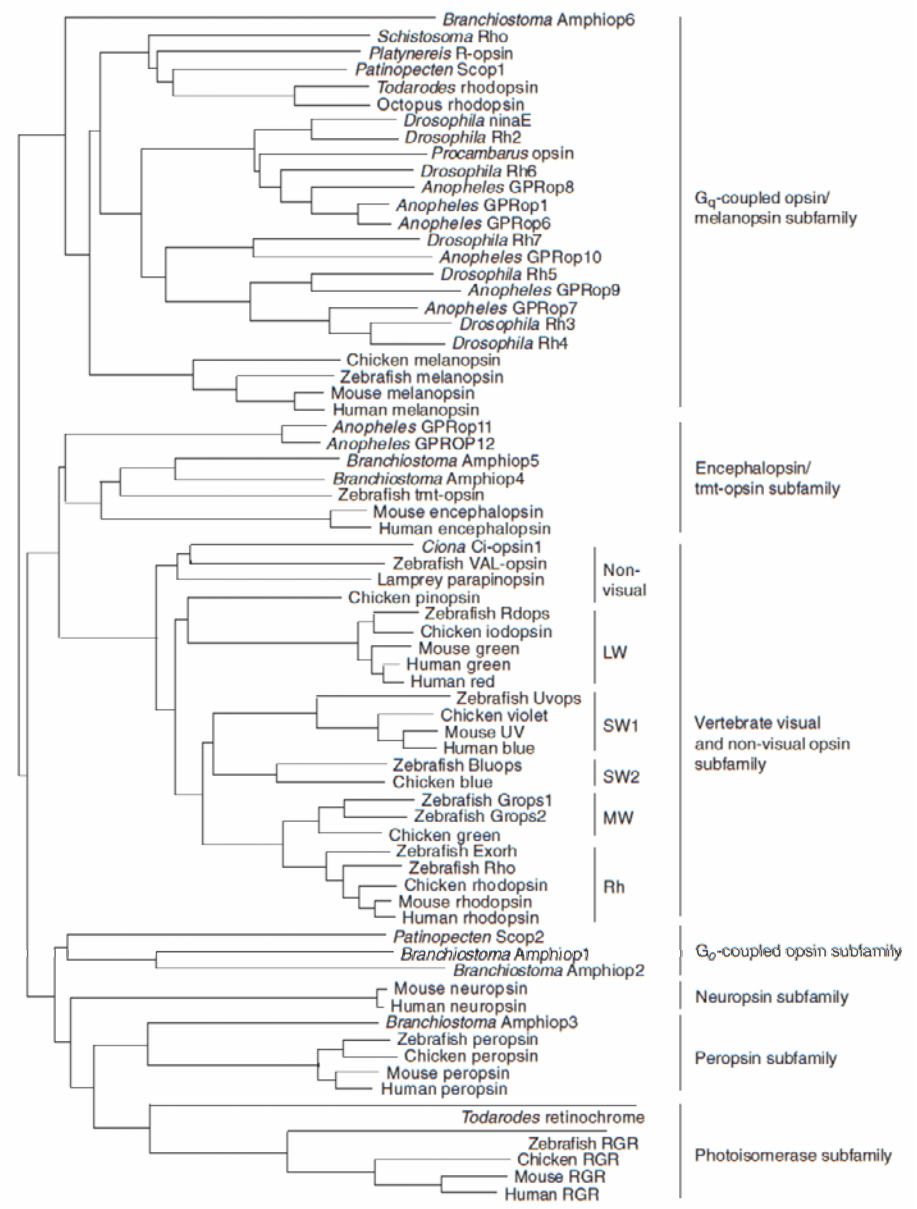


Figure 2: *The opsin subfamilies: the vertebrate visual and non-visual opsin subfamily, the encephalopsin/tmt-opsin subfamily, the G_q-coupled opsin/melanopsin subfamily, the G_o-coupled opsin subfamily, the neuropsin subfamily, the peropsin subfamily, and the retinal photoisomerase subfamily (TERAKITA, 2005).*

1.2 Encephalopsin/tmt-opsin

Opsins belonging to the encephalopsin/tmt-opsin subfamily were described in mouse and human (BLACKSHAW and SNYDER, 1999) and in the teleosts zebrafish, Fugu (*Fugu rubripes*; MOUTSAKI *et al.*, 2003) and medaka (FISCHER *et al.*, 2013).

The TMT-Opsins and Encephalopsins are sister groups; this was observed due to phylogenetic analyses (*Figure* 3; FISCHER *et al.*, 2013).

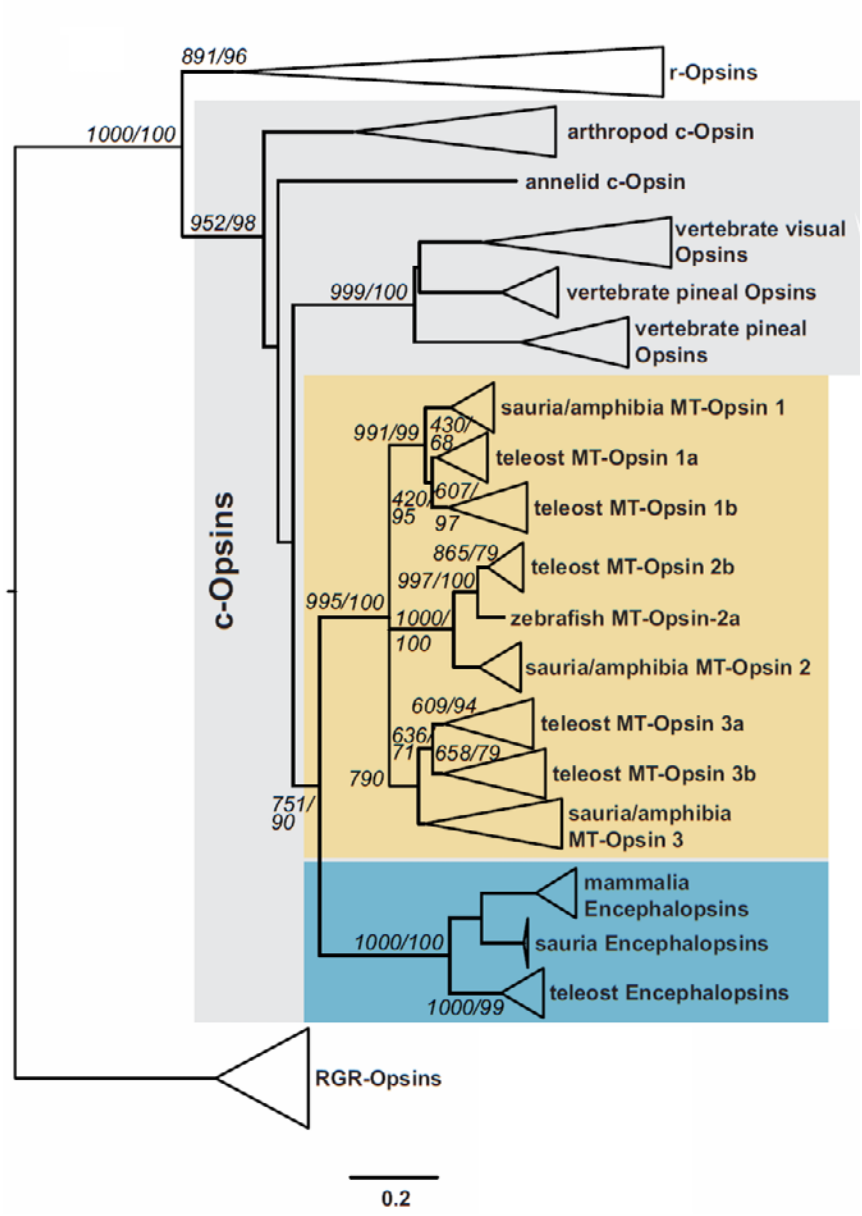


Figure 3: *Phylogenetic tree of TMT-Opsins and Encephalopsins showing their close relationship within the opsins (FISCHER et al., 2013; modified).*

Moutsaki and co-workers identified a new opsin (Moutsaki *et al.*, 2003). Until their findings, rod and cone cells in the retina were considered the only light-sensitive cell types in the vertebrate eye (Soni *et al*., 1998). The novel opsin is expressed in several tissues of zebrafish and *Fugu*. Hence, the authors called the new opsin teleost multiple tissue (tmt) opsin. Expression of TMT-opsins was described in zebrafish in the eye, brain, kidney, heart, and in the embryonic cell line, as well as in the telencephalon, the optic tectum, and the hindbrain of dissected brains (Moutsaki *et al.*, 2003).

A recent publication by Fischer *et al.* revealed that tmt-opsins are specifically expressed within the nervous system in brain nuclei, the eye, and the pineal. Interestingly, tmt-opsins are expressed within brain regions that are associated with deep brain photoreceptors. Furthermore, expression was reported in the forebrain, midbrain, and hindbrain (Fischer *et al.*, 2013). Fischer *et al.* observed *tmtops1b* and *tmtops2* expression in amacrine cells. In addition, *tmtops2* is expressed in the pineal organs, the annular ligament of the eye, and the iris. These cell types are usually associated with light sensory organs (Fischer *et al.*, 2013).

Whereas Moutsaki *et al.* assume tmt-opsins to be involved in photic regulation of peripheral clocks (Moutsaki *et al.*, 2003), Fischer *et al.* provide evidence that tmt-opsins render neuronal cells light-sensitive. Moreover, their results may indicate the existence of ancestral “sensory-inter-motorneurons’’ in brain evolution (Fischer *et al.*, 2013).

The opsins of the encephalopsin/tmt-opsin subfamily are also found outside teleosts and birds. Based on analyses of genomes and transcriptomes, opsins of the encephalopsin/tmt-opsin subfamily were also identified in amphibians and reptiles. Orthologs of tmt-opsin2 were observed in marsupials: in the Opossum (*Monodelphis domestica*), in the Platypus (*Ornithorhynchus anatinus*), and in the Wallaby (*Macropus eugenii*; Fischer *et al.*, 2013). However, Eutheria seem to lack tmt-opsins completely, probably due to their nocturnal ancestors; Encephalopsins, on the contrary, are also present in placental mammals, including humans (Fischer *et al.*, 2013).

1.3 Valop

Soni and Foster identified an opsin in the retina and amacrine cells of Atlantic salmon (*Salmo salar*); they called it vertebrate ancient (VA) opsin (Soni and Foster, 1997), which is a functional photoreceptor *in vitro* (Soni *et al.*, 1998). Kojima *et al.* identified a splice variant of VA opsin in zebrafish, whose cytoplasmic tail is 67 amino acids longer; thus, Kojima and coworkers called it VA-*Long* (VAL) opsin (Kojima *et al.*, 2000). When VAL-opsin binds to 11-*cis*-retinal, it acts as photopigment with an excitation maximum in green light (λ = 500 nm; Kojima *et al.*, 2000). It is expressed within cells of the thalamus (Kojima *et al.*, 2000).

VA and VAL opsins are also not unique to teleosts; they were in addition identified in the hypothalamus of chicken (Halford *et al.*, 2009).

A recent publication of our lab demonstrated that VAL-opsin and tmt opsins are mostly expressed within the same cells, suggesting a complex ability to perceive light (Fischer *et al.*, 2013). Moreover, this could mean that vertebrate inter- and motorneurons modulate their output in a light-dependent manner (Fischer *et al.*, 2013).

1.4 Zebrafish and Medaka

Zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are both freshwater teleosts and well established molecular model organisms. Both species are separated by about 300 million years of evolution (Kasahara *et al.*, 2007); this is almost the same time period as between birds and mammals (about 310 million years; Kumar and Hedges, 1998). Furthermore, zebrafish and medaka dwell in two different habitats; whereas the subtropical zebrafish lives in the Indian Ganges and its tributaries and distributaries, medaka dwells in shallow waters in Japan, like rice paddies (the scientific name *Oryzias latipes* – "ricefish" – refers to its habitat). In comparison to zebrafish, medaka is exposed to steady change in the length of the day over the course of the year. Therefore, it is interesting to compare the functions of opsins in these two species. In addition, medaka has a reduced number of non-visual opsins compared to zebrafish lowering possible functional redundancy.

1.5 TALEN

Transcription Activator-Like Effector Nucleases technology is a method for site specific cleavage. TALENs originate from the plant pathogen *Xanthomonas ssp*. They induce transcription by binding to promoter sequences of their host. TALENs are proteins that consist of a DNA binding domain and an artificially linked catalytic *Fok*I domain (*Figure 4*). The DNA binding domain can be designed for any sequence of interest. TALENs bind DNA as dimers. When the *Fok*I domains are close enough to each other, the DNA is cut (Christian *et al*., 2010; Li *et al*., 2011). This double-strand break is repaired by non-homologous end joining (NHEJ). Erroneous NHEJ leads to mutations like insertions or deletions (indels) resulting in non-functional gene products. To screen for these mutations, TALENs are designed around a restriction site, which can afterwards be used as readout. When indels alter the restriction site, the DNA remains uncut within a restriction enzyme digest.

We use *Fok*I heterodimers to increase the specificity of the TALEN activity, which lowers the possibility of off-target effects.

Our lab used TALEN successfully to knock out different genes in zebrafish, medaka, and *Platynereis*.

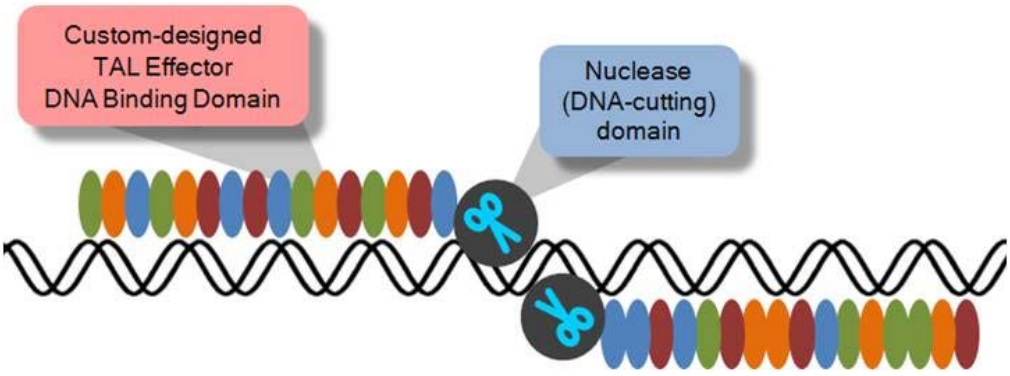


Figure 4: *Scheme of TALEN pair (www.pnabio.com). The DNA binding domain can be designed arbitrarily. The FokI nuclease domain depicted as scissors cuts the DNA double-strand as dimer.*

2. Aim of the study

It has been known for decades that light pervades the bony skull and reaches inner brain regions even of mammals (Brunt *et al.*, 1964). Furthermore, early studies postulated that the brain itself reacts to light (Frisch, 1911; Scharrer, 1927). However, hitherto the light receptors, the light-receptive cell types, and the function of light-sensory neurons is still unknown. With my studies I aimed to investigate the function of zebrafish and medaka inner brain opsins by creating knock-outs and by expressing fluorescent dyes *in vivo* under control of the opsin promoter and gene regulatory sequences.

3. Material and Methods

3.1 Media

SOC medium

For 400 mL:

Tryptone	8 g
Yeast extract	2 g
NaCl	0.2 g
KCl 0.25 M	4 mL
$MgCl_2$ 2 M	2 mL
Glucose 1 M	8 mL

to 400 mL ddH_2O

Adjust pH to 7.0 and autoclave.

LB medium

For 2 L:
20 g NaCl
20 g tryptone
10 g yeast extract

Fill up with ddH_2O to a total volume of 2 L, dissolve completely and autoclave.
For plates add 15 g bacto agar per liter LB.

3.2 Solutions

50x TAE
242 g Tris·HCl
57.1 mL glacial acetic acid
100 mL of 0.5 M EDTA, pH 8.0
fill up to 1 L with autoclaved ddH_2O

10 x PBS, pH 7.4-7.5
300 mL of 10x PBS for preparation of 3 x 1 L 1x PTW
15 PBS tablets
to 300 mL ddH_2O
adjust pH to 7.4–7.5

1x PTW
1x PBS with 0.1 % Tween-20

Ethidium bromide staining bath
60 µL of ethidium bromide stock solution in 600 mL 1x TAE

16 % PFA stock solution

What you need:

- heating plate
- balance/scale
- 1000 mL beaker
- thermometer
- paraformaldehyde powder (stored at 4 °C)
- tap water and autoclaved ddH2O
- magnetic stirrer
- 250 mL glass bottle
- 10 M NaOH/10 M HCl
- weighing dish and spatula
- pipettes/ pasteur pipettes
- pH test strips

- fill about 200 mL of tap water into the 1,000 mL glass beaker, place on heating plate
- fill up 250 mL glass bottle with 190 mL autoclaved ddH_2O
- heat up the water to 65 °C
- weigh 32 g of paraformaldehyde powder into a weighing dish under the fume hood while wearing a mask
- transfer powder into the 65 °C hot water in the bottle
- stir at 65 °C for at least 30 min
- After 30 min the solution will still be cloudy; add one drop of 10 M NaOH to the solution and let stir for several minutes, if it is not completely clear add 1 or 2 additional drops
- When the solution is clear, measure the pH with test strips (pH 7.2 to 7.5)
- adjust pH with 10 M HCl or 10 M NaOH
- let the bottle of PFA cool down to room temperature
- fill up to exactly 200 mL
- sterile-filter with 250 mL Stericup® Filter Units

10x ERM

10 g NaCl
0.3 g KCl
0.4 g $CaCl_2 \cdot 2\ H_2O$
1.63 g $MgCl_2 \cdot 6\ H_2O$
170 mL 1M HEPES
0.001 % methylene blue
to 1 L ddH_2O

10x E3

50 mM NaCl
1.7 mM KCl

3.3 mM $CaCl_2 \cdot 2\ H_2O$
3.3 mM $MgSO_4$
170 mL 1M HEPES
0.001 % methylene blue
to 1L ddH_2O

Lysis buffer

25 mL 1M Tris·HCl pH 8.0
0.5 mL EDTA pH 8.0
2.5 mL 10 % ($^{w}/_{v}$) SDS
10 mL 5 M NaCl
to 250 mL ddH_2O

3.3 Antibiotics

Chloramphenicol stock solution 30 mg/mL in EtOH, f.c. 1:2000.
Kanamycin stock solution 15 mg/mL in ddH_2O, f.c. 1:1000.
Ampicillin stock solution 50 mg/mL in ddH_2O, f.c. 1:1000.
Hygromycin stock solution 100 mg/mL, f.c. 1:1000.
Anhydrotetracycline stock solution 200 mM, f.c. 1:1000.
LB plates were prepared by adding 15 g agar to 1 L low salt-LB (5 g NaCl, 10 g tryptone, 5 g yeast extract).

3.4 Marker

2-log DNA marker (cat. No. N3200L, NEB)

3.5 Cloning

Cloning Kits

We used the pGEM®-T Easy Vector Cloning Kit (Promega) for cloning fragments amplified by HotStarTaq Plus DNA Polymerase (QIAGEN).
For blunt end cloning the CloneJET PCR Cloning Kit (Thermo Scientific) was used.

Ligation

When pGEM-T easy or pJET1.2 vectors were ligated with inserts, it was done according to the instructions of the manual in the corresponding kit.
For ligations of other vectors we used T4 DNA Ligase (NEB) and ligated 1 hour at room temperature or at 16 °C overnight following the instructions of NEB.

Competent Cells

We used the One Shot® TOP10 Chemically Competent *E. coli* from Invitrogen™.

Transformation

- Thaw competent cells on ice for 10 min
- add 3 µL of plasmid to 50 µL of one shot TOP10 competent cells
- incubate on ice for 30 min
- heat shock at 42 °C for 30 sec
- cool down on ice
- add 200 µL SOC
- regenerate at 37 °C for 30 min
- plate on selective plates

Colony PCR

- pick colonies from plate and inoculate in 50 µL ddH_2O (no medium)
- perform PCR using primers of backbone vector flanking the insert (e. g. pJET-F and pJET-R)
- take 1 µL of inoculated colony as template
- load amplified products on agarose gel to analyze length of the insert

Agarose gel

We chose the concentration of the agarose depending on the size of the expected fragments. Typically we used the following guideline: For fragments less than 1 kb we prepared 2 % agarose, for fragments between 1 and 3 kb 1.5 %, and for fragments longer than 3 kb 1 % agarose.

3.6 DNA preparation

Miniprep Buffers

Resuspension buffer P1 (sterile filter and store at 4 °C), 1 L
10 mM Glucose, 25 mM Tris·HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mg RNase A

Alkaline Lysis buffer P2 (freshly mixed)
0.2 M NaOH, 1 % SDS

Neutralization buffer P3 (store at RT), 500 mL 3 M Potassium acetate,
57.5 mL Glacial acetic acid

Miniprep

- inoculate colony in 2 mL LB-Amp
- incubate at 37 °C shaking at 165 rpm overnight
- spin down culture at full speed for 2 min
- resuspend pellet in 200 µL buffer P1
- lyse cells with 200 µL P2
- neutralize with 200 µL P3
- spin down cell debris by centrifuging at full speed for 10 min
- transfer supernatant to 1.5 mL tube

- add 450 µL isopropanol
- spin down at full speed for 10 min
- discard supernatant
- wash DNA pellet by adding 1 mL 70 % ethanol
- centrifuge at full speed for 5 min
- discard ethanol
- let pellet dry
- resuspend DNA pellet in suitable amount of water (30 – 50 µL)
- keep DNA on ice

Midi- and Maxiprep

Midi- and Maxipreps were performed using Qiagen-Midi and Maxi kits according to the instructions of the manual.

BAC mini-prep

- centrifuge 1.5 mL overnight culture in a 2 mL tube at 5 krpm for 8 min
- remove supernatant and repeat
- resuspend pellet in 600 µL P1
- add 600 µL of P2 and incubate at RT for 5 min
- add 600 µL of P3 and invert gently
- incubate tube on ice for at least 5 min
- centrifuge at full speed at 4 °C for 10 min
- transfer 840 µL of supernatant to new 2 mL tube (i.e. two new tubes per sample)
- add 700 µL isopropanol
- precipitate DNA at -20 °C overnight
- centrifuge at full speed at 4 °C for 10–20 min
- aspirate off supernatant
- add sample of second tube
- centrifuge at full speed at 4 °C for 10–20 min
- aspirate off supernatant
- add 1 mL 70 % ethanol
- centrifuge at full speed at RT for 5 min
- aspirate off supernatant
- add 50 µL EB or TE when pellet turns glassy
- dissolve at RT

3.7 Injections

Needle pulling

For injections in zebrafish and medaka two different needles are required. The needles for zebrafish embryos have to be long and thin, which became flexible; the tip of medaka needles is short and rigid.

We pulled glass capillaries using a needle puller (Sutter Instrument Company, model P-97, Flaming/Brown Micropipette Puller).

For injection needles for zebrafish a glass capillary with an inner diameter of 0.78 mm and an outer diameter of 1 mm was pulled in the needle puller using the following parameters: heat 490, pull 120, velocity 100, and time 150.

For medaka we used glass capillaries with an inner diameter of 0.58 mm and an outer diameter of 1 mm. The parameters were: heat 470, pull 40, velocity 45, and time 200.

1 µL TRITC (3 % TRITC-dextran in 0.2 M KCl; injection marker dye, SIGMA; stored at -20 °C) was added to the injection solution to be able to see easily if the injection worked properly; the injected embryo is then visible under the fluorescence binocular.

- 1 µL TRIT-C
- 40 ng/µL TALEN mRNA each
- 40 ng/µL ssDNA Oligo
- to 20 µL ddH_2O

The next day we checked under the fluorescence binocular to see if all the embryos have been properly injected using TRITC fluorescence as read-out.

Injection

Medaka males and females were separated the evening before injection. The next morning one male was placed back in a tank with three females. 35 min later we collected the eggs by scraping them off of the females and transferring them into chilled ERM. Chilling the collected eggs delays further development; this is beneficial because we only inject single cell staged embryos.

For injections in zebrafish we placed one male and one female separately into a mating chamber in the evening. The next morning we removed the spacer. The male starts to chase the female, which finally releases the eggs. To collect the fertilized eggs we removed the fish with the upper compartment of the mating chamber and poured the eggs into a petri dish containing E3.

3.8 Genotyping

Genotyping Embryos

To genotype medaka offspring we collected zygotes by scraping off the fertilized eggs of females in the morning. The larvae hatch after 7 days. Subsequently, we euthanized the

larvae with liquid nitrogen, added 10 µL lysis buffer and 0.5 µL Proteinase K per embryo, and homogenized the sample with a pestle. We incubated the sample at 56 °C for 2 to 3 hours. Proteinase K was inactivated by heating the sample to 99 °C for 10 min. Afterwards, we diluted the sample 1:20 with ddH_2O and used 1 µL as template in a PCR reaction.

Fin Clipping

The fish were anesthetized with 4 mL 1 % tricaine in 80 mL fish water. A small part of the tail fin was cut off using a fresh scalpel. The tissue was then incubated in 20 µL lysis buffer containing 4 µL Proteinase K at 56 °C for 3 hours. After Proteinase K inactivation (99 °C for 10 min) the sample was diluted 1:20 and 1 µL was used as template in a PCR reaction.

Genotyping PCR and Restriction Digest

The gene locus of interest was amplified by PCR. The PCR product was digested with the restriction enzyme recognizing a restriction site within the spacer region of the TALEN pair.

The locus around *tmt-1B* exon 1 was amplified by PCR using the primers 910 and 1943 (the numbers of the primers refer to our plasmid stock data base), giving a 967 bp fragment. Subsequently, 10 µL PCR product were digested with *Bss*HII (*Pau*I). When the TALEN pair works properly, the restriction site of *Bss*HII is altered, and therefore not recognized by the restriction enzyme. The expected band sizes for wt are 371 + 347 + 257 bp, for mutants 714 + 257 bp (due to indels the 714 bp fragment can be longer or shorter).

When screening for *tmt-2* mutants we amplified the exon 1 of *tmt-2* with 995 and 996. The resulting 500 bp fragment was either digested with *Hinf*I or *Bsa*HI depending on testing for the left or the right TALEN pair respectively. Mutants show an undigested band whereas wild type is completely cut (*Hinf*I: 405 + 98 bp, *Bsa*HI: 279 + 223 bp).

3.9 Two-Color Medaka *in situ* Hybridization with BM Purple and Fast Red

- anesthetize fish in 4 mL 1 % tricaine in 80 mL H_2O
- decapitate fish with scalpel
- dissect brain
- fix in 4 % PFA in 1x PTW at 4 °C overnight
- wash 5x 5 min in 1x PTW
- wash in 100 % MeOH for 5 min
- replace with fresh 100 % MeOH and put at -20 °C overnight

- 5 min 75 % MeOH
- 5 min 50 % MeOH
- 5 min 25 % MeOH
- 2x 5 min in 1x PTW
- prepare fresh 10 µg/mL Proteinase K solution in 1x PTW
- digest brains without shaking for 20-30 min at room temperature
- rinse 2x 2 min in 2 mg/mL glycine on a shaker
- post fix for 20 min in 4 % PFA on a shaker
- wash 5x 5 min in 1x PTW on a shaker
- Prehybridization/Hybridization at 65 °C in water bath
- replace 1x PTW with Hyb+ solution and incubate for 2-4 h
- (brains can be stored afterwards for months in Hyb+ at -20 °C)
- prepare probe in Hyb+ and denature probe for 10 min at 90 °C
- replace prehybridization Hyb+ with probe
- incubate embryos in probe overnight (>12 h)
- Washing at 65 °C in water bath
- take off probe and save at -20 °C for reuse
- 2x 45 min in 50 % Formamide/2x SSCT
- 1x 45 min in 2x SSCT
- 2x 45 min in 0.2x SSCT
- melt 3 % agarose in 1x PBS
- embed brains in agarose and cut them out when agarose is solidified
- cut brains at vibratome in 100 µm thin slices
- collect brain slices with brush and transfer in 1x PTW
- block brain slices in 1x MABT + 2 % DIG block at room temperature for 1 hour
- incubate embryos in fresh α-Fluorescein-AP antibody for 1 hour at room temperature (1:1000 dilution in MABT + 2 % DIG block)
- take off antibody
- wash 5 x 15 min in MABT
- Equilibrate 15 min in 0.1 M Tris·HCl (pH 8.2), 0.1 % Tween-20
- transfer to 24-well plate
- replace with Fast Red substrate for color reaction
- develop to desired intensity in the dark without shaking (3 min - 48 h)
- stop the reaction by washing 2 x 5 min with 1x PTW
- incubate 3 x 5 min in 0.1 M Glycine/HCl, pH 2.2, 0.1 % Tween-20
- wash 1x 5 min with MABT
- block 1h in MABT + 2 % DIG block
- incubate embryos in anti-DIG-AP antibody for >2 hours at room temperature or overnight at 4 °C (1:2000 - 1:4000 dilution in MABT + 2 % DIG block)
- take off antibody and save for reuse
- wash 5 x 15 min in MABT
- transfer to 24-well plate
- replace MABT with BM Purple for color reaction
- develop to desired intensity in the dark without shaking (3 min–48 h)
- stop reaction by briefly washing 3 x 5 min in PTW
- refix 45 min in 4 % PFA /1x PTW at 4 °C overnight
- wash 2 x 5 min in 1x PTW
- mount in Aquamount (Fisher Scientific) or in DABCO
- store in dark at 4 °C

Solutions:

- 4 % PFA in 1x PTW
- 75 %, 50 %, 25 % MeOH in 1x PBS 10 µg/ml Proteinase K diluted 1:2000 from 20 mg/ml stock in 1x PTW
- Hyb+: 50 % Formamide, 5x SSC (pH=6.0), 0.1 % Tween-20, 5 mg/ml torula (yeast) RNA, 50 µg/ml Heparin
- 20x SSC: 175.3 g NaCl, 88.2 g Trisodium citrate in 1 L ddH_2O, pH 6.0 with 1 M citric acid
- SSCT: SSC + 0.1 % Tween-20
- MAB: 100 mM Maleic acid, 150 mM NaCl, adjust to pH 7.5 with NaOH; never autoclave
- MABT: MAB + 0.1 % Tween-20
- DIG-block: 2 % blocking reagent (Roche) dissolved in MABT, needs to be heated to 65 °C to dissolve
- Anti-Digoxigenin-antibody-AP: Fab fragments (Roche) diluted in 2 % DIG-block
- Fast Red substrate: dissolve 1 tablet in 2 mL 0.1 M Tris·HCl (pH 8.2), 0.1 % Tween-20 (never filter, precipitate is necessary for efficient reaction)

3.10 Zebrafish and Medaka Husbandry

We keep our fish at 28 °C on a 16 hours light/8 hours dark cycle in a constant recirculating system (Fischer *et al*., 2013). The fish are fed with flakes (TetraMin) and living brine shrimp (*Artemia*).

3.11 Primers

The numbers refer to our primer stock data base in the lab.

910	GGGATCGTGTCTCCATCACT
995	CGGTGAGCGATGTGACTG
996	GGGAGATCTTTGTCCAGGTG
1372	TAATACGACTCACTATAGGG
1693	GGATCACTCTCGGCATGGAC
1943	CAGGTCAGAGCGGATCTCAT
1986	ATGGTGAGCAAGGGCGAGG
1987	AGAGGAGCTCCCGCGGTTACTTGTACAGCTCGTCCATG
1988	TTTCAAACAAGAAGTGACCAAACC
1989	AGCTTTTTGCTCCTAGAGGTC
1990	TCGGGAGTAGTGGAGCTGTC
2047	CCACCTCCAAGAGTGTTTCCACCTAATTCTTCTTTGACT GGACTGCAAATATGCCACAAGATCCACTCGATACTAGC
2048	GAAGACAGTTTCAGAAAGCAAAGGTGGTGCAGGAG GAGCGTAGGAAACACGTCTTTATAATCCTCGAGC
2156	TAGGGATAACAGGGTAATCACCCACAATAAACGGATGCTG
2157	TAGGGATAACAGGGTAATCCAGGTGCTGAACGCTCTTC
2158	TAGGGATAACAGGGTAATACGGGTTCGACGCCTTTAGC
2159	TAGGGATAACAGGGTAATGAACGTTGGCGTGGAGTGAG
2160	TAGGGATAACAGGGTAATTCGGGAGTAGTGGAGCTGTC
2200	TAGGGATAACAGGGTAATCACCCACAATAAACGGATGCTGCTACTCACC
2201	TAGGGATAACAGGGTAATCCAGGTGCTGAACGCTCTTCAGGTACATG
2202	TAGGGATAACAGGGTAATACGGGTTCGACGCCTTTAGCGCACAGCGG

2203	TAGGGATAACAGGGTAATGAGTTGCTGATGGGAACGTTGGCGTGG
2204	TAGGGATAACAGGGTAATTCCTAGAGGTCAGTCGGGAGTAGTGGAG
2205	TAGGGATAACAGGGTAATACGGGTTCGACGCCTTTAGCGCACAGCGG
2206	ATCGGTCGACGTGCCAGGGCGTGCCCTTGGGCTCCCC GGGCGCGTAAAAGCTTAAATCAGTTGTGTTAAATAAG
2207	ATCGTCTAGATTACTTGTACAGCTCGTCCATGCC
2290	CGCGGATCCATGGACACGTACGCGGGTGC
2291	CCGGAATTCCTAAACCTTCCTCTTCTTCT
attP-F	GGTAACCTTTGAGTTCTCTCAGT
attP-R	CAACTGAGAGAACTCAAAGGTTA
pGEM-F (89)	CCATGGCGGCCGCGGGAATTC
pGEM-R (90)	CAGGCGGCCGCGAATTCACTAGTG
pJET1.2-F (141)	CGACTCACTATAGGGAGAGCGGC
pJET1.2-R (140)	AAGAACATCGATTTTCCATGGCAG
ssDNA Oligo	ACGCGAGCCTGAGCTGCGCGGCCCCAACTGGGGTAACCT TTGAGTTCTCTCAGTTGGGGGCACTGTGACGGGGACGCA

4. Results and Discussions

4.1 Precise Genome Editing of genomic loci by Combining TALEN Technology and Homologous Recombination in Medaka

4.1.1 Aim

Prior results showed that the TALEN pair for exon 1 *tmt-1B* designed by RUTH FISCHER, a PhD student in our lab and my supervisor, cut DNA very efficiently (>90 %). We combined TALEN technology with homologous recombination using ϕC31, attP, and attB to knock-out *tmt-1B* and express GFP under the control of the promoter of *tmt-1B*.

Normally the double-strand break caused by TALENs is repaired by NHEJ. However, when ssDNA with homology arms is provided, the cell uses homology-directed repair (HDR; *Figure* 5). This leads to either precise insertion of the ssDNA or insertion with an additional indel (BEDELL *et al*., 2012). We co-injected TALEN mRNA with an ssDNA Oligo. The Oligo possesses short homology arms and an attP site, which is recognized by the ϕC31 integrase.

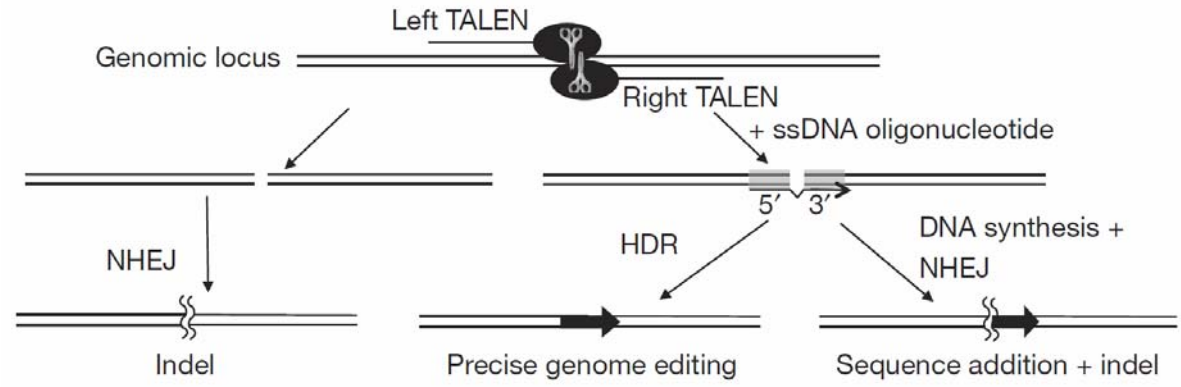


Figure 5: *Scheme of DNA double-strand breaks caused by TALENs and subsequent repair by NHEJ or HDR (BEDELL et al., 2012).*

The ϕC31 based system originates from the phage ϕC31, whose DNA contains an attP site, which is integrated by the ϕC31 recombinase into attB of *Streptomyces sp*. After recombination, the former attP and attB sites are altered into attL and attR; those sites cannot be recognized any more by the integrase. This is a considerable benefit compared to other recombination systems: recombination is unidirectional (*Figure* 6; CHALBERG *et al*., 2006).

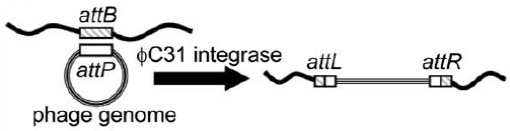


Figure 6: *Scheme of the unidirectional recombination catalyzed by φC31 integrase (*CHALBERG *et al., 2006).*

After the knock-out we want to label the cells expressing *tmtopsin-1B* with the fluorescent dyes eGFP and mCherry *in vivo*. This will be very useful for further research. First, we can express eGFP and mCherry under the control of the promoter of *tmtopsin-1B in vivo*. When the *tmtopsin-1B* expressing cells are labeled we can study its exact expression pattern. This allows further characterization of *tmtopsin-1B.* Second, the labeled cells alleviate electrophysiology assays.

4.1.2 Results

RUTH FISCHER, a PhD student in our lab and my supervisor, designed a TALEN pair binding in exon 1 of *tmt-1B* of medaka fish.

We injected 40 ng/µL mRNA of the TALEN pair (L2/R2) together with 40 ng/µL ssDNA oligo containing an attP40 site flanked by short homology arms (20 and 18 nt). The 27 injected embryos of the first injection round were tested by amplifying the *tmt-1B* locus with the primers 910 and 1943 and digesting the PCR product with *Bst*EII. 2 of the 27 embryos (7 %) show digested bands of the expected sizes (388 + 624 bp). Moreover, a PCR using one outer primer and one primer binding within attP confirmed our result: PCR I (910 + attP-R: 407 bp), PCR II (attP-F + 1943: 625 bp), and as positive control 910 + 1943 (1007 bp; *Figure 7*).

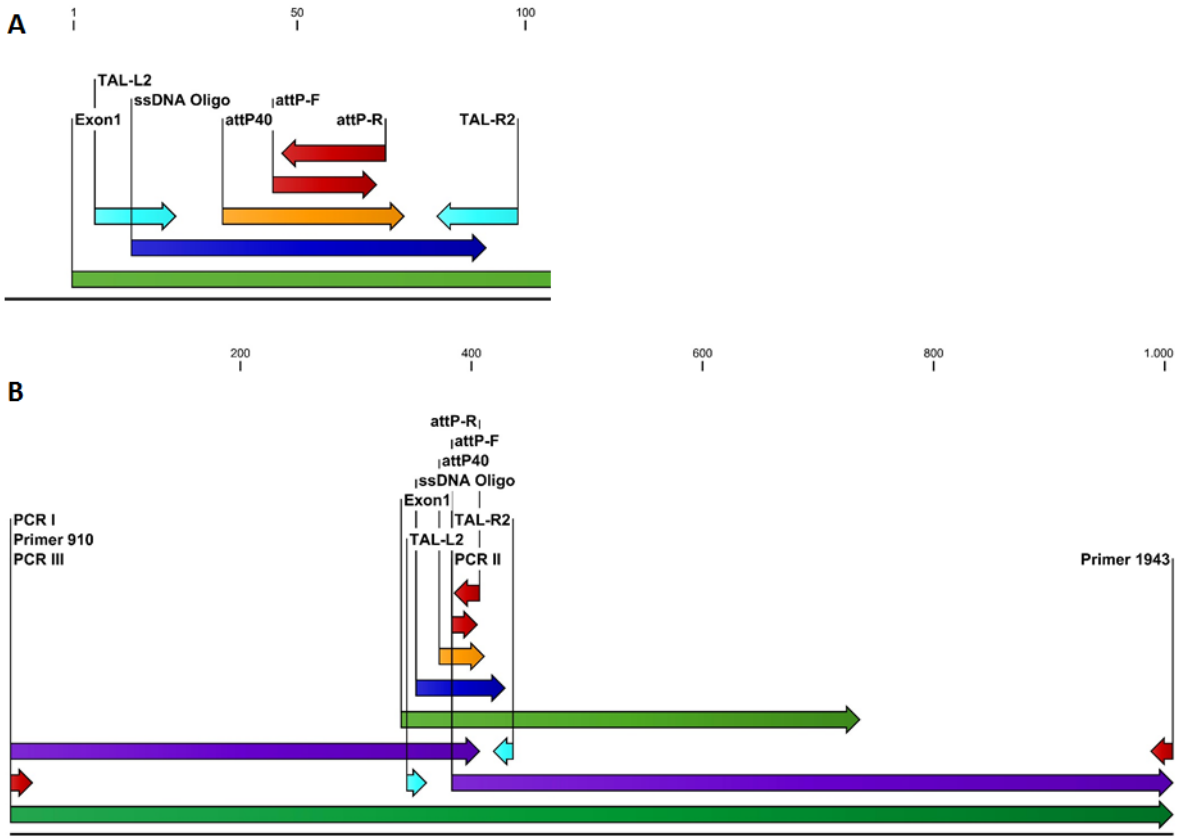


Figure 7: *attP locus in injected embryos. (A) The primers attP-F and attP-R (red arrows) bind within the attP40 site (orange), which is part of the ssDNA Oligo (blue). attP40 is flanked by homology arms of 20 nt on the left side and 18 nt on the right side. The TALEN binding sites are indicated with light blue arrows. In green is the beginning of exon 1 of tmtopsin-1B depicted. The exon is in total 398 bp long. The number of bp is set relative to the beginning of exon 1. (B) Confirmation of attP40 recombination by PCR amplification. The expected PCR products are depicted in purple (PCR I: 407 bp; PCR II: 625 bp). The 1007 bp long PCR III in dark green serves as positive control. The primers 910 and 1943 are indicated by red arrows.*

The two embryos we identified by restriction digest gave PCR products of expected lengths, whereas wt only shows the band of the positive control (*Figure* 8).

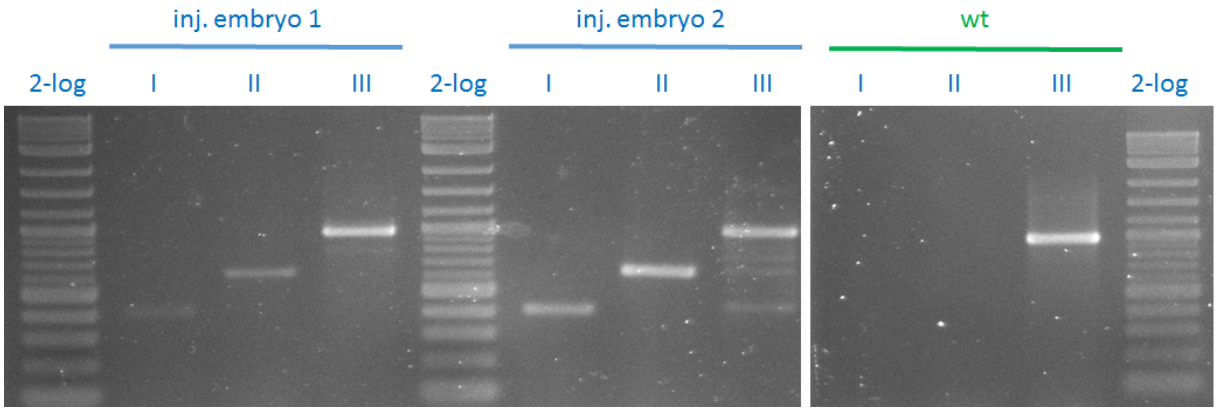


Figure 8: *PCR to confirm attP recombination in injected embryos. Both injected embryos show PCR products of the expected band size: PCR I 407 bp, PCR II 625 bp, PCR III 1007 bp. The wt sample shows only the band of the positive control (PCR III).*

We injected in total 96 embryos. 57 were raised to adulthood. Fin clipping of 30 F0 fish and screening with *Bst*EII digest revealed a recombination rate of about 20 % (7 fish with recombination found). 2 of those fish did not transmit the recombination.

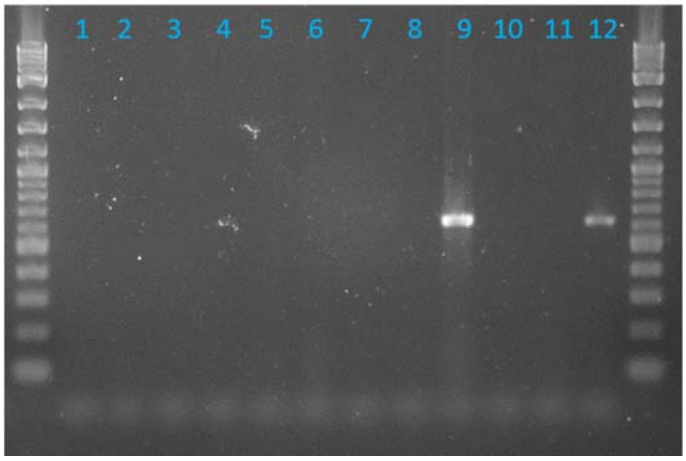


Figure 9 *PCR products of screened embryos of F1 of Gislind. Each sample represents a single embryo. Used primers are attP-F + 1943, which give 625 bp. The embryos #9 and #12 show a band.*

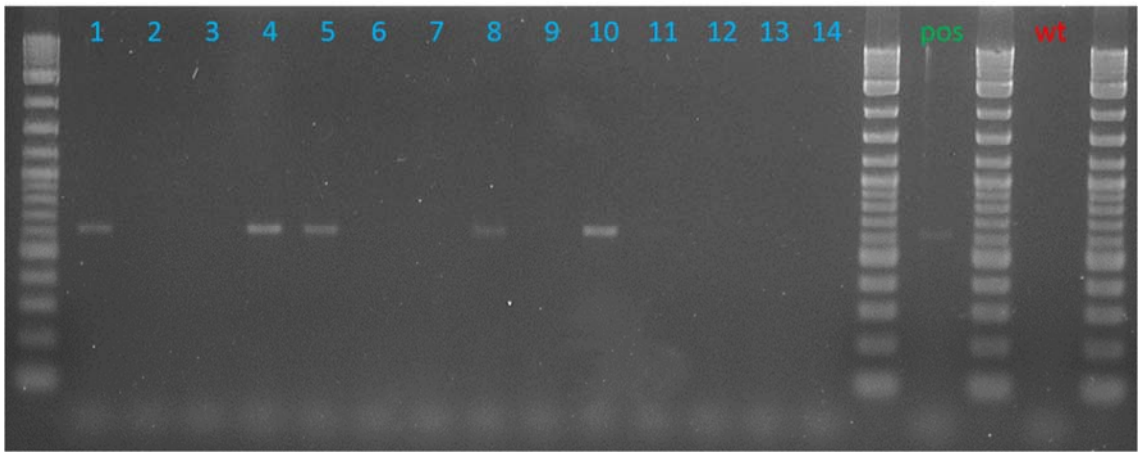


Figure 10 *PCR products of screened embryos of F1 of Ursulus. Each sample represents a single embryo. Used primers are attP-F + 1943, which give 625 bp. Six embryos show a band of the expected size (#1, 4, 5, 8, 10, 11). The positive control is the injected embryo #1 (FigureXXX), wt is the negative control.*

The mutation of three founder fish was characterized by subcloning and sequencing (*Figure* 11). To make things easier we gave those three fish names: Udelurf, Gislind, and Ursulus.

Udelurf, a male fish, has an almost perfect integration of the attP site. The only difference is one mismatch in the right TALEN binding site. The female fish Gislind has an additional insertion of 22 nucleotides at the 5' end of the recombination site. The male fish Ursulus has an insertion of 48 nt at the 5' end of the recombination site, generating a stop codon in the coding frame upstream of the recombination site (*Figure* 11).

Although all three fish are suitable candidates for recombination with attB, we chose Udelurf and his offspring due to his almost perfect recombination. However, the offspring of Gislind already reached adulthood when we started to collect Udelurf's offspring. Therefore, we decided to continue work on both lines.

Udelurf has been outcrossed with three wild-type Cab females. Their offspring will be

Figure 11 Sequencing results of the attP locus of the three founder fish Udelurf, Gislind, and Ursulus compared to wt and predicted attP40 integration. The TALEN binding sites are shown in green, the BssHII restriction site in wt in light blue, the BstEII restriction sites in blue, the attP construct in pink, insertions in yellow, stop codons in red, and the point mutation is red and bold.

raised. After reaching adulthood we will test for the recombination by screening with fin clipping.

We crossed Gislind with a wild type male and fin clipped their offspring when they reached maturity. We amplified the attP locus by PCR using the primers 910 and 1943 and digested the PCR products with *Bst*EII (*Figure* 12). Fin clipping and screening via *Bst*EII digest of F1 of Gislind revealed that 4 of 32 fish (12.5 %) show the recombined attP cassette. We crossed the two positive males with three wild type females each and raised their offspring.

In the next step we wanted to inject ϕC31 integrase mRNA together with an attB construct. Peter Duchek of the Institute of Molecular Biotechnology (IMBA) endowed us with a template of ϕC31 integrase (see Appendix II.a for sequence). The template was amplified with the primers 2290 and 2291, which contain a *Bam*HI and an *Eco*RI restriction site re-

spectively. The PCR product was gel extracted. The ϕC31 integrase and pCS2+ were digested with *Bam*HI and *Eco*RI. After purification, both constructs were ligated and transformed into one shot TOP10 competent cells. Sequencing of the insert confirmed the sequence Peter Duchek sent us (Appendix II.a).

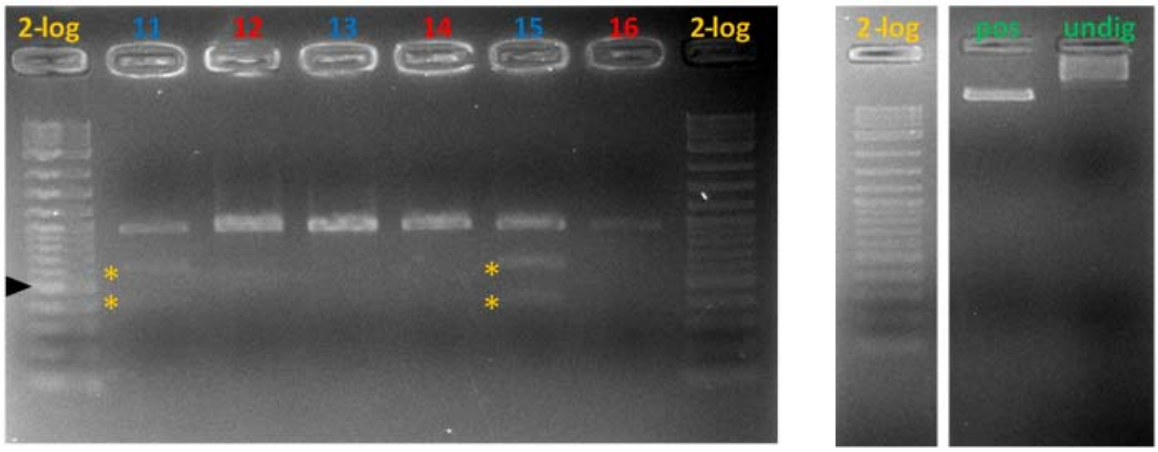


Figure 12: *BstEII digest of PCR amplified fin clips of F1 of Gislind. Depicted are six fish (11 – 16), males in blue and females in red. The asterisks indicate the digested bands of male #11 and male #15. The black arrowhead indicates the 500 bp band of 2-log. As a positive control we used the digested and undigested plasmid #1030, which contains a BstEII restriction site.*

4.1.3 Discussion

We demonstrated efficient homologous recombination technology in medaka and stable inheritance of the inserted attP cassette in 10 % of the offspring. While I was writing this thesis, RUTH FISCHER designed an attB construct containing a 36 bp attB site, the heart specific *cardiac myosin light chain 2* promoter (cmlc2; HUANG *et al.*, 2003), and the red fluorescent dye mCherry in a pCS2+ backbone vector. This construct was injected together with mRNA of ϕC31 integrase. The ϕC31 integrase recombines attP and attB unidirectionally. Finally, RUTH FISCHER checked the injected embryos under fluorescent light and observed mCherry expression within the heart muscle (unpublished data).

This demonstrates that the integration of attB worked. Therefore, an attB-eGFP construct containing loxP sites will be designed and injected. The loxP sites can be used to remove the plasmid back bone and its features, e.g. the antibiotics resistance gene.

By using primers binding at attL and attR, Ruth demonstrated successful single integration of attB-eGFP. We raise those fish and want to use their GFP expression to investigate the expression pattern of tmt-1b *in vivo*. However, in case the GFP signal is too weak, we want to clone an eGFP construct containing multimeric GFPs flanked by ribosomal skipping sites (P2A, F2A, T2A). With this cassette we will amplify the signal and then hopefully see specific GFP expression in tmt-1B expressing cells.

This is very useful for further studies. On the one hand, it is interesting to see where *tmtopsin-1B* is expressed, which eases further characterization. On the other hand, these labeled cells will facilitate the work on electrophysiology assays. Bruno Fontinha, a postdoc in our lab, uses the so-called patch clamp technique to measure changes in membrane potential of putative light-sensitive neurons within the optic tectum of fish brains upon light delivery. Bruno observed that about 25 % of the cells of the optic tectum are light-responsive (Fischer *et al*., 2013). Unfortunately, they are indistinguishable by normal light microscopy. Labeling these cells *in vivo* would therefore alleviate Bruno's work. In addition, labeling tmtopsin-expressing neurons reveals their projections, which means that we are able to see which areas they project and from which areas they receive inputs.

However, this method has limitations. GFP and tmt-opsins are both excited by light of blue wavelength. When we want to test putative light-sensitive neurons for GFP expression the neurons become already activated. To overcome this limitation, fluorescent dyes excited by different wavelengths can be used, e.g. mCherry, which has its excitation maximum in red light.

The lab of J. Wittbrodt recently reported the use of the attP/attB system in medaka (Kirchmaier *et al.*, 2013). The ϕC31 integrase, attP, and attB have been used successfully in other organisms as well: in the fission yeast *Schizosaccharomyces pombe* (Thomason *et al.*, 2001), in barley (*Hordeum vulgare*; Kapusi *et al.*, 2012), in *Arabidopsis thaliana* (Thomson *et al.*, 2010), in the fruit fly *Drosophila melanogaster* (Groth *et al.*, 2004; Venken *et al.*, 2006; Venken *et al.*, 2011; Yonemura *et al.*, 2012), in the silk worm *Bombyx mori* (Yonemura *et al.*, 2012), in the frog *Xenopus laevis* (Allen and Weeks, 2005), in murine embryonic stem cells

(Belteki *et al.*, 2003), in human cells (Groth *et al.*, 2000), and in zebrafish (Hu *et al.*, 2010; Lister, 2010; Lu *et al.*, 2011; Mosimann *et al.*, 2013).

Those numerous reports in the literature and our own results are promising that this technology will serve well for labeling also other opsins *in vivo*. The ϕC31 integrase could also be used to label other gene products like ChAT2, providing an alternative to the fosmid recombineering technique (cf. 4.3. Labeling Cholinergic Cells *in vivo* Using Fosmid Recombination).

4.2 Creation of Single and Double Knock-outs for *tmt-2* and *tmt-1B*, *tmt-2* in Medaka

4.2.1 Aim

Like *tmtopsin-1B*, *tmtopsin-2* is a non-visual photoreceptor of the ETO family, present in medaka fish (*Figure* 2 phylogeny). To characterize *tmtopsin-2*, we knocked out *tmtopsin-2* using TALEN technology. Our goal is to clarify the function of inner brain opsins. Hence, we want to knock out all functional inner brain opsins (Ol-tmtopsin-1B, Ol-tmtopsin-2, Ol-Valop). Subsequently, we expect a distinct behavioral phenotype compared to wt fish.

4.2.2 Results

Two TALEN pairs binding in exon 1 (357 bp) of *tmt-2* were designed by Ruth Fischer (*Figure* 13). We injected the left TALEN pair [100 ng/µL] into medaka zygotes. Screening of the medaka larvae revealed that 10 of 11 (91 %) samples showed incomplete digestion, i.e. indicative of functioning TALENs (*Figure* 14).

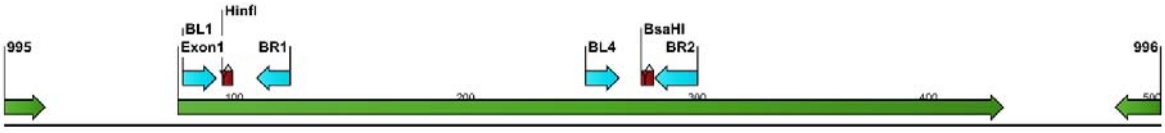


Figure 13: *500 bp PCR product with the primers 995 + 996 flanking exon 1 (depicted as green arrow) of tmt-2. The binding sites of the TALEN are indicated by light blue arrows. The two restriction sites (red) are flanked by TALEN pairs.*

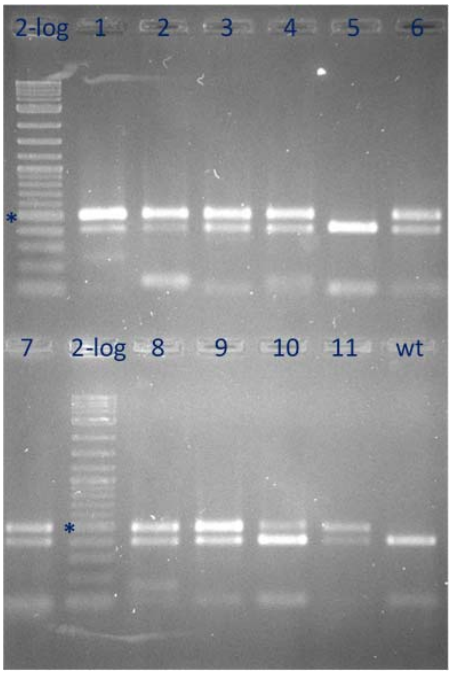


Figure 14: *HinfI digest of single embryos injected with left TALEN pair binding in exon 1 of tmt-2. The wt is digested completely. 10 of 11 samples show incomplete digest; only sample 5 is totally digested like the wt control. Asterisks indicate 500 bp band of 2-log.*

We raised the injected embryos to adulthood, set up crosses with wild type and screened their offspring to identify TALEN mutations. *Figure* 15 shows identified mutations. These indels lead to a frame shift and generate a stop codon, which results in an early truncated and therefore non-functional gene product.

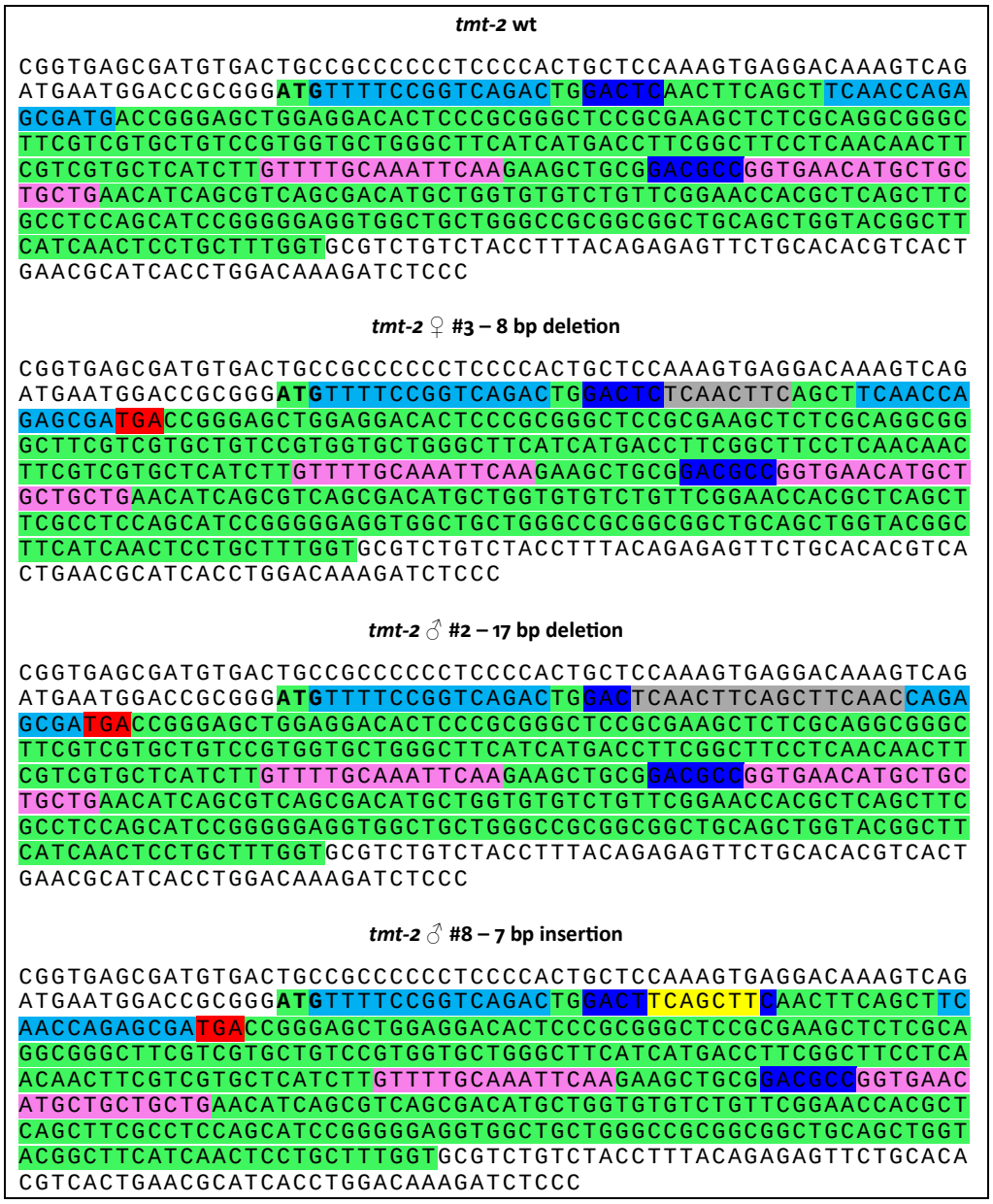


Figure 15: *Sequencing results of PCR product (995 + 996) of three founder fish compared to wt. The exon 1 (357 bp) is shown in green, the start codon is bold, the left TALEN binding sites are shown in light blue, the right TALEN binding sites in pink, the restriction sites of HinfI and BsaHI are depicted in dark blue, deletions in gray, insertions in yellow, and stop codons in red.*

All three identified mutations depicted in *Figure* 15 generate a stop codon and are hence suitable founders with knocked-out *tmtopsin-2*. However, the screening for mutated offspring requires a restriction digest, which is expensive and time consuming. Thus, we injected both *tmtopsin-2* TALEN pairs together. If both TALEN pairs cut, the DNA between

them is cut out. This deletion is visible on an agarose gel as shorter band. This facilitates screening for mutants, because the expensive digest with a restriction enzyme becomes obsolete. The region between both TALEN sites is about 200 bp long; however, due to indels the PCR product can be longer or shorter. We knew already that the left TALEN pair cuts with an efficiency of about 90 %. We injected 100 ng/µL of the left and 200 ng/µL of the right TALEN pair and observed deletions in about half of the screened single embryos. We raised the injected embryos and fin clipped them when they reached adulthood. We performed a PCR using the primers 995 and 996 and observed that 4 samples show a shorter PCR product of about 300 bp (*Figure* 14). We crossed those fish with wild type and screened their offspring for deletions. Furthermore, we set up incrosses, i.e. crosses with two injected fish.

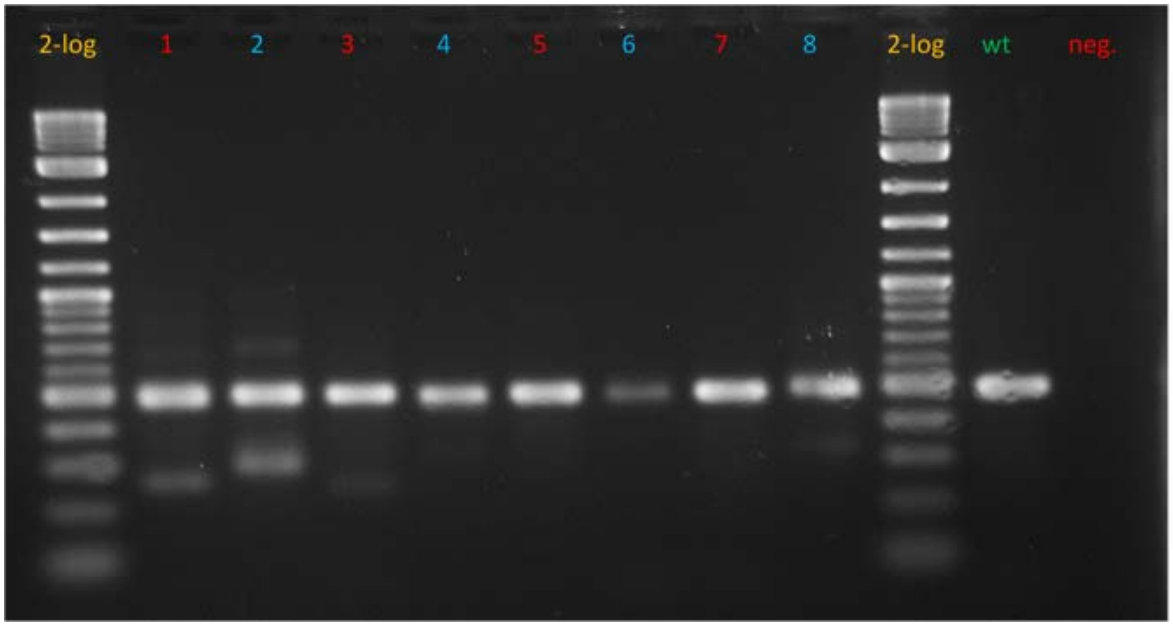


Figure 16: *PCR of fin clipped F0 TALEN tmt-2 injected fish showing deletions. Fish 1, 2, 3, and 8 show deletion bands. Red digits indicate females, blue digits males.*

We observed that the deletion is transmitted through the germ line in one outcross and in one incross (*Figure* 15). The deletion band of the incross seems very faint. However, when we screen fin clips of the offspring, the deletion band should be as strong as the wt band due to their heterozygosity. 16 embryos screened from a couple of two injected fish and 20 embryos were screened from the female #3 (*Figure* 16) crossed with a wt male. Only

one of those embryos showed this deletion band, therefore we expect that about 5 % (6.25 % and 5 %) of the offspring show the deletion (*Figure* 17). To identify the founder we crossed both injected fish with wt and screened their offspring.

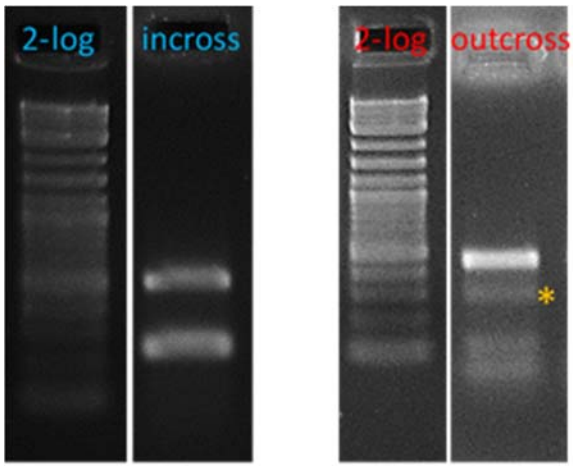


Figure 17: *PCR of F1 of incross and outcross couples showing a deletion band. The outcross consists of an injected female and a wt male. The asterisk indicates the deletion band. Both samples are pools of embryos: incross 6x, outcross 9x.*

So far we have created two fish which transmit deletions allowing facilitated screening. RUTH FISCHER created *tmtopsin-1B* homozygous fish using TALEN technology and subsequent breeding. To create fish mutant for both TMTopsins, we injected TALEN for *tmtopsin-2* into zygotes of *tmtopsin-1B* -/- couples. We raised the injected embryos and screened their fin clips. 8 of 17 fish (47 %) showed *tmtopsin-2* mutations in the soma. We crossed tmtopsin-1B -/- fish, which were injected with TALEN mRNA against tmtopsin-2 with tmtopsin-1B -/- fish. Screening their offspring revealed that one fish transmits deletions (*Figure* 18). This band is very faint, however, when we fin clip the F1 each fish will show two bands of the same intensity, one mutant allele (deletion band) and one wt allele.

We subcloned the deletion band shown in *Figure* 18 and identified the mutation, a deletion of 257 bp (*Figure* 19) leading to a frame shift and finally to a stop codon after 104 amino acids. Therefore, we expect a truncated and thus non-functional gene product.

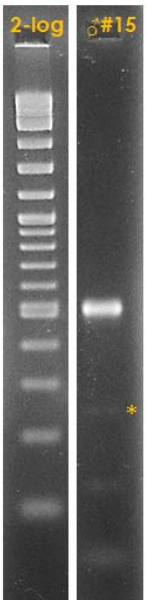


Figure 18: *PCR of F1 of tmt-1B -/- and TALEN for tmt-2 injected male crossed with tmt-1B -/- female. The asterisk indicates the deletion band. The sample is a pool of 6 embryos.*

***tmt-2* wt**

CGGTGAGCGATGTGACTGCCGCCCCCCTCCCCACTGCTCCAAAGTGAGGACAAAGTCAG
ATGAATGGACCGCGGGATGTTTTCCGGTCAGACTGGACTCAACTTCAGCTTCAACCAGA
GCGATGACCGGGAGCTGGAGGACACTCCCGCGGGCTCCGCGAAGCTCTCGCAGGCGGGC
TTCGTCGTGCTGTCCGTGGTGCTGGGCTTCATCATGACCTTCGGCTTCCTCAACAACTT
CGTCGTGCTCATCTTGTTTTGCAAATTCAAGAAGCTGCGGACGCCGGTGAACATGCTGC
TGCTGAACATCAGCGTCAGCGACATGCTGGTGTGTCTGTTCGGAACCACGCTCAGCTTC
GCCTCCAGCATCCGGGGGAGGTGGCTGCTGGGCCGCGGCGGCTGCAGCTGGTACGGCTT
CATCAACTCCTGCTTTGGTGCGTCTGTCTACCTTTACAGAGAGTTCTGCACACGTCACT
GAACGCATCACCTGGACAAAGATCTCCC

***tmt-1B -/-* + *TALEN tmt-2* inj. male**

CGGTGAGCGATGTGACTGCCGCCCCCCTCCCCACTGCTCCAAAGTGAGGACAAAGTCAG
ATGAATGGACCGCGGGATGTTTTCCGGTCAGACTGGACTCAACTTCAGCTTCAACCAGA
GCGATGACCGGGAGCTGGAGGACACTCCCGCGGGCTCCGCGAAGCTCTCGCAGGCGGGC
TTCGTCGTGCTGTCCGTGGTGCTGGGCTTCATCATGACCTTCGGCTTCCTCAACAACTT
CGTCGTGCTCATCTTGTTTTGCAAATTCAAGAAGCTGCGGACGCCGGTGAACATGCTGC
TGCTGAACATCAGCGTCAGCGACATGCTGGTGTGTCTGTTCGGAACCACGCTCAGCTTC
GCCTCCAGCATCCGGGGGAGGTGGCTGCTGGGCCGCGGCGGCTGCAGCTGGTACGGCTT
CATCAACTCCTGCTTTGGTGCGTCTGTCTACCTTTACAGAGAGTTCTGCACACGTCACT
GAACGCATCACCTGGACAAAGATCTCCC

***tmt-2* wt**

MFSGQTGLNFSFNQSDDRELEDTPAGSAKLSQAGFVVLSVVLGFIMTFGFLNNFVVLIL
FCKFKKLRTPVNMLLLNISVSDMLVCLFGTTLSFASSIRGRWLLGRGGCSWYGFINSCF
GIVSLISLVILSYDRYSTLTVYNKGGLNYRKPLLAVGGSWLYSLFWTVPPLLGWSSYGL
EGAGTSCSVSWTANTAQSHAYIICLFIFCLGLPILVMIYCYSRLLLAVKQVGKIRKTAA
RKREYHILFMVLTTAACYLLCWMPYGVVAMMATFGPPNIISPVASVVPSLLAKSSTVIN
PLIYILMNKQFYRCFLILFHCDHWSSENGNTSVPSKTTVIPLNRRIYTNTVAQISTDNA
N*

***tmt-1B -/-* + TALEN *tmt-2* inj. male**

MFSGQTGRLQHPGEVAAGPRRLQLVRLHQLLLWYRFADLSGDPLLRPLQYSDCIQQRWA
QLPKAPTSCWRLLALLLVLDSAPSPGLEQLWLRGGWNKLFCLLDS*

Figure 19: *Sequencing results of F1 of tmt-1B -/- and TALEN tmt-2 injected male crossed with tmt-1B -/- female. Sequences represent PCR product amplified with the primers 995 and 996. Exon 1 of tmt-2 is depicted in green, the binding sites of the left TALEN pair are light blue, of the right pair pink. Dark blue are the restriction sites for HinfI (left TALEN pair) and BsaHI (right TALEN pair). Deletion is indicated in grey, the start codon ATG is depicted in bold letters. Moreover, mRNA of tmt-2 is translated into protein for wt and tmt-1B -/- + tmt-2 inj. male. The first methionine is depicted in green, the stop codon in red. The altered protein sequence due to the frame shift is underlined.*

In total we created three founder fish transmitting deletions, two have wt background and one fish is also homozygous mutant for *tmtopsin-1B*. The screening for deletion bands makes the subsequent screening efforts faster and cheaper.

4.2.3 Discussion

BRUNO FONTINHA and CHEN SHANG-FU, his bachelor student, collected and screened the offspring of the two outcrossed putative founders. They identified the male as founder. When the offspring reaches maturity, they will identify the *tmt-1B* +/- fish by fin clipping. Subsequent interbreeding will finally generate *tmtopsin-1B* -/- *tmtopsin -2*-/- fish.

By using TALEN technology we created single and double knock-outs for *tmt-2* and *tmt-1B*. Knocking out those tmt-opsins is very useful for their functional investigation. BRUNO FONTINHA and RUTH FISCHER performed a behavioral assay with *tmt-1B* -/- medaka larvae. Their preliminary data revealed that the knock-out of *tmt-1B* can be linked to a distinct phenotype (unpublished data).

It will be interesting to see how fish homozygous for both tmt opsins behave phenotypically. Our aim on the long run is to create fish lacking all TMT opsins and encephalopsins; we. The optic tectum is involved in processing vision (NEVIN *et al.*, 2010). Therefore, we expect those fish to have a strong behavioral phenotype, in which altered vision is involved.

4.3 Labeling Cholinergic Cells *in vivo* Using Fosmid Recombination

4.3.1 Aim

Amongst other cell types we are interested in cholinergic cells, which constitute about 5-10 % of the cells in the optic tectum (in the goldfish: TUMOSA *et al*., 1986). BRUNO measures changes in membrane potential of these cells. To facilitate BRUNO'S work we want to label the cholinergic cells by tagging them with GFP. Thus, we used homologous recombination to substitute exon 2 of choline acetyltransferase 2 (ChAT 2), a marker for cholinergic cells (CLEMENTE *et al.*, 2004), with fluorescent dyes using fosmid recombineering.

We used Red/ET Recombination (COHEN *et al.*, 1973; MULLIS *et al.*, 1973; MUYRERS *et al*., 1999, 2000, 2001; ZHANG *et al*., 1998, 2000) to substitute exon 2 of ChAT2 with a GFP containing cassette. This technology is based on *in vivo* homologous recombination in *E. coli*. In the first step a fosmid carrying strain is transformed with pRedFlp4, a hygromycin resistance gene containing expression plasmid. Subsequently, L-rhamnose is used to induce expression of genes mediating Red/ET while incubating the cells at 30 °C. Afterwards, the bacteria are prepared for electroporation with our target cassette (EGFP-F2A-tdTom-FRT) flanked by homology arms. Red/ET recombines target cassette and exon 2 of ChAT2; only cells with proper recombination are able to grow on selective plates containing kanamycin. It is important that the cells are incubated at 30 °C; otherwise, the pRedFlp4 plasmid gets lost. In the last step the kanamycin resistance is removed inducing expression of Flp recombinase with anhydrotetracycline (AHT). Finally the temperature shift to 37 °C will remove the pRedFlp4 plasmid.

4.3.2 Results

Cloning of pR6K-EGFP-F2A-tdTom-FRT

We digested the vector 894 (pR6K-E2N-FRT) with *Eco*RV-HF and *Sac*II (NEB, Buffer CutSmart, 30 min, 37 °C) and amplified the insert tdTomato by PCR using the primers 1986 and 1987 (adds *Sac*II restriction site to PCR product). The correct sequence of tdTomato was confirmed by sequencing (LGC genomics, using primer 1693 and 1372). Both, the am-

plified insert and the digested vector were gel extracted (QIAGEN gel extraction kit). Subsequently, we digested tdTomato with *Sac*II (NEB, Buffer CutSmart, 30 min, 37 °C). After cleaning the reaction using the PCR cleanup kit of QIAGEN, we ligated tdTomato into 894 and transformed it into PIR1 cells. This is crucial because only PIR1 cells express the π element required for the R6K origin of replication. On the next day we picked 20 colonies, inoculated them in 2 mL LB-Amp and incubated them shaking at 37 °C overnight. Subsequently, we miniprepped them and confirmed the right sequence by an analytical digest with *Eco*RI/*Stu*I and by sequencing.

Target Cassette Amplification

We set up 200 µL PCR reaction using Phusion polymerase and the HF buffer (NEB). We used the primers 2047 and 2048. 35 cycles with Tm of 60 °C and Elongation time of 2 min. Subsequently, we gel extracted the amplified cassette and eluted in 30 µL ddH_2O (no elution buffer, the salt deteriorates electroporation results).

The fosmid GOLWFn0526_i22 was plated on LB-Cm and one colony was inoculated in 1 mL LB-Cm shaking at 37 °C overnight. The next day two 1.5 mL tubes containing 1.4 mL LB-Cm and 30 µL of the pre-culture were incubated at 37 °C at 1 krpm for 3 h. Afterwards, the culture was centrifuged at 11 krpm at 2 °C for 30 sec. The supernatant was discarded and the pellet resuspended in 10 % pre-chilled (at least 2 hours on ice) glycerol. We repeated the centrifugation and resuspension and finally pooled the cultures of both tubes. After another centrifugation step we discarded the supernatant, resuspended the pellet in the residual liquid and added 10-70 ng of the pRedFlp4 vector (893). Subsequently, we transferred the sample to a pre-chilled electroporation cuvette. We electroporated at 2500 V, 25 µF, and 200 Ω (Bio-Rad Electroporator, 2 mm electroporation cuvette). When the electroporation was successful, the time constant should be between 4.5 and 5. Immediately after the electroporation we added 1 mL LB without antibiotics and transferred the bacteria to a punctured 1.5 mL tube. We incubated them at 30 °C shaking at 1 krpm for 70 min. It is crucial that the temperature is not higher, otherwise the pRedFlp4 vector will be lost. Afterwards, we plated 100 µL on agar plates containing hygromycin and chloramphenicol and incubated them at 30 °C overnight.

The next day we picked one colony from the plate and inoculated it in 1 mL LB-Hyg and Cm and incubated the tube at 30 °C at 1 krpm overnight. Expression of Red/ET recombination proteins was induced by adding 50 µL 25 % L-rhamnose and shaking at 37 °C for 1 hour. We transformed 200 ng of the amplified target cassette by electroporation. After incubating the cells at 37 °C for 70 min, cells were plated on selective plates with LB-Kan and Hyg. The plates were incubated at 30 °C overnight.

To verify correct recombination we amplified the cassette using outer primers (1988 + 1989).

– 2.5 µL 1988
– 2.5 µL 1989
– 2.5 µL Coral load 10x
– 5 µL Q Solution
– 0.5 µL 10 mM dNTPs
– 1 µL template (colony in 50µL ddH_2O)
– 0.125 µL HotStar Plus Taq
– to 25 µL ddH_2O

– 95 °C 5'
– 95 °C 20"
– 60 °C 30"
– 72 °C 4'
} 40x
– 72 °C 10'
– 10 °C ∞

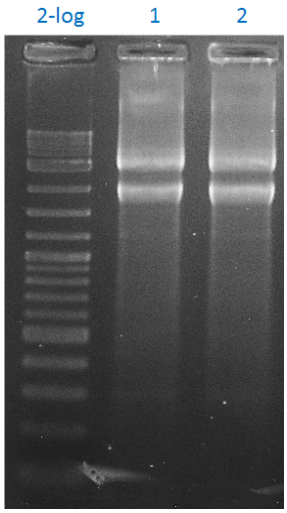


Figure 20: *Colony PCR of two colonies on day 5. The wt band is gone.*

We incubated the tested colonies in fresh medium for 5 days to get rid of the wt band (500 bp). On the fifth day the wt band was gone (*Figure* 20). Interestingly the clones showed two strong bands. The expected band size is about 3 kb.

After flipping out the kanamycin cassette with AHT, we observed that the PCR product is shorter than expected. The missing 700 bp can probably be explained by the loss of one of the fluorescent dyes. Apparently the recombinase recombined tdTomato and EGFP as well; their sequences might be too similar. Therefore, we decided to repeat the fosmid recombineering with the 'old' cassette, i.e. containing EGFP and nitroreductase (NTR). NTR can be used to abolish NTR-expressing cells. After treatment with metronidazole, nitroreductase produces cytotoxic products, which lead to cell death (Curado *et al.*, 2008).

After flipping out the neomycin cassette, we amplified different loci upstream of ChAT2. By this, we want to amplify the exon 1 of ChAT2 together with regulatory sequences. We amplified upstream regions using primers containing I-*Sce*I restriction sites. The subcloned PCR fragments will be injected into medaka zygotes together with I-*Sce*I, which cuts the DNA at the I-*Sce*I restriction sites. Subsequently, our construct will be integrated into the medaka genome.

We tried to amplify several PCR products (2156 + 2157: 12.3 kb, 2158 + 2159: 11.1 kb, 2158 + 2160: 4.8 kb; *Figure* 21). We used two different PCR reaction buffers (NEB HF and GC buffer), two different template concentrations (10 and 100 ng/µL), and two different PCR programs (Tm 60 °C 35x and Tm 56 °C 5x + 72 °C 30x) to see which conditions give the best result. Unfortunately, none of the PCR reactions gave satisfying results. Therefore, we designed new extended primers of about 28 nt binding site and an I-*Sce*I restriction site (2200 + 2201: 12.3 kb, 2202 + 2203: 11.1 kb, 2204 + 2205: 4.8 kb, 2202 + 2204: 4.8 kb, 2200 + 2204: 11.7 kb, 2200 + 2203: 18 kb, 2205 + 2201: 5.4 kb). The expected band sizes ranged from 4.8 kb to 18 kb (*Figure* 21).

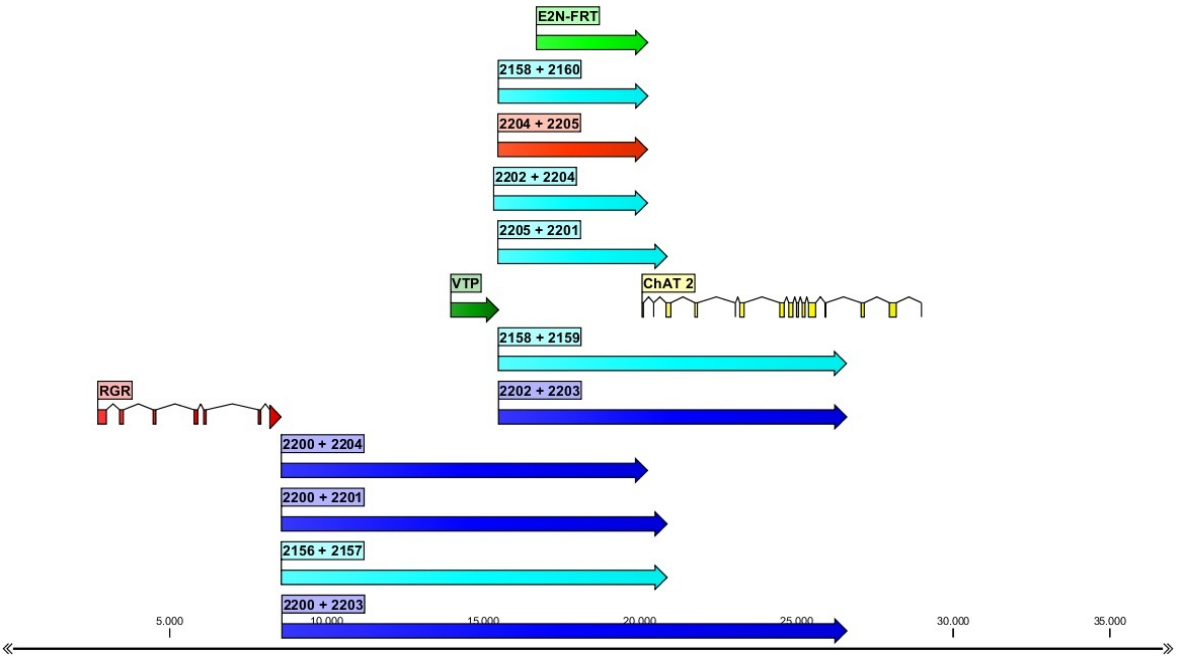


Figure 21: *Fosmid GOLWFn0526_i22 sequence. Indicated are the photoisomerase retinoid G protein-coupled photoreceptor (RGR) in red, the vesicular transporter protein (VTP) in green, and the choline acetyltransferase 2 (ChAT2) in yellow. The expected PCR products with primers of standard length (and an additional I-SceI restriction site) are indicated in light blue, whereas the expected PCR fragments amplified with prolonged primers are depicted in dark blue. The EGFP-F2A-NTR cassette containing the neomycin resistance flanked by FRT sites is indicated in light green. Note that all expected PCR products include this cassette. The working PCR product amplified with the primers 2204 and 2205 is depicted in orange.*

Only the PCR with the primers 2204 + 2205 generated a PCR product. We expected a band size of about 6 kb. However, the sequence of the used fosmid was partially unknown. Therefore, we continued with the generated 4.8 kb fragment although we expected a larger PCR product. We gel extracted the fragment, cloned it into pGEM-T easy, and transformed it into competent cells (one shot TOP10, Invitrogen). We inoculated the grown colonies in 2 mL LB-Amp and incubated them shaking at 37 °C overnight. We mini-prepped the DNA of the overnight-cultures and confirmed the plasmid by an analytic digest.

We performed a maxi-prep and injected the plasmid and I-*Sce*I into medaka zygotes.

– 20 ng/µL plasmid DNA
– 2 µL TRIT-C
– 1 µL I-*Sce*I (stored at -80 °C)
– 1 µL 10x I-*Sce*I buffer
– to 20 µL nuclease-free ddH_2O

We added TRITC dextrane to the sample we injected. TRITC dextrane is visible as orange dye under red light. This visualizes if the embryos were injected properly.

We observed an extremely high lethality (about 90 %) among the injected embryos (n=59). The surviving embryos showed orange fluorescence (TRITC dextrane), however they did not have any GFP staining in the brain.

4.3.3 Discussion

We used the fosmid GOLWFno526_i22 and fosmid recombineering to substitute exon 2 of ChAT 2 with a cassette containing GFP and the red fluorescent dye tdTomato. When we sequenced the cassette after flipping out the neomycin resistance gene, we realized that 700 bp were lost. The sequences of the two fluorescent dyes are similar. The ϕC31 integrase probably recombined their sequences, too. Thus, we repeated the recombineering using a cassette, which contains only one fluorescent dye (GFP) and nitroreductase (NTR). Nitroreductase can be used to ablate cells. After treatment with metronidazole, nitroreductase forms cytotoxic products, which lead to cell death in NTR-expressing cells. This method was successfully used in zebrafish (Curado *et al*., 2008; White *et al*., 2011; Fernandes *et al*., 2012) and in the marine bristle worm *Platynereis dumerilii* (Veedin-Rajan *et al*., 2013). It could be useful in future studies when we want to ablate cholinergic cells.

After successful substitution of exon 2 of ChAT 2 with eGFP-F2A-NTR (E2N), we tried to amplify upstream regions of ChAT 2 containing the E2N cassette. Our aim was to amplify regulatory sequences of ChAT 2 expression. The expected PCR products ranged from 6 to 18 kb. In the beginning none of the reactions gave satisfying results. Most PCR reactions did not give any bands at all. After prolonging the primers to 26-28 nt, the PCR with the primers 2204 and 2205 gave a band of 4.8 kb. Although we expected a larger fragment (about 6 kb) we continued our work. The fragment was ligated into a backbone vector and injected together with I-*Sce*I into medaka zygotes. About 90 % of the injected embryos (n = 59) died within the first days for unknown reasons. However, in the surviving and hatched larvae was no GFP visible.

We want to express GFP under the control of the ChAT 2 promoter. Therefore, we amplified the inserted cassette together with upstream regions. The regulatory sequences for ChAT 2 are

not known. They might be even more upstream than the amplified region. This might be a possible explanation for the absence of GFP expression in the screened larvae.

When looking at our combined results, it seems that according to our findings using TALEN technology in combination with attP recombination might be a more promising strategy for inserting reporter genes (cf. 4.1. Precise Genome Editing of Non-visual Opsins Genomic Loci by Combining TALEN Technology and Homologous Recombination in Medaka).

4.4. Is *ValopA* Expressed in GABAergic Cells? Double *in situ* Hybridization with *ValopA* and *gad65/67* in Zebrafish Brains

4.4.1 Aim

We want to characterize the cells expressing inner brain opsins. To see exactly where gene products are expressed we use labeled antisense probes. These antisense probes are labeled with digoxigenin (DIG), which is a hapten. After hybridization, we detect the probes with an antibody against DIG, which has alkaline phosphatase attached. After treatment with NBT/BCIP substrate (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate), the alkaline phosphatase hydrolyzes BCIP, which forms with atmospheric oxygen a blue dye. NBT serves as an oxidant and gives also a blue dye (Trinh *et al*., 2007).

When we want to see if two gene products are expressed within the same cells we perform a double *in situ* staining. One antisense probe is labeled with DIG and the antisense probe of a second gene product is labeled with fluorescein. Antibodies against DIG and fluorescein detect the labeled probes. The alkaline phosphatase, which is attached to the antibodies, catalyzes the formation of blue dye for DIG probes (NBT/BCIP) and of red dye for fluorescein probes (Fast Red tablets; Speel *et al*., 1992).

4.4.2 Background

Superficial interneurons (SINs) in the outer cellular layer of the optic tectum are GABAergic (Nevin *et al*., 2010) (*Figure* 22). Prior *in situ* hybridizations revealed that ValopA is expressed within this region (*Figure* 23). To further characterize those cells and to find out if ValopA is

expressed in GABAergic SINs, we incubated zebrafish brain slices with labeled antisense probes (DIG-ValopA and Fluo-GAD65/67).

GAD65/67 is a marker for GABAergic cells (Erlander *et al.*, 1991). GAD65/67 (Glutamic Acid Decarboxylase) decarboxylates glutamate and thereby forming GABA (γ-aminobutyric acid, Kaufman *et al.*, 1991), which is an inhibitory neurotransmitter (Martin *et al.*, 1998); however, in early development GABA is excitatory as well (Cherubini *et al.*, 1991).

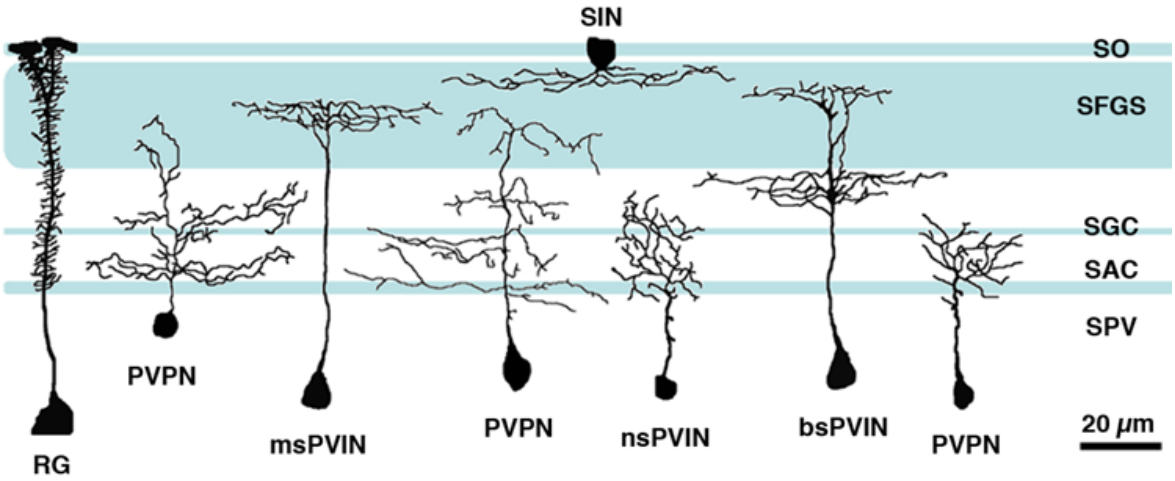


Figure 22: *Different types of neurons in the tectum of zebrafish larvae (Nevin et al., 2010). Abbreviations: PVINs, periventricular interneurons; bsPVINs, bi-stratified PVINs; msPVINs, mono-stratified; nsPVINs, non-stratified; PVPNs, periventricular projection neurons; RG, radial glia; SAC, stratum album centrale; SFGS, stratum fibrosum et griseum superficiale; SGC, stratum griseum centrale; SIN, superficial interneuron; SO, stratum opticum; SPV, stratum periventriculare.*

Although the amino acid sequences of GAD65 and 67 are very similar, their molecular weight (65 and 67 kDa) and their interaction with the GAD co-factor pyridoxal phosphate is different (Erlander *et al.*, 1991; Katarova *et al.*, 2000). Both isoforms are co-expressed in almost all GABAergic neurons and can therefore be used as marker for GABAergic cells (Katarova *et al.*, 2000).

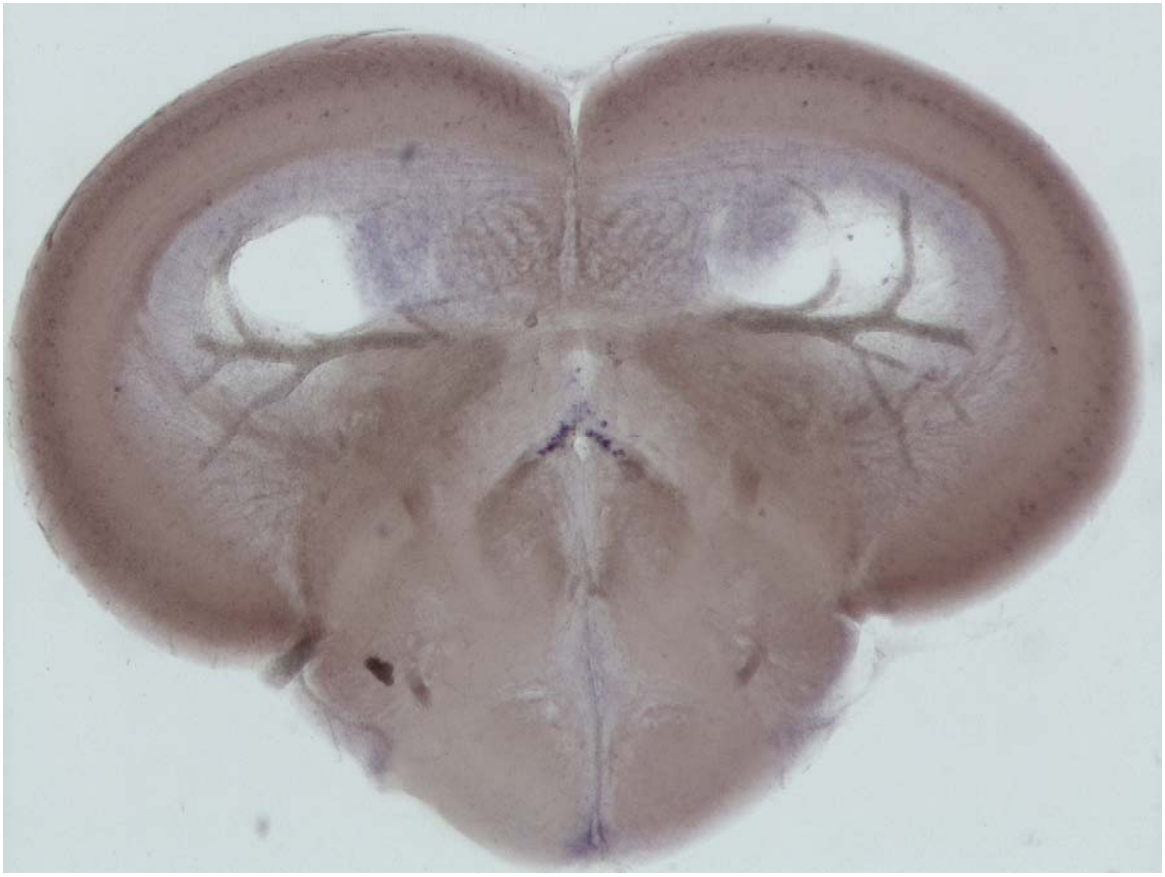


Figure 23 Slice of adult medaka brain stained with α-DIG labeled ValopA antisense probe. Note the expression in the outer layer of the optic tectum.

4.4.3 GABA in the literature

HIGASHIJIMA *et al.* published the expression pattern of GAD65/67 in zebrafish larvae (*Figure* 24; HIGASHIJIMA *et al.*, 2004). The authors sliced the hindbrain and spinal cord of larvae at three different time points (20-24 hpf, 30-32 hpf, 4-5 dpf), which are correlated with specific embryonic movements. The larvae contract their bodies spontaneously, which results in lashing from side to side 20-24 hpf (KIMMEL *et al.*, 1995). 30-32 hpf the unhatched embryos show bending movements (HIGASHIJIMA *et al.*, 2004). 4-5 dpf the zebrafish hatch and swim freely (HIGASHIJIMA *et al.*, 2004).

The spinal cord and hindbrain slices were stained with α-DIG-GAD65/67. HIGASHIJIMA and co-workers described stripe-like clusters of GAD65/67 expressing neurons within the hindbrain of zebrafish larvae (*Figure* 24; HIGASHIJIMA *et al.*, 2004).

In situ hybridizations revealed heavily expression of GAD65/67 within the optic tectum and other parts of the brain (medial and ventral regions of the telencephalon, the nucleus preopticus, the nucleus recessus lateralis of the hypothalamus, and within the Purkinje cell layer of the cerebellum) of goldfish (*Carassius auratus*; MARTYNIUK *et al.*, 2007).

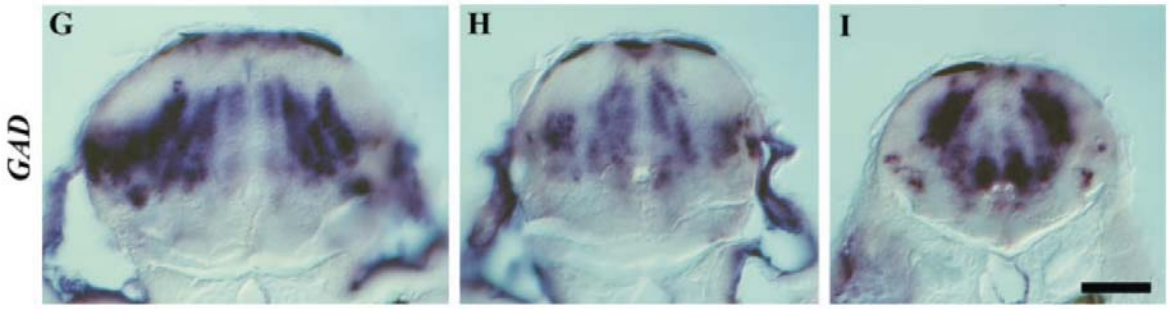
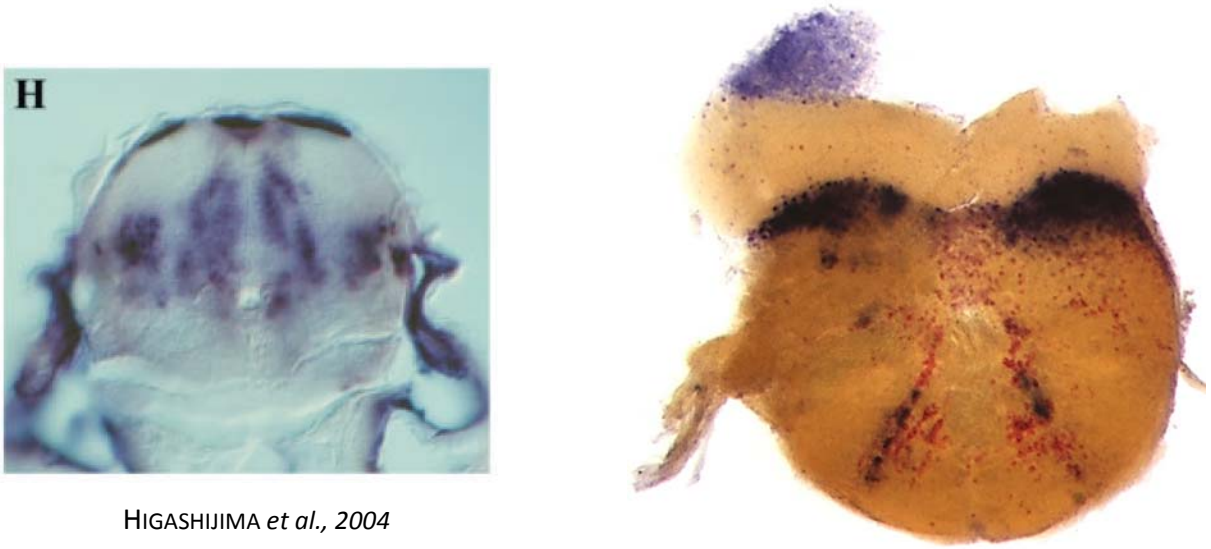


Figure 24: *GAD65/67 expression pattern in hindbrain of zebrafish larvae (4-5 dpf). (G) rostral hindbrain, (H) middle hindbrain, (I) caudal hindbrain. Scale bar = 50 µm (HIGASHIJIMA et al., 2004).*

4.4.4 Results

We cut the brain of adult zebrafish in 100 µm thin slices and performed *in situ* hybridization using a DIG-labeled antisense probe for ValopA and a Fluo-labeled antisense probe for GAD65/67. ValopA and GAD65/67 are both expressed within the outer layer of the optic tectum. In the hindbrain, both gene products are expressed in a striped pattern as reported in the literature (HIGASHIJIMA *et al*., 2004; see 4.4.5 Discussion).

The double staining revealed that in some cells we see co-expression of ValopA and GAD65/67 (*Figure* 27 (D) and (E)). However, the larger part of the stained cells is not co-expressed. GAD65/67 is expressed within a more distal region of the optic tectum than ValopA (*Figure* 26 and 27 (B) and (C)).



HIGASHIJIMA *et al., 2004*

Figure 25: *Comparison of published GAD65/67 expression with our results. The left picture shows a 4-5 dpf larval rostral hindbrain stained with α-DIG-GAD65/67 (HIGASHIJIMA et al., 2004). The right picture shows an adult hindbrain stained with α-DIG-ValopA (blue) and α-Fluo-GAD65 (red).*

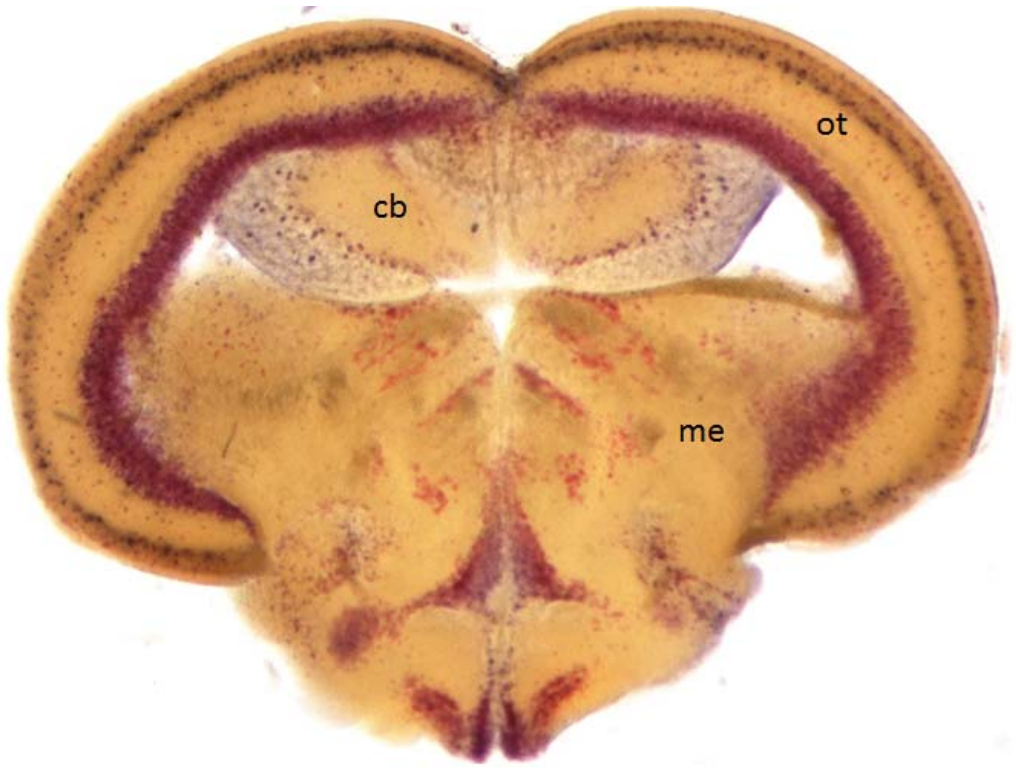


Figure 26: *Brain slice of adult zebrafish stained with α-DIG-ValopA (blue) and α-Fluo-GAD65 (red). cb, cerebellum; me, mesencephalon; ot, optic tectum.*

4.4.5 Discussion

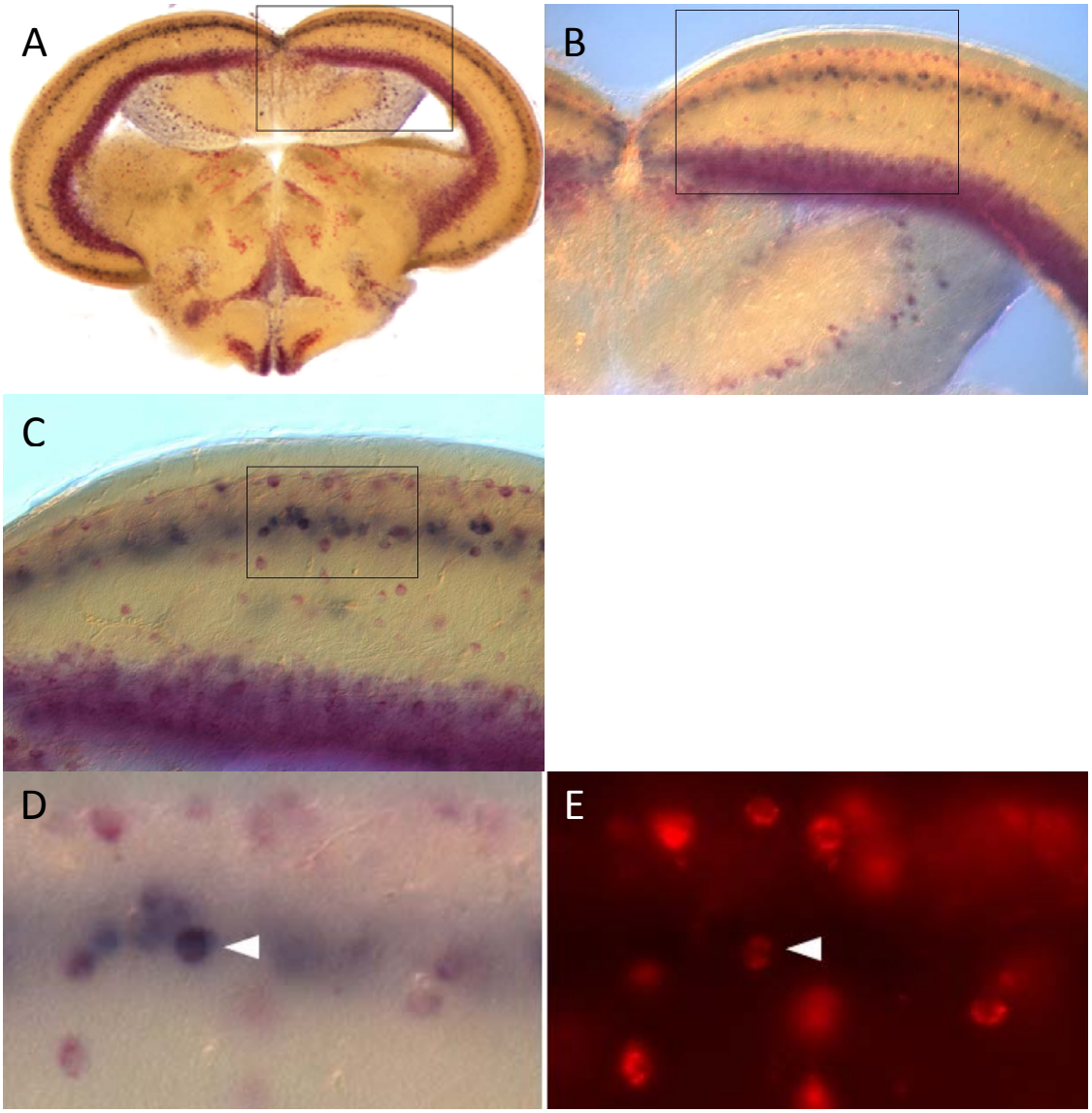


Figure 27: *(A) Optic tectum under binocular. (B) – (D) magnifications of (A) under microscope, (D) and (E) show stained cells. Single cell indicated by white arrowhead shows co-expression of blue Valop staining in (D) and red GAD65/67 staining under fluorescent light in (E).*

Although the expression pattern of zebrafish larvae and adults is hard to compare, our results are very similar to prior observations (*Figure* 25). HIGASHIJIMA and coworkers studied the staining of GAD65/67 exclusively in the larval hindbrain and spinal cord; the authors reported a striped expression pattern. The GABAergic cells co-express GLYT2, a marker for glycinergic neurons (HIGASHIJIMA *et al.*, 2004). HIGASHIJIMA *et al.* published the expression pattern of GAD65/67

only in zebrafish larvae (*Figure* 21; HIGASHIJIMA *et al.*, 2004). The authors sliced the larval hindbrain and spinal cord at three different developmental time points (20-24 hpf, 30-32 hpf, 4-5 dpf), which are correlated with specific embryonic movements. The larvae contract their bodies spontaneously, which results in lashing from side to side 20-24 hpf (KIMMEL *et al.*, 1995). 30-32 hpf the embryos are still in their chorions but show bending movements (HIGASHIJIMA *et al.*, 2004). 4–5 dpf the zebrafish hatch and swim freely (HIGASHIJIMA *et al.*, 2004).

The spinal cord and hindbrain slices were incubated with DIG-labeled antisense probes. HIGASHIJIMA and co-workers describe stripe-like clusters of GAD65/67 expressing neurons within the hindbrain of zebrafish larvae (*Figure* 21; HIGASHIJIMA *et al.*, 2004).

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With our double *in situ* hybridization we tried to further characterize the cells, which express ValopA. SINs (superficial interneurons) are GABAergic neurons at the position of the stratum fibrosum et griseum superficiale and the stratum opticum (NEVIN *et al*., 2010; *Figure* 22). Moreover, a transgenic zebrafish line with labeled SINs was reported (NEVIN *et al*., 2010). Hence, it is important to know if ValopA is expressed within GABAergic cells.

If ValopA were expressed exclusively or mostly in GABAergic cells, labeling GABAergic cells would facilitate work on ValopA expressing cells when performing electrophysiology assays. Unfortunately, co-expression of ValopA and GAD65/67 could only be observed in a minor portion of cells. Therefore, labeling GABAergic cells *in vivo* will not ease measuring the light-sensitivity of ValopA expressing cells in an electrophysiology assay. However, the staining revealed that at least a small fraction of GABAergic neurons co-expresses ValopA.

To further characterize neurons which express ValopA, one could continue study co-expression with neuronal markers. Double *in situ* hybridization with ValopA and VGLUT 1 and 2 (Vesicular Glutamate Transporter) could demonstrate if ValopA expression is correlated with glutamatergic cells; the glycine transporter GLYT2 is a marker for glycinergic neurons (HIGASHIJIMA

et al., 2004). Double staining with probes against choline acetyltransferase serve as cholinergic markers (CLEMENTE *et al.*, 2004) and was successfully used in medaka, too (AKAZOME *et al.*, 2011).

FISCHER *et al.* demonstrated that ValopA and tmt-opsins are co-expressed including inter- and motorneurons; this suggests that neurons possess a complex capacity to react to light (FISCHER *et al.*, 2013).

Further experiments on co-expression of ValopA and other inner brain opsins will hopefully help to finally reveal the function of non-visual opsins.

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II. Appendix

II. a) ϕC31 Integrase Sequence

ATGGACACGTACGCGGGTGCTTACGACCGTCAGTCGCGCGAGCGCGAAAATTCGAGCGCAGCAAG
CCCAGCGACACAGCGTAGCGCCAACGAAGACAAGGCGGCCGACCTTCAGCGCGAAGTCGAGCGCG
ACGGGGGCCGGTTCAGGTTCGTCGGGCATTTCAGCGAAGCGCCGGGCACGTCGGCGTTCGGGACG
GCGGAGCGCCCGGAGTTCGAACGCATCCTGAACGAATGCCGCGCCGGGCGGCTCAACATGATCAT
TGTCTATGACGTGTCGCGCTTCTCGCGCCTGAAGGTCATGGACGCGATTCCGATTGTCTCGGAAT
TGCTCGCCCTGGGCGTGACGATTGTTTCCACTCAGGAAGGCGTCTTCCGGCAGGGAAACGTCATG
GACCTGATTCACCTGATTATGCGGCTCGACGCGTCGCACAAAGAATCTTCGCTGAAGTCGGCGAA
GATTCTCGACACGAAGAACCTTCAGCGCGAATTGGGCGGGTACGTCGGCGGGAAGGCGCCTTACG
GCTTCGAGCTTGTTTCGGAGACGAAGGAGATCACGCGCAACGGCCGAATGGTCAATGTCGTCATC
AACAAGCTTGCGCACTCGACCACTCCCCTTACCGGACCCTTCGAGTTCGAGCCCGACGTAATCCG
GTGGTGGTGGCGTGAGATCAAGACGCACAAACACCTTCCCTTCAAGCCGGGCAGTCAAGCCGCCA
TTCACCCGGGCAGCATCACGGGGCTTTGTAAGCGCATGGACGCTGACGCCGTGCCGACCCGGGGC
GAGACGATTGGGAAGAAGACCGCTTCAAGCGCCTGGGACCCGGCAACCGTTATGCGAATCCTTCG
GGACCCGCGTATTGCGGGCTTCGCCGCTGAGGTGATCTACAAGAAGAAGCCGGACGGCACGCCGA
CCACGAAGATTGAGGGTTACCGCATTCAGCGCGACCCGATCACGCTCCGGCCGGTCGAGCTTGAT
TGCGGACCGATCATCGAGCCCGCTGAGTGGTATGAGCTTCAGGCGTGGTTGGACGGCAGGGGGCG
CGGCAAGGGGCTTTCCCGGGGGCAAGCCATTCTGTCCGCCATGGACAAGCTGTACTGCGAGTGTG
GCGCCGTCATGACTTCGAAGCGCGGGGAAGAATCGATCAAGGACTCTTACCGCTGCCGTCGCCGG
AAGGTGGTCGACCCGTCCGCACCTGGGCAGCACGAAGGCACGTGCAACGTCAGCATGGCGGCACT
CGACAAGTTCGTTGCGGAACGCATCTTCAACAAGATCAGGCACGCCGAAGGCGACGAAGAGACGT
TGGCGCTTCTGTGGGAAGCCGCCCGACGCTTCGGCAAGCTCACTGAGGCGCCTGAGAAGAGCGGC
GAACGGGCGAACCTTGTTGCGGAGCGCGCCGACGCCCTGAACGCCCTTGAAGAGCTGTACGAAGA
CCGCGCGGCAGGCGCGTACGACGGACCCGTTGGCAGGAAGCACTTCCGGAAGCAACAGGCAGCGC
TGACGCTCCGGCAGCAAGGGGCGGAAGAGCGGCTTGCCGAACTTGAAGCCGCCGAAGCCCCGAAG
CTTCCCCTTGACCAATGGTTCCCCGAAGACGCCGACGCTGACCCGACCGGCCCTAAGTCGTGGTG
GGGGCGCGCGTCAGTAGACGACAAGCGCGTGTTCGTCGGGCTCTTCGTAGACAAGATCGTTGTCA
CGAAGTCGACTACGGGCAGGGGGCAGGGAACGCCCATCGAGAAGCGCGCTTCGATCACGTGGGCG
AAGCCGCCGACCGACGACGACGAAGACGACGCCCAGGACGGCACGGAAGACGTAGCGGCGCCTAA
GAAGAAGAGGAAGGTTTAG

II. b) Abstract

About 100 years ago KARL VON FRISCH and ERNST SCHARRER demonstrated that minnows react to light even without eyes and pineal. Experiments with quails confirmed the reaction to light in ophtal- and pinealectomized animals. About 50 years ago BRUNT *et al*. reported that measurable amounts of light penetration even reach into deep inner brain parts in mammals.
Hitherto, several photoreceptors in the eye and in specific brain regions have been identified. However, their function is still unclear.

We are working with the two teleosts zebrafish and medaka, which live in two different habitats and are exposed to different kinds of seasonality: medaka is exposed to a steady change in day length over the year, whereas zebrafish experiences monsoon season. Both fish species have been evolving independently of each other for about 300 million years; the same period as between birds and mammals. Interestingly, both fish express G protein-coupled photoreceptors (tmt-opsins and Valop) within their brains, suggesting functional importance. Furthermore, both teleosts possess encephalopsin, which is also known to be present in mammals. TMT-opsins and encephalopsin are closely related, which makes zebrafish and medaka interesting model organisms for studying the function of tmt-opsins and encephalopsin.

We used two different approaches to start elucidating the role of inner brain opsins: loss of function and labeling gene products *in vivo*. We used TALEN technology to knock out opsins. In addition, we tried to label cholinergic cells with fluorescent dyes using Red/ET recombineering.

One interesting project combined both approaches: a knock-out of *tmt-1B* with TALENs and a knock-in of eGFP to label *tmt-1B* expressing cells. Therefore, we used the ϕC31 integrase with attP and attB. We co-injected TALEN mRNA for exon 1 of *tmt-1B* together with an ssDNA Oligo containing attP40 flanked by short homology arms. The DNA is cut by TALENs, the ssDNA Oligo serves as a template, and the cell integrates attP via homology-directed repair. In the next step a construct of attB and eGFP will be injected together with ϕC31 integrase mRNA. After translation, the ϕC31 integrase will recombine attP and attB unidirectionally. The cells are now lacking *tmt-1B* and express EGFP instead, which can be traced under fluorescent light *in vivo*. Thus, we are able to observe where *tmt-1B* is expressed; moreover, the labeled cells ease patching in electrophysiology assays.

II. c) Zusammenfassung

Es ist nun schon etwa 100 Jahre her, dass KARL VON FRISCH und ERNST SCHARRER bei Elritzen Reaktionen auf Licht feststellten, auch wenn den Fischen Augen und Epiphyse operativ entfernt worden waren. Ähnliche Experimente an Wachteln führten ebenfalls zu der Erkenntnis, dass die Tiere nach wie vor auf Licht reagierten. Vor etwa 50 Jahren wurde darüber hinaus gezeigt, dass Licht sogar imstande ist, in die tieferen Gehirnregionen größerer Säugetiere wie z.B. Schafe vorzudringen. Obwohl diese Phänomene schon seit Jahrzehnten bekannt sind, ist die Funktion des Gehirns auf Licht zu reagieren weiterhin unklar. In unserem Labor arbeiten wir unter anderem mit Zebrabärblingen und Medaka. Beide Fischarten leben in unterschiedlichen Lebensräumen und sind verschiedener Saisonalität ausgesetzt. Während Medaka sich an sich ändernde Tageslängen über das Jahr angepasst hat, ist der Zebrabärbling an die An- und Abwesenheit vom Monsun angepasst. Beide Tierspezies entwickeln sich seit ungefähr 300 Millionen Jahren unabhängig voneinander; das entspricht etwa derselben Zeitspanne wie zwischen Vögeln und Säugetieren. Es ist interessant, dass beide Fischarten dennoch dieselben Photorezeptoren in ihren Gehirnen exprimieren, lässt es doch auf deren Wichtigkeit schließen. Säugetiere besitzen Encephalopsin, das der Subfamilie der TMT-Opsine der Fische sehr ähnlich ist.

In der vorliegenden Arbeit verfolgten wir zwei verschiedene Ansätze, um der Funktion der Opsine auf die Spur zu kommen. Einerseits schalteten wir das Gen selbst aus, andererseits haben wir mit Fluoreszenz-Farbstoffen versucht, die Genprodukte zu markieren. Mit der TALEN-Technologie unterbanden wir die Expression von funktionellen TMT-Opsinen in Medaka. Unter Verwendung von Red/ET recombineering wollten wir den Marker für cholinerge Neuronen mit dem grünen Fluoreszenz-Farbstoff eGFP ersetzen.

In einem anderen Projekt haben wir beide Ansätze kombiniert: so zerstörten wir *tmt-1B* in Medaka mit TALEN, stellten allerdings gleichzeitig ein kurzes DNA-Stück mit homologen Enden zur Verfügung, das die Zelle, den Doppelstrangbruch reparierend, eingebaut hat. Die DNA enthält die Sequenz von attP. Im nächsten Schritt injizieren wir ein Konstrukt mit attB und EGFP gemeinsam mit mRNA der ϕC31-Integrase, die, erst einmal translatiert, attP and attB miteinander unidirektional rekombiniert, was eGFP unter den Promotor von *tmt-1B* exprimiert. Dadurch

lässt sich einerseits das Expressionsmuster von *tmt-1B in vivo* nachvollziehen, andererseits erleichtert es das Patchen cholinerger Zellen bei elektrophysiologischen Arbeiten.

Zusammenfassend hoffe ich, mit meiner Arbeit einen Beitrag geleistet zu haben damit schließlich die Funktion der Opsine des Zebrabärblings und von Medaka aufgeklärt werden kann.

II. d) Acknowledgments

I would like to extend my gratitude to the members of the lab of KRISTIN TESSMAR-RAIBLE and FLORIAN RAIBLE at the Max F. Perutz Laboratories of the University of Vienna. It was a tremendous pleasure for me to do my master thesis in our lab. Without your help and support I would not have learned as much as I did.

Special thanks to RUTH FISCHER, who did a great job in supervising me. In addition, I would like to thank KRISTIN TESSMAR-RAIBLE for giving me the opportunity to work in her lab and for her steadily support und supervision.

I sincerely enjoyed the time in our lab and the fruitful discussions especially with RUTH, KRISTIN, FLORIAN, SVEN SCHENK, STEPH BANNISTER, and MARCUS DEKENS.

Last but not least I would like to thank my sister MANUELA TONDL and my friends RENATE BEROUN, CHRISTIAN RIEDL, and RYAN SANDERS, who were essential for my work-life balance.

II. e) Curriculum Vitae

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