HOCHSCHULE MITTWEIDA
UNIVERSITY OF APPLIED SCIENCES

CRTD
Center for Regenerative Therapies Dresden

BACHELORARBEIT

Frau
Mandy Schönbrodt-Rühl

Characterization of the zebrafish larval mutant *knörf*

Mittweida, 2012

Fakultät MNI

BACHELORARBEIT

Characterization of the zebrafish larval mutant *knörf*

Autor:
Frau

Mandy Schönbrodt-Rühl

Studiengang:
Biotechnologie/Bioinformatik

Seminargruppe:
BI09w2-B

Erstprüfer:
Prof. Dr. Röbbe Wünschiers

Zweitprüfer:
Dr. Christopher Antos

Einreichung:
Mittweida, 27.08.2012

Verteidigung/Bewertung:
Mittweida, 2012

Abstract

The larval zebrafish mutant *Knörf* has got a not yet identified gen, which is lethal after 14 dpf in a homozygous state. The mutation courses various degenerations and the loss of the regeneration ability. One of these degenerations was first discovered in the retina by a histological section. The mutant`s retinas show gaps in the IPL at 7 and 8 dpf which number increases during the maturation of the larva. In recent studies a pax 6 staining was performed, which showed that amacrine cells are affected. Different types of amacrine cells were tested and it was shown that the parvalbuminergic amacrine cells disappear. The staining was performed in a time course. At 5 dpf is no difference between the number of parvalbuminergic amacrine cells in siblings and mutants but then the degeneration starts. At 2 dpa there is the first significant difference which increases at later stages and leads nearly to a full disappearance of these cells in the eye. Parvalbumin is not only present in the retina, therefore the brain as another central nervous system structure was examined. In the telencephalon these cells disappear already at 2 dpa. The parvalbuminergic cells are also present in the skeletal muscle of the tail. Here the degeneration starts approximately at the half of the tail and intensifies to distal areas. It was shown, that parvalbuminergic cells in the muscle disappear until 4dpa.
The role of parvalbumin is seemed in the binding of calcium and therefore it supports the adjustment of the resting potential after an excitation in the central nervous system. In muscles it assists in the slowing of relaxing after a contraction of a muscle.

Danksagung

Ich möchte mich an dieser Stelle bei all denen bedanken, die mich bei der Anfertigung meiner Bachelorarbeit so kräftig unterstützt haben.

Besonderen Dank gilt dabei der gesamten Antos Gruppe des CRTD, die mich mit Ratschlägen unterstütz haben und immer ein offenes Ohr für Fragen hatten.

Ganz besonderer Dank gilt dabei meiner direkten Betreuerin Judith Konantz, von der ich sehr viel lernen konnte. Ich danke ihr dafür, dass sie sich immer Zeit für mich genommen hat, wenn es nötig war und dass sie mich auf einem langen Weg voller nicht funktionierender Experimente und neuer Daten unterstütz hat.

Weiterhin möchte ich mich bei Dr. Christopher Antos bedanken, dass ich meine Arbeit in seiner Gruppe schreiben konnte. Er hat mich immer mit guten Ratschlägen unterstütz und Kritik an den richtigen Stellen walten lassen.

Denise Theil möchte ich für die Unterstützung im Bereich der Histologie danken und für die Zeit die sie aufgewendet hat um mich in diesen Bereich einzuführen.

Ich bedanke mich ebenfalls bei der Light Microscopy Facility, ganz besonders bei Hella Hartmann, die mir immer bei Mikroskop Problemen geholfen haben. Ohne sie wären die vielen schönen Bilder nicht möglich gewesen.

Ein weiteres Dankeschön gilt der Histology Facility, besonders Susanne Weiche und Anja Menge, die immer für mich Zeit hatten, wenn Probleme mit den Kryostaten oder dem Mikrotom auftraten.

Herrn Prof. Dr. Wünschiers von der Hochschule Mittweida danke ich für die Betreuung meiner Arbeit und für die Zeit, die er sich für meine Fragen genommen hat.

Ganz besonderer Dank gilt meinen Eltern, die mich über die ganze Zeit meines Studiums finanziert und so diese Arbeit ermöglicht haben. Ebenfalls möchte ich mich bei meinem Freund bedanken. Diese Menschen haben mich immer in schwierigen Situationen unterstützt und in Situationen in denen ich schwierig war zurechtgewiesen.

Table of Contents

List of figures

List of tables

List of charts

Abbreviations

dUTP	2'-deoxyuridine 5'-triphosphate
DAPI	4',6-diamidino-2-phenylindole
BS	beamsplitter
BSA	Bovine serum albumin
CNS	central nerve system
dpa	days post amputation
dpf	days post fertilisation
EM	Emission
MESAB	ethyl-m-aminobenzoate methanesulfonate + di-sodium hydrogen phosphate
EX	Excitation
GFP	green fluorescent protein
OH	hydroxyl functional group
INL	inner nuclear layer
IPL	inner plexiform layer
ONL	outer nuclear layer
OPL	outer plexiform layer
PFA	Paraformaldehyde
PBS	phosphate buffered saline
PBST	phosphate buffered saline + tween 20
rpm	revolutions per minute
R&CL	rod and cone layer
RT	room temperature
TUNEL	Terminal deoxynucleotidyl transferase [TdT] dUTP nick end labeling

1 Introduction

1.1 The zebrafish as a model organism

The zebrafish (*Danio rerio)* became one of the most important vertebrate model organisms due to its numerous advantageous properties. The fresh water fish has a short generation time. They become sexually mature after 12-16 weeks [Dinort, 2008]. They are breeding throughout the whole year and the females lay up to 300 eggs per week so you can easily perform statistical analyses and so you produce reproducible data. Furthermore, the husbandry and breeding are facilitated due to the small size of the animal. The adult zebrafish is about three to four centimeter long; thus one can keep many animals on little space. The larvae are beneficial for optical analyses, because of their transparency, rapid growth and external development outside their mother [Fethob]. Most organs, cells and genes which exist in humans are also present in the zebrafish, which is one reason why lots of research groups are using the zebrafish as a model organism to examine human diseases. Additionally, the genes and tissues have similar or the same functions as in humans so that the gained perceptions can be assigned to develop novel therapies [URL Nr. 2].

1.2 Regeneration

The zebrafish has the ability to regenerate. It is able to regrow limbs, tail, eye structures, jaw, optic nerve, the spinal cord and the heart muscle [Poss et al, 2003].
Mammals, like humans, contain several organ systems which can regenerate, such as blood and liver but their regeneration potential is weaker, so most organs and tissues heal by scarring.
After injury, there are two major ways of healing the wound. One is the repair of the injured tissue. It is characterized by an inflammation response and formation of collagen-rich tissue which ends in a permanent scar, usually resulting in the loss of function of the injured tissue [Poss et al, 2003]. The second injury response is regeneration. In zebrafish amputated fins, the first inevitable step of regeneration is the formation of a wound epidermis until 12 hours post amputation (hpa). It is the rapid migration and rearrangement of the epithelial cells from amputated regions around the stump which cover the surface of the cut. This if followed by adding several additional layers of epidermal tissue, which form the apical epidermal cap. Subsequently, a blastema is formed, a major hallmark of epimorphic regeneration. The blastema is a mass of proliferative, mainly mesenchymal-like cells but also consists of cells that retain their tissue origin. It is believed that the blastema forms through disorganization and migration of mesenchymal tissue, which migrates to the distal region and initiates proliferation. In adult zebrafish the blastema becomes clearly visible at 2 days post amputation (dpa) [URL Nr. 1]. Following, the blastema matures and the regenerative outgrowth finally ends in the restoration of the original size and

shape of the injured tissue [Akimenko et al, 2003]. It takes 7 to 14 days in adult zebrafish [Poss et al, 2003] to regenerate a lost fin.
Not only adult zebrafish are capable of regenerating their fins, but also zebrafish larvae are able to regenerate their caudal fin after amputation by similar mechanisms as adult fish [Kawakami et al. 2004]. Although the larvae are still developing, they activate the same regeneration genes as adult fish, whose expression patterns are different from the normal developmental processes.

1.3 The Retina

The retina is a light sensitive tissue which lies in the back of the eye. Since it develops out of the brain, it belongs to the central nervous system. It comprises a huge diversity of neuronal cell types. A mammalian retina for example consists of 55 distinct cell types which are organized in different layers. The outermost layer consists of horizontal cells of the pigment epithelium, which protects the retina from excess incoming light. The next layer is the photoreceptor layer, containing the light sensitive cells – the rods and cones. The outer nuclear layer contains cell bodies of the rod and cones, the axons of the bipolar and the horizontal cells lie in the outer plexiform layer, the inner nuclear layer contains the cells bodies of all the retinal interneurons like amacrine, bipolar and horizontal cells and the inner plexiform layer contains the axons of amacrine and ganglion cells. The signals from the photoreceptors arrive in the brain via the optic nerve. Figure 1 shows the organization of a vertebrate retina [URL Nr. 3].

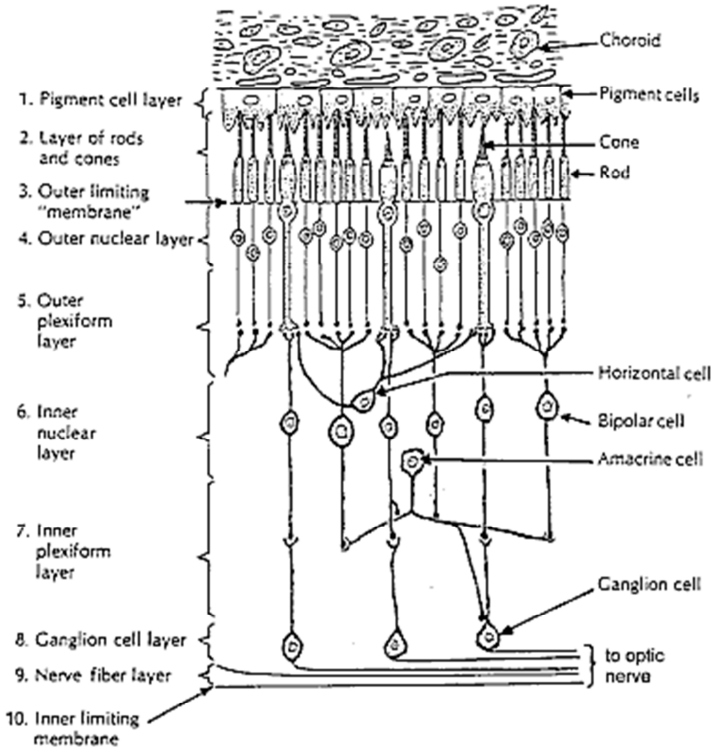


Figure 1: Organization of the retina [URL Nr. 6]

From the photoreceptors (rod and cones) light is transmitted through the different layers. The outer nuclear layer contains cell bodies of horizontal cells, the axons of bipolar cells lie in the outer plexiform layer, the inner nuclear layer contains bipolar and amacrine cells and the inner plexiform layer contains the axons of bipolar, amacrine and ganglion cells. The ganglion cell layer contains the cell bodies of ganglion cells. Via the optic nerve the signal is transmitted to the brain.

Table 1: Layers and cells of the retina

Layer	Cells in it
photoreceptor cell layer	Rod and cones
outer nuclear layer	cell bodies of the rods and cones
outer plexiform layer	axons of horizontal and bipolar cells
inner nuclear layer	cell bodies of bipolar, horizontal and amacrine cells
inner plexiform layer	axons of amacrine and ganglion cells

The photoreceptor cells absorb photon energy from different wavelengths of light and initiate signal transduction to the visual system of the brain where this information is converted into image information. There are two types of photoreceptors: the rod-photoreceptor cells and cone photoreceptor cells. The cones are mainly used during daylight and used for color vision, roughly from 10^6 cd/m^2 to 10 cd/m^2 (photopic vision) whereas the rods are mainly used for vision at dim light from 10^{-2} cd/m^2 to 10^{-5} cd/m^2 (scotopic vision) [Eysel, 2007]. In a mammalian retina, the rods out numbers the cones by approximately 20-fold, the human eye has about 120 million rods and 6 million cones [Masland, 2001]. The cone signal is transmitted by the cone bipolar (cone-driven bipolar cells) cells to the ganglion and amacrine cells. The cone bipolar

cells have branches at different levels of the inner plexiform layer where they connect to different amacrine and ganglion cells. There are 3 different types of bipolar cells: the on-cone bipolar, the off-cone bipolar and the rod bipolar cells. A distinction is made between the ways of the signal transduction: depending on the light intensity, the signal is transduced in different ways towards the ganglion cells. It is either transmitted directly along photoreceptors, bipolar and ganglion cells or laterally along interneurons (horizontal and amacrine cells). The horizontal cells transmit the signal between the adjacent photoreceptors. Another class of interneurons is the amacrine cells class, which consist of 29 distinct cell types [Masland, 2001]. One of the important ones is the cone amacrine cell which is needed for the movement vision. During the scotopic vision they transmit the signal from rod bipolar to cone bipolar cells. A second important class are the dopaminergic amacrine cells. Their purpose is to switch between the photopic and the scotopic vision [Eysel, 2007]. The ganglion cells are the output of the retina and transmit the signal coming from the photoreceptors via the optic nerve into the brain.

1.4 Neurogenesis of the Zebrafish Retina

The retina is a region of the central nervous system which is structured relatively simple. It is a part of the body which forms and becomes functional very early in development. The neurogenesis of the zebrafish retina is basically complete at 60 hpf. There is a small field between the telencephalic and the diencephalic precursor fields, where the retina originates during the early gastrulation of the zebrafish embryo. The retinal field is separated in two primordia by the anterior migration of diencephalic precursors. Thereby, it takes the form of a solid rod, the so called neural keel. At 11.5 hpf the optic lobes, which are the equivalent to the optic vesicles in higher vertebrates, become present as a bilateral thickening of the neural keel. Their posterior portion starts to separate from the brain at 13 hpf, the anterior portions remain attached. At the same time the optic lobes turn so that its lower surface becomes directed towards the brain and the upper surface towards the outside environment (see fig. 2 A). This outside surface will then form the neural retina. At 15 hpf, the medial layer becomes thinner because cells migrate from there to the lateral epithelial layer of the optic lobe. This forms the retinal pigment epithelium [Avanesov et all, 2004]. On the lateral surface of the optic lobe forms an invagination which is accompanied by the thickening in the epithelium, which lies over the optic lobes (see fig. 2 C). Both become more prominent over time, when the optic lobes transform into the optic cup. Until 24 hpf the lens placode continues to grow and the choroid fissure forms in the rim of the optic cup, until it is detached from the epidermis. At about 24 hpf, melanin granules appear in the pigment epithelium. Shortly afterwards there is an expansion of the ventral diencephalon, the eye rotates so that the choroid fissure is directed towards the heart. In this period the stalk becomes less prominent. The optic stalk provides support for the axons of ganglion cells, which start to differentiate at the first half of day 2. At this point the optic cup consists of the pseudostratified columnar neuroepithelium and the cuboidal pigment epithelium which are two closely connected sheets.

The nuclear divisions and cytokinesis of cells that are about to divide take place at the apical surface of the neuroepithelium. Approximately at 27 hpf the first ganglion cell precursors start to become postmitotic. The first other cells in the INL become postmitotic 10 h after the cell cycle exit of the first ganglion precursors and at 60 hpf more than 90 % of neurons in the central retina are postmitotic and the different layers are morphologically distinguishable. In teleosts and in larval amphibian, the proliferation in the retinal margin continues throughout the lifetime [Avanesov et all, 2004].

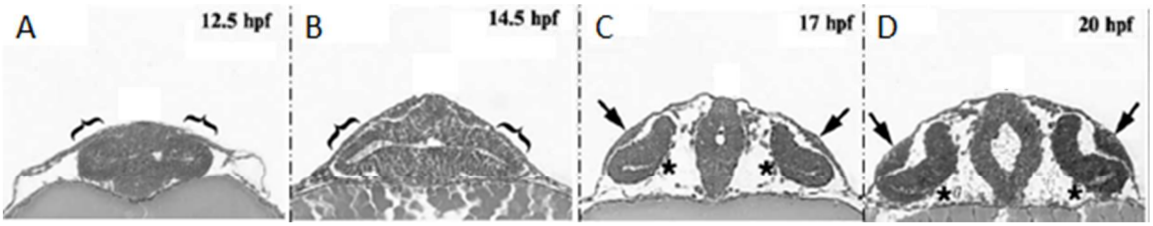


Figure 2: Early development of the retina [Avanesov et all, 2004]

The retina originates from one single field of cells. A , B: The picture at 12.5 and 14.5 dpf shows a transverse plastic section through the anterior portion of the neural keel and optic lobes (brackets)
C: Approximately at17 hpf: section through the neural keel and optic lobes during the reorientation. At about the same time, lens rudiments start to form (arrows) and the medial layer of the optic lobe becomes thinner as it begins to differentiate into the pigmented epithelium (asterisks). The lateral surface of the optic lobe starts to invaginate.
D: section through the anterior neural keel during optic cup formation at 20 hpf. Lens rudiments are quite prominent here.

2 The zebrafish larval mutant *knörf*

Zebrafish larvae are able to regenerate their caudal fin after amputation. Figure 3 shows a non-amputated caudal fin at 5 dpf. By amputation, the distal part of the fin is removed (Fig. 4). After that, a bulge forms in the middle of the amputation plane (Fig. 4 A) which is then covered by finfold-tissue over time (Fig. 4 C-F) until the original size and shape of the lost fin is reconstructed. This process takes about 4-5 days.

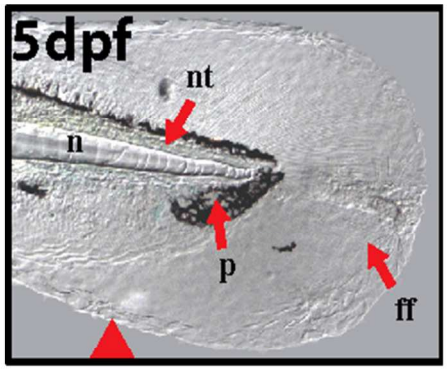


Figure 3: Fin of a 5-day old larva [Konantz, 2012]

Fin of a 5 day old larva before the amputation. The red triangle shows the prospective amputation plan. The notochord (n), finfold (ff), neural tube (nt) and the pigment (p) are visible.

The zebrafish larval mutant *knörf* was found during a screen to identify regeneration mutants at the MPI in Tübingen 2005 (Dr. Christopher L. Antos, Dr. Caghan Kizil). Due to the mutation in a particular gene which has not yet been identified, the mutant lost its ability to regenerate the caudal fin. After amputation, they also form a bulge-like structure (Fig.4 H-N), but no regenerative outgrowth of the finfold takes place indicating that they fail to initiate regeneration.
In addition to the regeneration phenotype the mutants are lethal in a homozygous state. The larvae die at around 5 to 7 dpa, which corresponds to day 10-12 of development. Mutants that are amputated earlier during development (at 3 dpf) are able to partially regenerate their caudal fin but at 5 dpf they lose this ability completely. The expression of different regeneration genes such as ApoE, a gene essential for the normal catabolism of triglyceride-rich lipoprotein constituents, Shh, involved in cell proliferation and the formation of various skeletal elements, MsxD and MsxB, which are essential for normal craniofacial, limb and ectodermal organ morphogenesis, were tested in amputated mutant and wild-type fins. Although they are all expressed in mutants, they show expression patterns of earlier time points of wild-type regeneration. That suggests that mutants can initiate but not maintain the regeneration program. [Konantz, 2012].

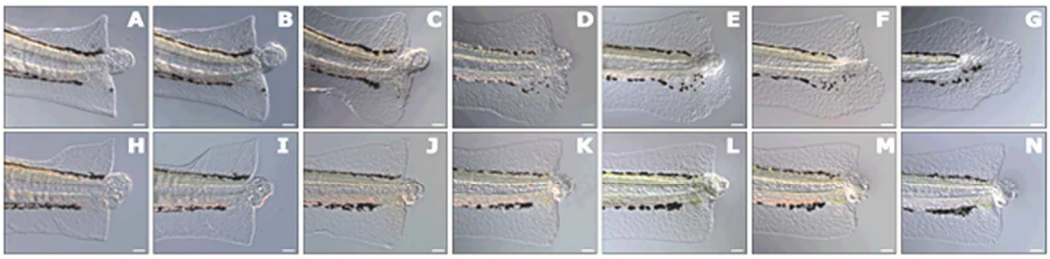


Figure 4: Regeneration in wild-type and *knörf* zebrafish [Konantz, 2012]

(A-G) Regeneration of wild-type larvae: After amputation a bulge forms in the middle of the amputation plane (A ;B) which is covered by epithelial cells over time (C-F) until the fin is completely restored (G).(H-N) Mutant phenotype. The mutants form the bulge structure, but there is no outgrowth of finfold tissue as observed in wild-types.

However the disability to regenerate is not the only phenotype of *knörf*. In addition to the regeneration phenotype, mutants display degeneration of skeletal muscle and retina tissue, starting at 2 dpa (7 dpf). The muscle degeneration starts in the distal trunk and progresses more proximal over time. Additionally, they also degenerate without amputation indicating that the observed phenotypes do not occur due to the injury of the animals

In the retina, gaps occur in the inner plexiform and inner nuclear layer. (Fig. 5) Due to the location of the degenerating cells (Fig. 6) it's most likely amacrine cells that are affected. The gaps occur for the first time at 7 dpf and their number is increasing over time.

Figure 5 shows the difference between wild-type and mutant retinas at different stages of development.

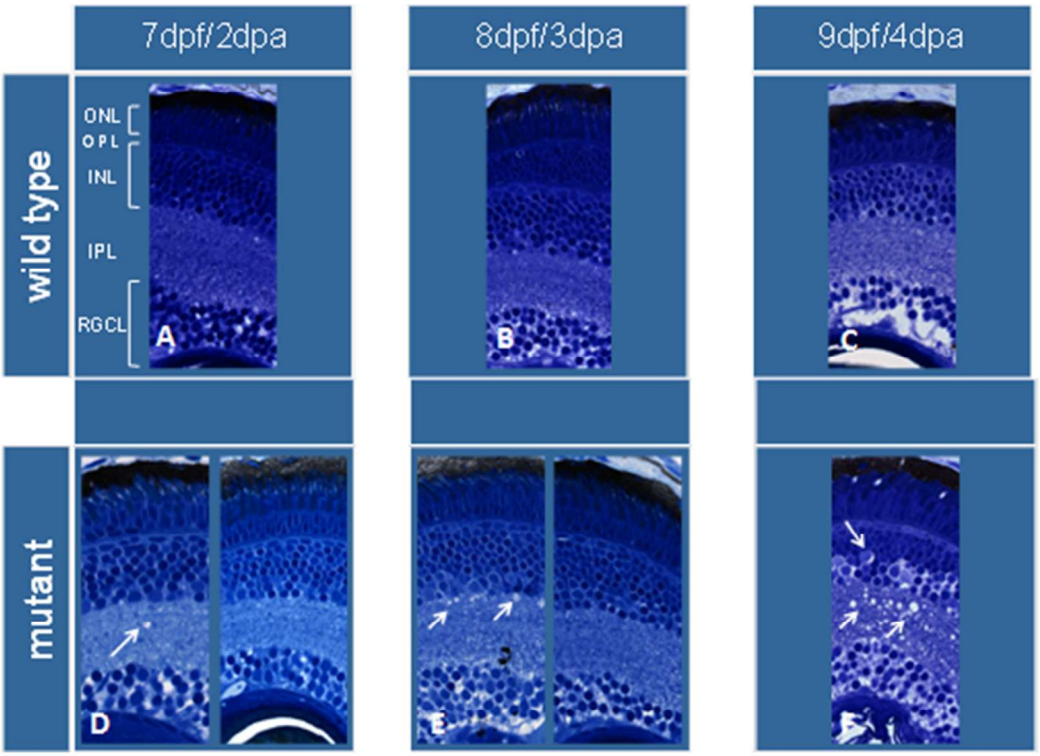


Figure 5: Retina of a wild-type and a mutant larva [Konantz, 2012].

A retina is organized in different layers: (ONL) outer nuclear layer; (OPL) outer plexiform layer, inner nuclear layer, inner plexiform layer, rod and cone layer. These layers are horizontally arranged. (A-C) wild-type retina at 7-9 dpf display nicely organized neuronal layers. . (D-F) mutant retinas how holes in the IPL at 7 and 8dpf (D; E, arrows) which become bigger at 9dpf (F, arrows). The number of gaps increases during the maturation of the larva.

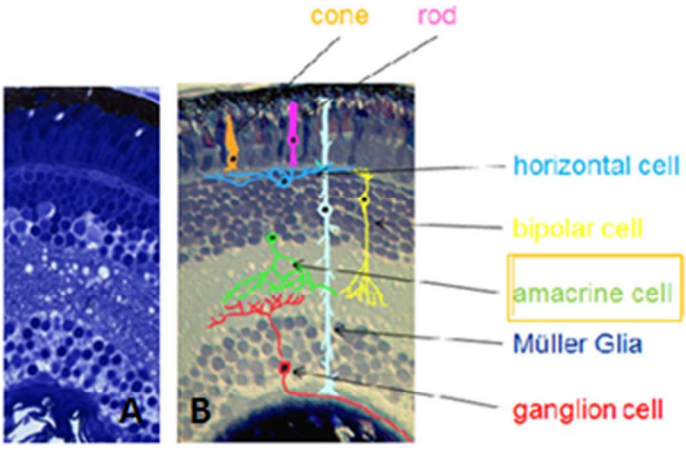


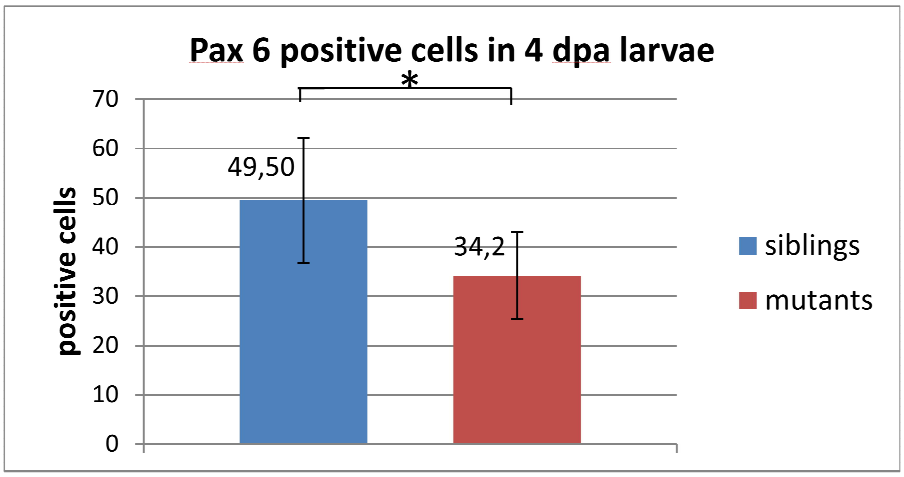
Figure 6: Cells in the retina [Konantz, 2012].

A: Mutant retina. In the inner plexiform and inner nuclear layer of mutants gaps are visible, in the area where the amacrine cells are located. B: Schematic view, showing the location of the different cell types of the retina.

3 Preliminary findings

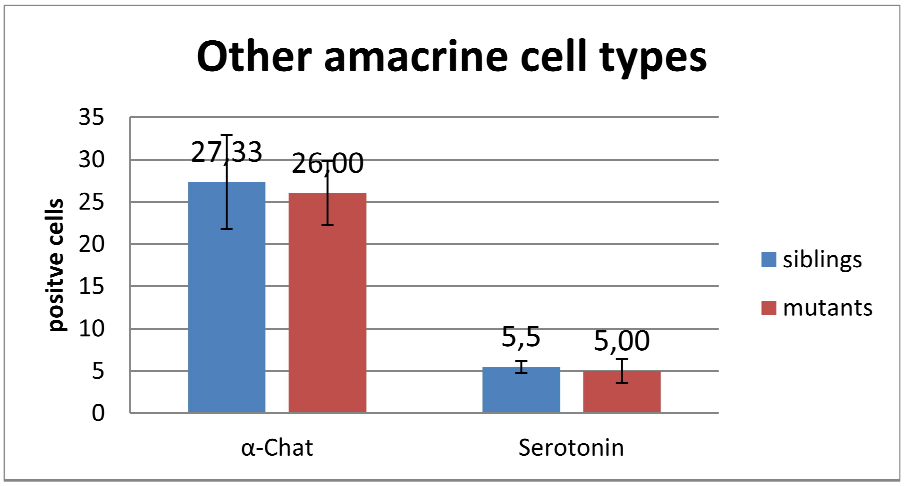
The zebrafish mutant *knörf* shows, besides others, degeneration of skeletal muscle and retina tissue. The gene causing the phenotypes in not yet identified and is homozygous lethal. The larvae degenerate in special tissues and die around 14-17 days post fertilization. One of these phenotypes is the occurrence of gaps in the retina in the area of the IPL. The location of the holes indicates that amacrine cells are affected. Therefore, I stained mutant and sibling retinas for pax 6, a protein which is present in all amacrine cells. I then counted the positive cells in sections containing the optic nerve. Thereby, I found out that mutants have 30 % fewer amacrine cells than the siblings, indeed indicating that amacrine cells are lost in the mutants. Chart 1 shows the number of pax 6 positive cells in siblings and in mutants.
Additionally, I investigated by performing immunohistochemistry experiments which of the different types of amacrine cells are affected. A staining with α-Chat and a-serotonin antibody showed no significant difference between siblings and mutants. Chart 2 shows the exact numbers of the different stainings in siblings and mutants. However, staining for parvalbumin, another marker for a certain type of amacrine cells, revealed that the mutants have got none or only some parvalbumin positive cells compared to wild-types suggesting that these cells are affected by the mutation.
Parvalbuminergic cells are important interconnections in the retina and are involved in the rod visual pathway. The disappearance of these cells may lead to a reduction or loss of vision. So as to test how the cells are lost, I performed immunohistochemistry using an active caspase 3 antibody to detect apoptotic cells showing no difference between mutants and siblings. The reason for this might be, that caspase is not only involved in apoptosis but also in differentiation of cells. Therefore, the staining we detected might not show apoptotic but differentiating cells. Because of this, another method for detecting apoptosis was performed, the TUNEL staining. However, also with this method no apoptotic cells where identified, neither in siblings nor in mutants indicating that enhanced cell death is not the reason for the degeneration of amacrine cells.
Further investigations are needed to fully understand the different phenotypes of *knörf*, which was the task of my bachelor thesis.

Chart 1: counting of pax 6 positive cells



Counted sections: 8

Chart 2: counting other amacrine cells



Counted sections: 4

4 Task

My task was to further characterize the larval zebrafish mutant *knörf*. In recent studies I found out that amacrine cells are affected by the mutation. One of my tasks was to examine if other retinal cell types which lie in the area of degenerating cells are affected. Therefore, I will perform immunohistochemistry on tissue sections for bipolar cells, Mueller glia cells and also other amacrine cell types. For this purpose, I will stain them with different antibodies specific for those cell types and then count the total number of positive labeled cells in mutants compared to wild-types.
However, the most prominent phenotype I saw during my previous characterization of the mutant phenotype was a complete loss of parvalbuminergic amacrine cells at later stages of development (9dpf). To see at which time point the loss of parvalbuminergic cells starts and to investigate whether mutants develop the same amount of parvalbumin positive cells as wild-type siblings, I will perform a time course starting at earlier stages of development. Parvalbumin is a widely spread protein which is also present in the brain and in the muscle. Therefore, this part of the body will be considered, too.
So the second point of characterization will be the examination of muscles. Here I will produce histological sections of the muscles in wild-types and in mutants in different orientations of the larva. By using this method I can test which muscles are affected and in which area of the larvae. Then I will perform immunohistochemistry on tissue sections of the muscle to find out which types of muscles are affected and whether they also display loss of parvalbumin. Finally, I will also focus on the larval brain. I will also perform histological sections to check if there is also degeneration in the brain. Additionally, I will examine whether parvalbuminergic cells within the brain are also lost in the mutants. Taken together, these experiments will gain further insights into the mutant phenotype and will help to understand how the gene that is affected in the mutants is required for the maintenance of certain cell types of the retina and skeletal muscle.

5 Materials

Table 2: Chemicals

Chemicals	*Manufacturer*	*Lot#/Charge#*
3-aminobezoic acid	AppliChem	9H000086
4',6-Diamidin-2-phenylindol (DAPI)	Carl Roth	
Albumin fraction V (BSA)	Merck	K42179118 128
D(+) Sucrose	Carl Roth	45791175
Di-sodium hydrogen phosphate dohydrate	Merck	K3742980 724
Methanol	Merck	J600109 134
Natriumcitrat Dihydrat Ph.Eur.	Klinik-Apotheke of the Univeritätsklinikum	1708E-01900
Paraformaldehyde (PFA)	ROTH	398 100038
Tissue Freezing Medium	Leica microsystems	036562286
Triton-X-100	Serva	100146
Tween 20	Serva	100541
Vectashield mounting medium	Vector	W0909
Taq-DNA-Polymerase	peqlab	091411-1
Primer	Eurofins MWG Operon	Z21636
dNTP-Mix	peqlab	102411
10x reaction buffer Y	peqlab	040511
Loading buffer x	applicham	1126535
Technovit 7100	Kulzer	010284
Technovit powder + liquid	Kulzer	012911
Härter II	Kulzer	010182
Ethanol	Normapur	11F300517
Agarose	Serva	110695
Osmiumtetroxid	electron Microscopy Sciences	111017
Permount mounting medium	Fisher chemicals	072617
Ethidium bromide	ROTH	321175500

Table 3: Primary Antbodies

Antibody	*Manufacturer*	*Lot#/Catalog#*	*Host Species*
Anti-Parvalbumin	Millipore	NG1827406	mouse
Anti-GABA	Sigma-Aldrich	121M4830	rabbit
PKC α (C-20) Antibody	santa cruz biotechnology	L0811	rabbit

Table 4: Secondary antibodies

Antibody	*Manufacturer*	*Lot#/Catalog#*
Alexa Fluor 488 Donkey Anti-Goat IgG Antibody	Invitrogen	830720
Alexa Fluor 488 Goat Anti-Mous IgG Antibody	Invitrogen	564513
Alexa Fluor 488 Goat Anti-Rabbit IgG Antibody	Invitrogen	424344

Table 5: Appliances

Appliance	*Type*	*Manufacturer*
12-Well plates	Tissue Culture Testplate 12	TPP Techno Plastic Products AG
Cryotome	HM 560	Microm
Microscope Slides	Superfrost Ultra Plus, 25x75x1 mm	Thermo Scientific
Polymax	Polymax 1040	Heidolph
Rotator	SB3	Stuart
Histoformblock	S	Kulzer
Microtom	RM 2255	Leica
Illuminator		Peqlab
Gelkammer	BlueMarine 200	Serva
Cycler	Mastercycler epgradient	Eppendorf
Power Source	250 V	VWR
normale slides	geschnitten mit Mattrand, 76 x 26 mm	engelbrecht

Table 6: Buffers and Solutions

4 % PFA (Paraformaldehyde)	20 g PFA dissolved in 500 ml PBS
E3-medium	5 mM NaCl, 0,17 mM KCl, 0.33 mM CaCl2, 0.33 mM Mg SO4, 0.01 % Methylene blue
MESAB	4 mg/ml amonibenzoate acid, 1 % Na2HPO4 *2 H2O
PBS	20 mM phosphate, 150 mM NaCl
PBST	PBST, 0.1 % Tween 20
PBS-Triton-X-100	PBS, 2,5% Triton-X-100
PBDT	PBS, 1 % BSA, 1 % DMSO, 2.5 % Triton-X-100

Pictures

Table 7: Microskope

Apotom	Equipment	Zeiss - Axio Observer, Inverted stand, manual XY stage, motorized z-stage, fluorescence, transmitted light with manual DIC (Senarmont), b/w epifluorescence acquisition, Optical Sectioning (Apotome)
	Illumination	Fluorescence (Metal Halide, HXP, 120W) Transmitted Light (Halogen)
	Reflectors	DAPI: EX 360/40; BS 400; EM 460/50 GFP: EX 470/40; BS 495; EM 525/50
	Software	AxioVision Rel. 4.8.1 release 11.2009

	Used features	sequential imaging, Brightfield and Fluorescence overlay, z-stack, optical sectioning
Stemi 2000	Equipment	Zeiss - Stemi 2000, Stativ N,

Image processing: Fiji (http://fiji.sc/wiki/index.php/Fiji)
Plugin: Cell counter

6 Methods

6.1 Husbandry and preparing of the Larvae

Larvae maintenance

Maximum 40 Larvae were kept in one petri dish at 28 °C in E3 medium until they were five days old.

Amputation of the caudal fins of larvae

Before I amputated the tail fin, I anesthetized the larvae to immobilize them. 40 µg/ml MESAB was added to the E3 medium. The anesthetized five-day-old larvae were put on a dry petri dish and a part of the tail fin was amputated with a razor blade. For accurate amputation, the stereomicroscope Stemi 2000™ [Zeiss] was used. The cut larvae were stored in fresh E3 medium where they recovered for two to four days at 28 °C. Figure 7 shows a larva of a zebrafish and the amputation plane.

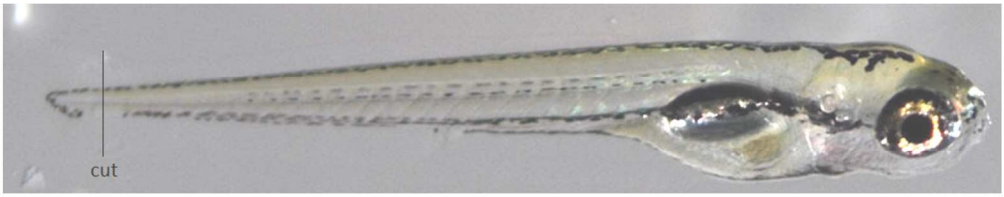


Figure 7: Lateral view of a 5 dpf zebrafish larva

Wild-type larva at 5 dpf. Black line shows the amputation plane.

Screening for mutant larvae

Since the mutation is recessive and the homozygous mutants die by 14 dpf, heterozygous parents have to be crossed to examine homozygous mutants. The progeny were then amputated to identify the mutants. 25 % of the progenies should show a mutant phenotype, 75 % should be wild-type. To recognize which larva is a mutant, they were anesthetized again at 2 dpa and sorted whether they show regeneration of the caudal fin (sibling) or not (mutant). Then they were stored separately in E3 medium at 28 °C.

Fixation und dehydration

Both mutants and wild-type siblings were fixed for further experiments at 2, 3, and 4 days post amputation. They were anesthetized in MESAB as described earlier and fixed with 4 % PFA/PBS in 12-well plates overnight at 4 °C on a polymax rocking table at 15 rpm. The next day the PFA was washed out five times in PBST for 15 min at room temperature (RT). To make the tissue more permeable and for storage they were then

dehydrated in graded series of methanol for 20 min each step on a rotating wheel (8 rpm) at RT as followed:

25 % methanol/PBST
50 % methanol/PBST
75 % methanol/PBST
3 x 100 % methanol

The dehydrated larvae were stored at -20 °C at least overnight.

6.2 Embedding in Tissue Freezing Medium and Sectioning

The larvae were rehydrated at RT by passing through:

75 % methanol/PBST, 10 min
50 % methanol/PBST, 10 min
25 % methanol/PBST, 10 min
3 x PBST, 10 min

To prepare the appropriate molarity for embedding they passed afterwards:

10 % sucrose/PBS, 30 min
20 % sucrose/PBS, 30 min
30 % sucrose/PBS, 30 min
1:1 30 % sucrose/Tissue freezing medium, 30 min
100 % Tissue freezing medium, 30 min

After embedding on dry ice, they were stored at -20 °C.
All steps were performed at a rotator at 8 rpm.

For the following immunohistochemistry, 20 µm sections of the retinas were made using a Cryotome. They were immediately transferred onto glass slides and used for further experiments.

6.3 Antibody staining on cryosections

Staining: Day 1

For the staining, mutants and their wild-type siblings were used to ensure the same genetic background. The sections were air-dried at 37 °C for 1 h. Then they were washed 5 times for 15 min at RT with PBS/2.5 % Triton-X-100 on a polymax (10 rpm) to permeabilize the tissue. 150 µl of primary antibody/PBS, 2.5 % Triton-X-100 in different dilutions (see table 8) were added and they were incubated overnight at 4 °C in a humid chamber, so that they do not dry out.

Table 8: Antibody dilutions

Primary antibody	Dilution	Stained cells
Anti Parvalbumin	1:500	parvalbuminergic amacrine cells
Anti GABA	1:750	GABAerig cells
Anti PKC	1:500	Bipolar cells
anit Tyrosine Hydroxylase	1:750	dopaminergic amacrine cells
Anti-Glutamin Synthetase	1:500	müller glial cells

Staining: Day 2/Day 3

The unbound primary antibody was washed out five times for 15 min at RT with PBS/ 2.5 % Triton-X-100. Following, the sections were incubated with the secondary antibody coupled with the fluorophore Alexa Fluor 488 diluted 1:500 in PBS/2.5 % Triton-X-100 at RT or overnight at 4 °C h in a dark humid chamber. The secondary antibody was washed out five or ten times for 15 min with PBS/2.5 % Triton-X-100. To stain the nuclei, DAPI was added with a concentration of 5 µg/ml in PBS/ 2.5 % Triton-X-100 for 15 min at RT in a dark humid chamber. After washing twice, a post staining fixation was performed. The tissue was fixed using 150 µl of 4 % PFA for 20 min and then the slides were washed again for three times with PBS/2.5 % Triton-X-100. Finally, the slides were overlaid with Vectashield mounting medium and covered with a coverslip, which was fixed to the slide using nailpolish.

6.4 Embedding in Technovit 7100

The larvae were incubated in Karnovsky´s fixative at 4 °C at least overnight. Then the fixation solution was washed out with PBS at RT, rinsed onece, then washed once for 5 min, then for 10 min, and then for 15 min. Then a postfixation in 1 % OsO4/PBS was performed on ice for 2 hours. After washing with PBS, 1x rinse, 2x 5min, the larvae were embedded in 2 % Agarose/H2O. Small pieces were cut around the larvae and were transferred into small glass vials. Then they were dehydrated by passing graded series of ethanol at RT:
30% ethanol/H2O, 15 min
50% ethanol/H2O, 15 min
70% ethanol/H2O, 15 min
90% ethanol/H2O, 15 min
96% ethanol/H2O, 15 min
2x 100% ethanol/H2O, 15 min
The samples were light protected and then infiltrated in Technovit 7100 by passing:
1:1 Technovit/100 % ethanol, 1h at RT
3:1 Technovit/ 100 % ethanol, 1 h at RT
Pure Technovit 7100, 1h at RT

Pure Technovit 7100 overnight

Technovit was warmed to RT. The Histoform was cleared by blowing or rinsing out. The block was placed under a stereoscope. 2 ml Technovit (including hardener I) were transferred to a 15 ml falcon and 133 µl hardener II were added. It was mixed by pipetting up and down before the mixture was filled in the molds of the histoblock. The samples were transferred into the molds and orientated via a toothpick. The block hardened at least fore 1 h before the histoblocks were mounted. Therefore the holders (Fischerschelle) were laid onto the polymerized histoblocks. 9ml of Technovit 3040 powder were mixed with 3 ml Technovit 3040-fluid in a petridish. The mixture was transferred with a pasteur pipette with shorted end into the histoblock and the holders were filled. It polymerized at RT over night before the block was levered out of the histoform. The samples were stored at 4 °C. 2 µm sections were prepared of the samples with a microtome. Every 5th section was transferred to a waterbath and put on a slide. The slides were dried on a hot plate at 37 °C and then stored at 4 °C.
For the staining, the samples where incubated in tolluidine blue for 6 minutes and then washed under running water until the Technovit becomes clear.
The dry slides were coverslipped with Permount mounting medium.

6.5 Determination of the genotype of 5 dpf larvae

Extraction of the genomic DNA

For the extraction of the genomic DNA the larvae were dehydrated in graded series of methanol (25 %, 50 %, 75 %, 100 % methanol/PBS) and then stored in 100 % methanol at -20°C at least one day. Each larva was divided into head and the rest of the body. The two parts were then transferred separately into 96 well-plates so that each head can be assigned to its counterpart. The heads were stored in 100% MeoH until sectioning whilst DNA was extracted from the trunks. Therefore, as much methanol as possible was removed. Residual methanol was evaporated at 70 ° C in an open PCR machine until the trunks were completely dry. Then, 25 µl of the digestion mix (2.25 ml TW buffer pH 8 with 1 mM EDTA+ 250 µl proteinase K [17mg/ml]) were added to each well. The tissue was then digested by incubating at 55 °C for 4 hours. Afterwards, the Proteinase K was degraded by incubating at 95 °C for 15 min. The samples were then stored at 8 °C until the next day.

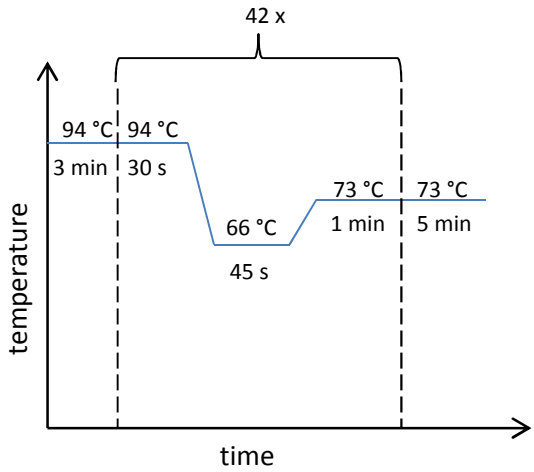
PCR reaction:

The genomic DNA was diluted 1:10 with H_2O and then the PCR reaction was prepared as followed:

2 µl Taq Buffer (10x)
0.4 µl 10 mM dNTP-mix
0.4 µl forward primer (50 pmol/µl)
0.4 µl reverse primer (50 pmol/µl)
0.4 µl Taq Polymerase

4 µl 5x PCR load
5 µl DNA (1:10 diluted)
7.4 µl water

This was mixed thoroughly and then placed into a PCR cycler. The appropriate



Genomic region was amplified as followed:
Primer sequences:
z21636 forward: CTCGCTCACATGAGGACTGA
z21636 reverse: AGTCTGATTGTGGCCGAGAG

Gelelectrophoresis:

The amplified PCR product was separated by gel electrophoresis. Therefore a 2 % TAE gel containing 1 x ethidium bromide was prepared and the total volumes of the PCR reactions were loaded on the gel. The electrophoresis was run at 140 V, 3 A 50 W for 30 min.
Then the gel was illuminated 312 nm to visualize and image the DNA-bands.

6.6 Imaging and cell counting

Z-stacks with a distance of 1 µm and a mosaic of two to six pictures were recorded with the black and white camera of the apotome microscope and the AxioVision software. The single mosaic pictures were merged to the final picture. DAPI was acquired with DAPI channel and other antibodies with the GFP channel.
Maximum intensity projections were generated with the Fiji software and the pictures were colored. For the examination of the eye degeneration the cells in the single layers containing the optic nerve were counted with the help of the cell counter plugin. For the brain degeneration every second section over the eye was used and the parvalbumin staining was analyzed. One section on the same level in siblings and mutants with most of the parvalbuminergic cells was used for counting.

7 Results

A complete loss of parvalbuminergic amacrine cells at later stages of development (9 dpf) was the most prominent phenotype I saw during my previous characterization of the mutant phenotype. To see at which time point the loss of parvalbuminergic cells starts and to investigate whether mutants develop the same amount of parvalbumin positive cells as wild-type siblings, a time course starting at earlier stages of development was performed.

7.1 Genetic distinction between siblings and mutants

In order to identify homozygous mutants at a developmental stage where they don´t show a phenotype, linkage analysis was performed. Thereby, recombination events were analyzed within each individual animal. One makes use of the so called simple sequence length polymorphisms (SSLPs). These are highly polymorphic regions containing CA repeats of different lengths which are distributed over the whole genome. These SSLPs differ between the different zebrafish strains that are used for this approach: the WIK (Wild-Type India Kalkutta) and the Tü (Tübingen) strain. SSLPs can be easily detected via PCR using primers flanking those regions. If a mutation is localized close to a certain SSLP on a chromosome, it will segregate together with this marker during meiosis. In order to perform linkage analysis parents being heterozygous for the mutation were crossed to the highly polymorphic WIK line. (see fig. 8). In the resulting F1 generation, 50% of the animals are heterozygous for the mutation. These heterozygous F1 carriers are then incrossed, which gives rise to an F2 generation with 75% phenotypically wild-type siblings and 25% mutants showing a phenotype. It was already shown by Judith Konantz that the mutation is linked to a certain marker (z21636) on chromosome 4. This means that this chromosomal region always segregates together with the mutation during meiosis and is not separated by recombination events. However, if a certain marker is not linked to a certain allele on a chromosome we observe recombination events, which is what we see in wild-type siblings. By performing gel electrophoresis using primers flanking the z21636 locus, it is now possible to distinguish between mutants and siblings by genotyping the animals. Mutants show one single band of a certain size which corresponds to the Tü allele. Wild-type siblings show either one band, the WIK allele, which differs in length from the Tü allele, or a double band for animals that are heterozygous for WIK and Tü.
Figure 8B shows the different band patterns of mutant and wild-type larvae. Mutants are homozygous for the Tü allele and have a bigger fragment (see fig. 8). Wild-type siblings are either homozygous for WIK or heterozygous for WIK and Tü. The WIK allele is shorter than the Tübingen allele as shown in fig 8. [Nüsslein, Dahm, 2002].

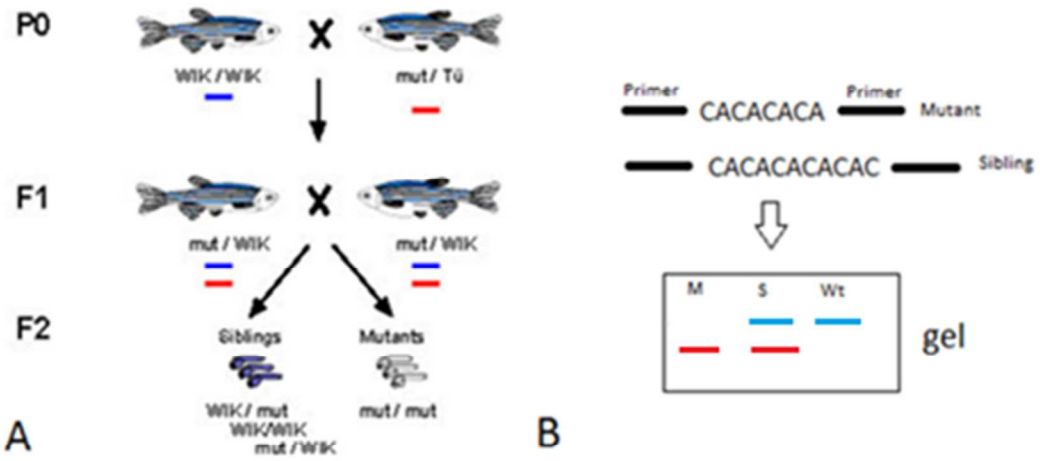


Figure 8: Genetic distinction between mutants and siblings [Nüsslein, Dahm, 2002]

A: Wild type (wik/wik) zebrafishs were cross with mutants (mut/tü) and so a heterozygous (mut/wik) f1 generated. If the f1 generation is cross again, 75 % siblings without phenotype and 25 % mutants with phenotype are generated.

B: Single fish PCRs for the scheme shown in fig .A. If a marker is linked to a mutation it will segregate together with the Tü allele (red band), whereas the wt-siblings will either show the WIK allele (blue band) or are heterozygous for the Tü and WIK allele (red and blue).

So as to investigate how the genotype of mutants and siblings looks like, larvae with known identity were tested first. To know on which height the different bands of siblings and mutants lie, I performed PCR and gel electrophoresis on larvae with known identity. Therefore, larvae were amputated to identify siblings and mutants and genomic DNA was extracted from each animal as described. After performing PCR for the z21636 locus the PCR products were separated and visualized via elelectrophoresis (see fig. 9). Mutants show only one band since they are homozygous for the Tü allele (see fig.9 left side). Siblings are either heterozygous for the Tü and Wik allele as shown by the occurrence of double bands, or homozygous for the wild-type Wik allele, which is smaller than the Tü allele (see fig. 9 right side). With this results it is possible to distinguish between mutant and wild-type larvae at a developmental stage where they don´t show a phenotype after amputation. Using this approach, the genetic distinction can be performed at earlier stages.

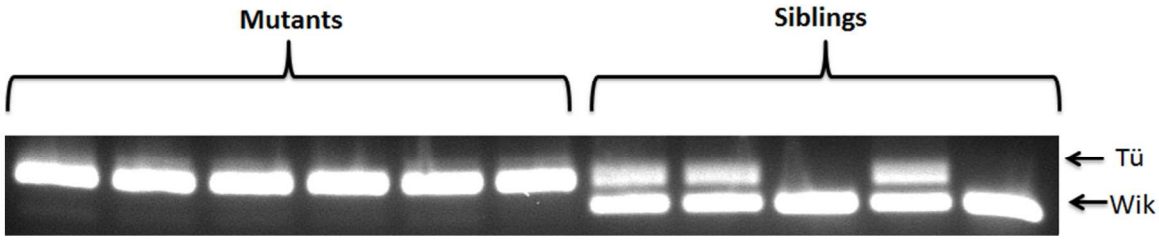


Figure 9: Genotyping with larvae with known identity

The left part of the images shows the genotypes of 6 different mutants which are homozygous for the Tü allele of the z21636 locus. On the left the genotype of wild-type siblings are shown. They are either heterozygous for the Tü and WIK locus (see double bands) or homozygous for the WIK locus.

After that the 5 dpf larvae were tested for their genotype. Altogether 48 larvae at were tested. Figure 9 shows an example of the different genotypes observed. All animals showing only the upper Tübingen band are referred to as mutants, whereas the others are wild-type siblings. With this method I could identify 6 larvae with a mutant genotype, which were then used for further analyses

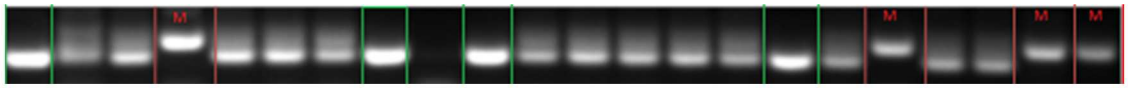


Figure 10: Single fish PCRs for the z21636 locus on larvae with unknown identity

Shown are 22 individual animals genotyped for the z21636 locus. Animals showing only the upper (Tü) band are mutants (red marked), all others are either heterozygous (double band) or homozygous (greed marked) wild-type siblings (lower band).

7.2 Characterization of the retina phenotype in *knörf* mutants

The results of the pax 6 staining revealed that there are less amacrine cells in mutants compared to wild-types. The parvalbuminergic (PV) amacrine cells were identified to be affected. In siblings, the parvalbumin positive cells in the IPL are clearly visible, whereas there are either very few or no parvalbuminergic cells in mutants at later stages of development. Because parvalbuminergic cells get lost, a time course of 5 dpf and then from 2 dpa to 4 dpa larvae was performed to identify at which time point the degeneration starts. Fig. 11 shows representative examples of mutant and wild-type retinas stained for parvalbumin at 2-4 dpa indicating gradual reduction of PV cells in mutants. Statistical analyses (see chart 3) of the number of parvalbuminergic cells showed that at 5 dpf the number of parvalbuminergic cells in mutants is the same as in wild-types. However, whereas we observe an increasing number of parvalbuminergic cells in siblings (starting from around 19 cells per section at 2 dpa to on average 23 cells at 4 dpa) mutants loose these cells over time with around 17 cells at 2 dpa to on average 6 cells at 4dpa (see chart 3). These results suggest that the mutants are able to

form the same amount of parvalbuminergic amacrine cells as wild-types, but then these cells disappear over time.
To examine if more amacrine cell types are affected by the mutation, different other stainings were performed.
Figure 12 shows the GABAeric positive cells. Counting of them also resulted in no significant difference between siblings with 38 and mutants with 35 positive cells.
The tyrosine hydroxylase is an enzyme which catalysis the conversion of tyrosine to the amino acid levodopa. The counting of these cells also displayed no difference. Chart 4 shows the results of the counting of single stacks in 4 dpa siblings and mutants. Siblings have on average 5 and mutants 4 tyrosine hydroxylase positive cells. Figure 12 shows that the single cells are clearly visible in the different larvae.

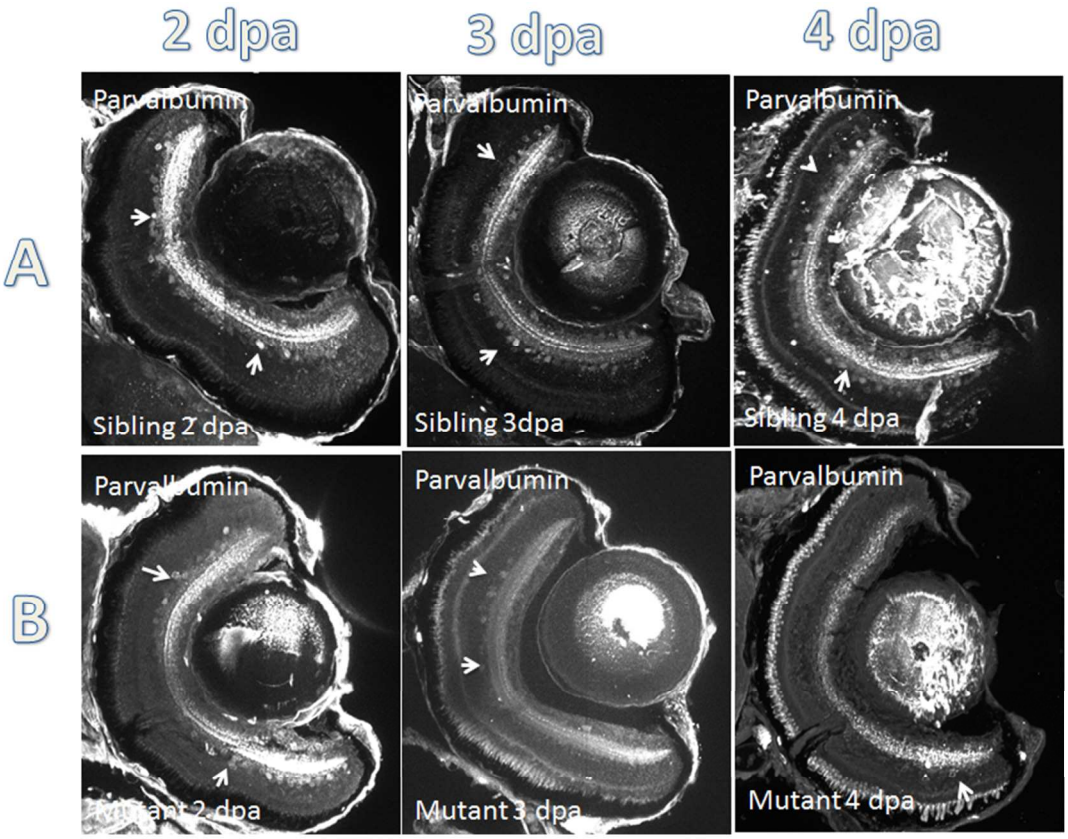
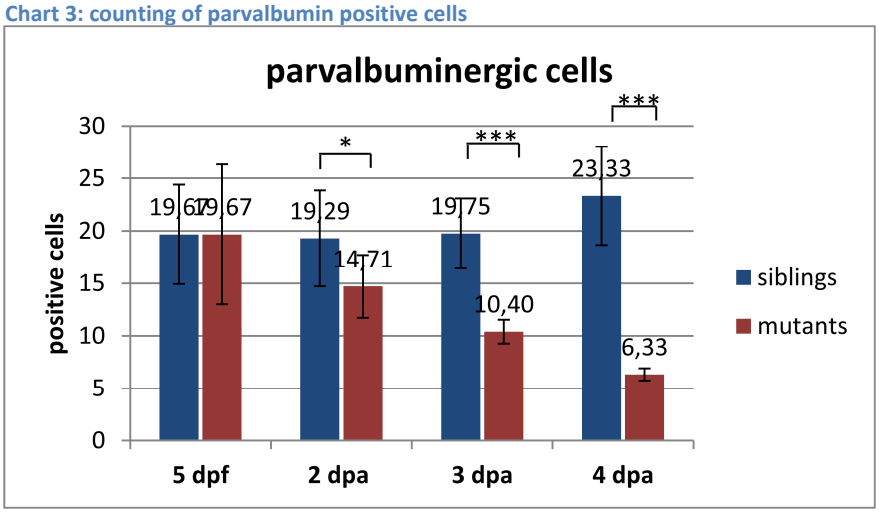


Figure 11: Time course of parvalbumin cells

The figure shows parvalbuminergic positive cells in siblings (A) and mutants (B). No clearly visible difference between mutants and wild-types at 2dpa. At 3dpa, one can surmise that there are less stained cells in mutants.at 4dpa nearly all parvalbumin stained cells in mutants are lost.

Chart 3: counting of parvalbumin positive cells



Counted sectionts: 5

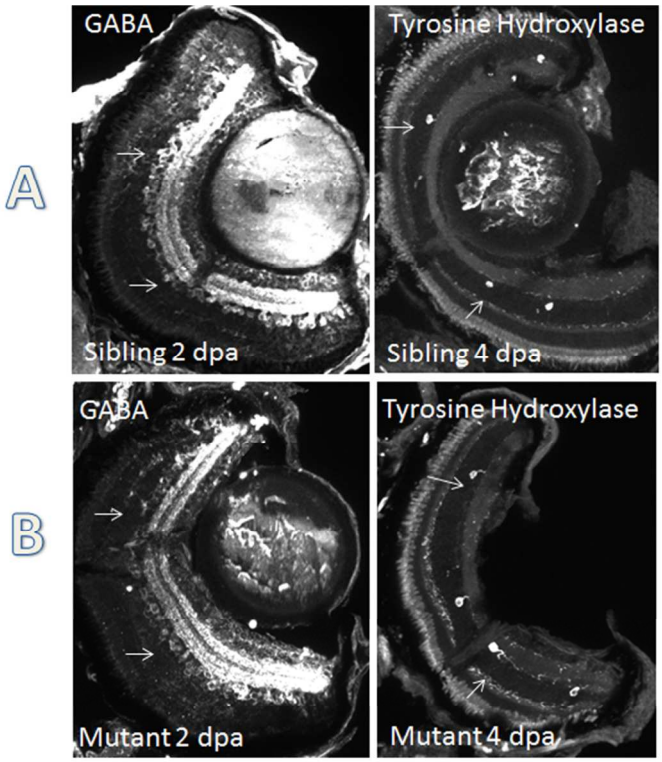
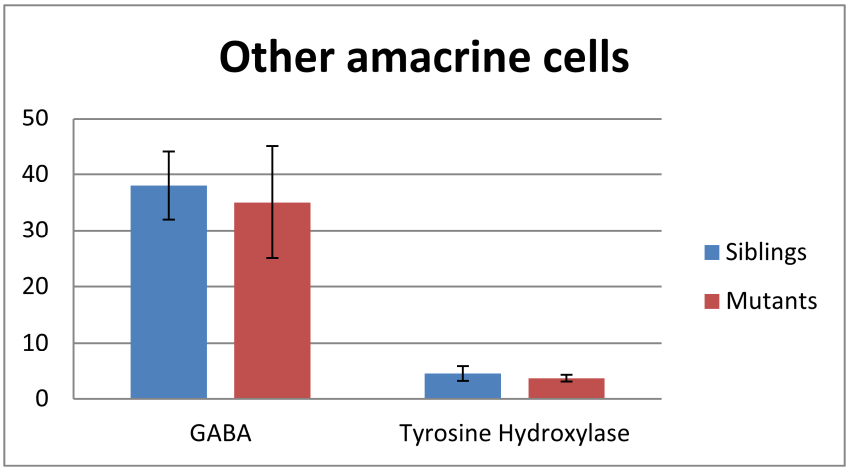


Figure 12: Other amacrine cell types
Visible are the GABAeric and the tyrosine hydroxylase positive cells in the INL. No difference is visible between siblings (A) and mutants (B).

Chart 4: counting of other amacrine cell types



Counted sections: 3

Other cells that localize to the INL of the retina are Müller Glia and Bipolar cells. Since the degeneration phenotype is present within this area, I tested if these two cell types are affected or not. Therefore, I analysed sections of 2dpa larvae. This early time point was chosen to see if, and when, which cell types disappear first. Figure 13 shows the maximum projection of 20 µm sections of sibling and mutant retinas. Bipolar cells were detected using an antibody against proteinkinase C alpha (PKC) as shown on the left

side of fig. 13. A great number of positive cells are visible in the INL of mutants and wild-types indicating no difference in the total number of bipolar cells. This was confirmed by statistical analyses showing that both mutants and wild-type retinas contain on average 91 cells per section (see chart 5). At 4 dpa this number increases to 121.75 in siblings and 101.5 in mutants.
Fig.13 (right panels) displays exemplary sections of siblings and mutants stained for Glutamin-Synthetase, a marker for Müller glia cells, whose cells bodies are also located in the IPL. Again, counting of the cells didn´t shows a difference between siblings with 35 and mutants with 38 cells on average (see chart 5).

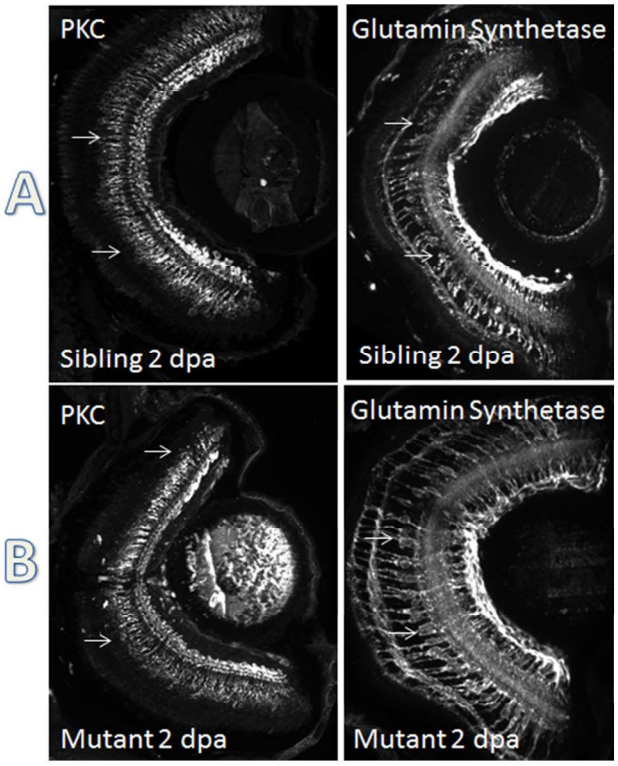
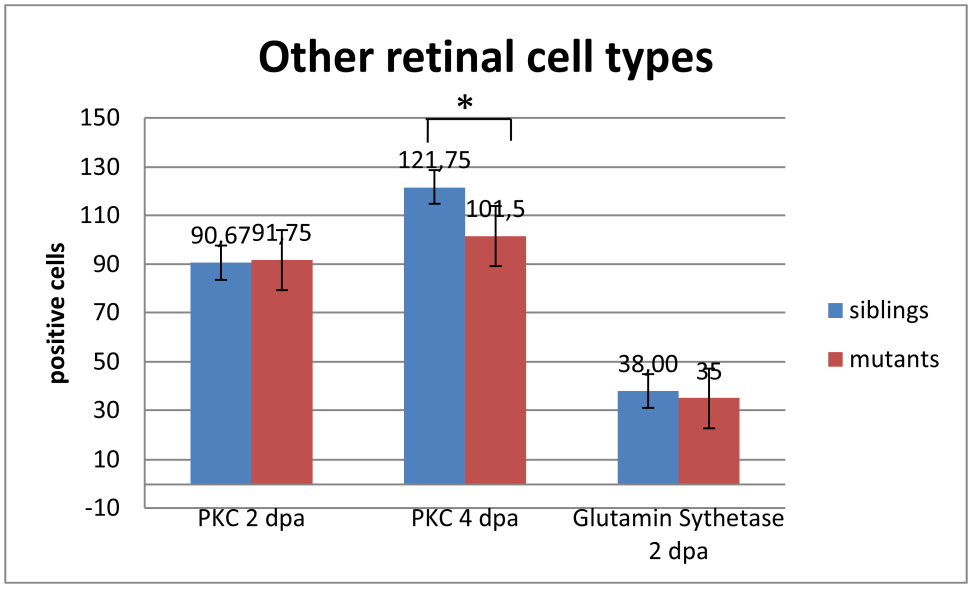


Figure 13: Bipolar (PKC) and Müller Glia (Glutamin Synthetase) staining of 2dpa mutant and wild-type retina sections.
(A) Siblings, (B) mutants. Left panels: a-PKC; right panels: a-Glutamin Synthetase

Chart 5: counting of Bipolar (PKC) and Müller glia (Glutamin Synthetase) cells



Counted sections: 4

7.3 Brain

To check if other parts of the central nervous system are affected parvalbumin staining in the brain was performed. Parvalbumin is also expressed in the zebrafish telencephalon. For that, every second section of the head at the level of the telencephalon was examined [Müller et all, 2011]. Figure 14 shows the location of the telencephalon in the larval head. At 2 dpa, the analyzed section of the brain of siblings contains on average 23 parvalbuminergic cells whereas the mutants only contain 3 cells (see chart 6 and fig. 15). This result indicates that the mutation not only affects the retina but also parts of the brain, especially parvalbuminergic cells.

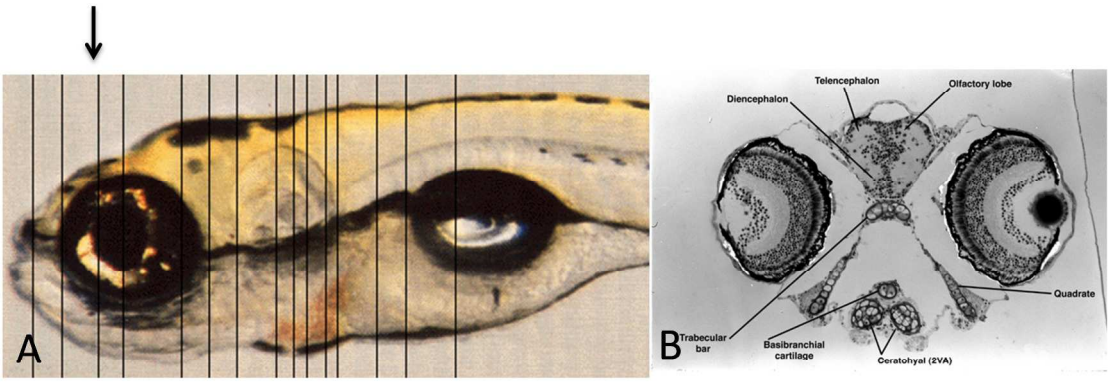


Figure 14: Location of the telencephalon

A: lateral view of a zebrafish larva. The location of the telencephalon is marked by the arrow. B: Section of a zebrafish head, which shows its different parts and the location of the telencephalon.

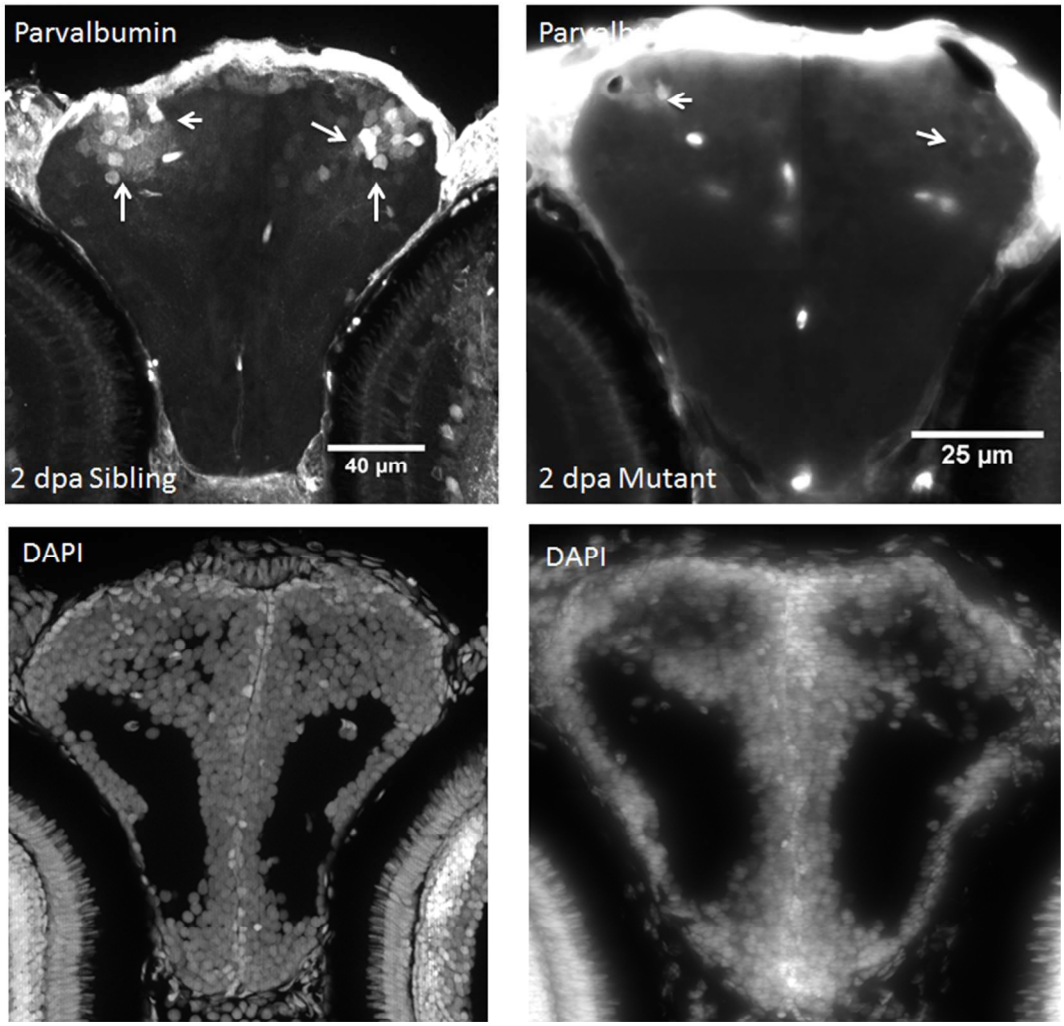
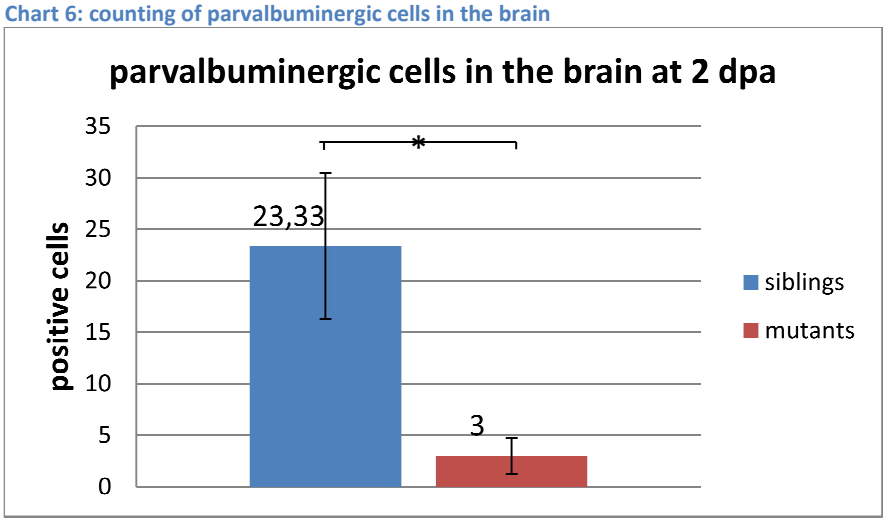


Figure 15: Parvalbumin staining in the brain

The parvalbumin staining at 2 dpa larvae shows significant differences between siblings (left picture) and mutants (right picture). Parvalbumin positive cells are nicely localized within the telencephalon of siblings (arrows) whereas these cells are nearly absent in mutants. DAPI staining shows that the sections are at the same level of the head.

Chart 6: counting of parvalbuminergic cells in the brain



Counted sections: 4

7.4 Muscle

As it was shown before, the mutants also display degeneration of skeletal muscle. For the purpose of further analyzing the phenotype, I performed additional histological sections. However, due to upcoming difficulties with this method and lack of time I further analyzed the sections which were prepared already by my supervisor Judith Konantz.

Figure 16 shows coronal sections of sibling (fig. 16 A) and mutants (fig. 16 B) at 4 dpa. Wild-type trunk muscles are densely packed whereas mutant muscles seem to detach from one another and show distinct fissures. Apparently, the degeneration of mutant muscle is stronger in the distal parts of the trunk than more proximal.

Parvalbumin is a protein which is also present in skeletal muscle [Berquin, Lebacq, 1992]. Therefore the muscles of the tail also were examined. The staining on sections of the trunk muscle shows that the protein localizes to the skeletal muscle in wild-types at 4 dpa, while there seems to be less protein in the mutants (see fig. 16).

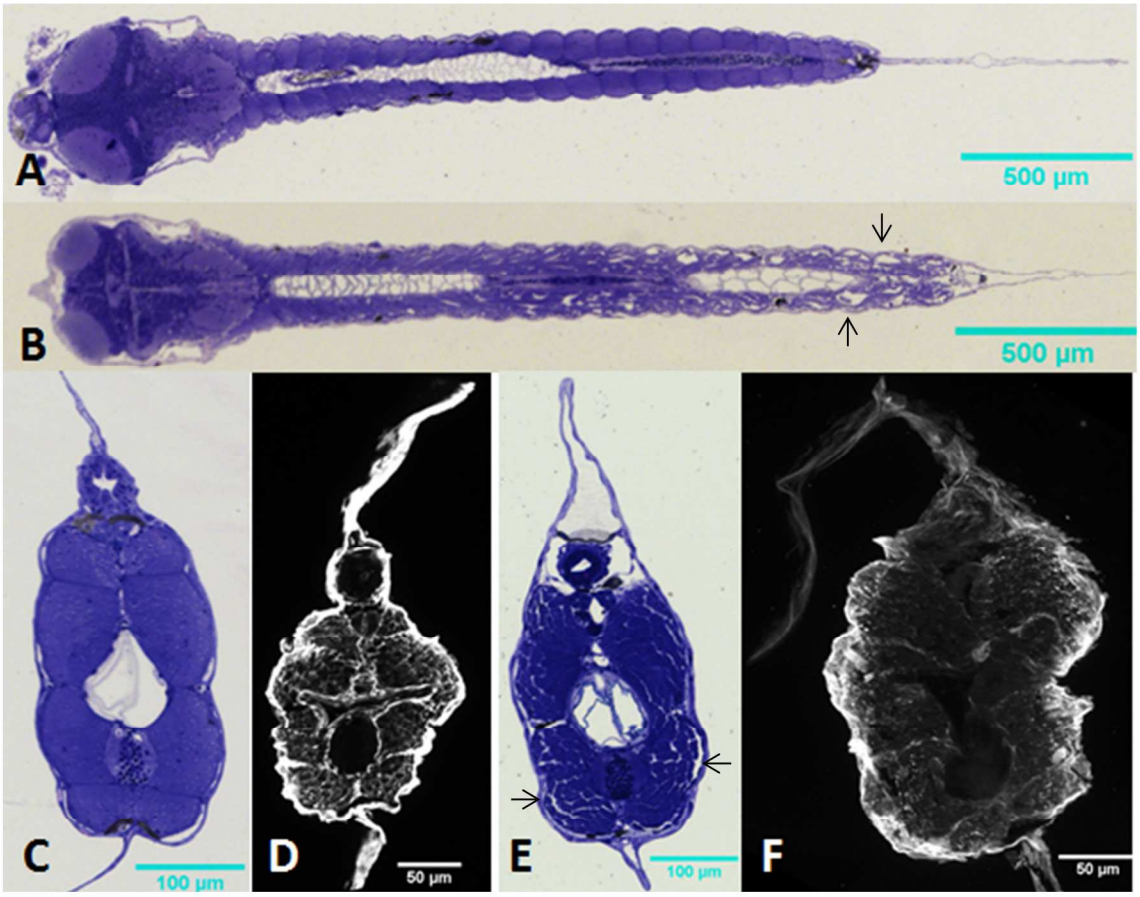


Figure 16: Degeneration of the muscle

A, B, C, E: 2 µm histological sections of the larval zebrafish muscle (D,F) Parvalbumin staining on 20 µm sections. (A-B) The dorsal view of larvae shows significant differences of the muscles in siblings (A) and mutants (B). Siblings have a continuous staining and mutants show distinct fissures in the muscles. (C; E) Cross sections show nicely arranged muscle fascicles in siblings (C) whilst mutant muscles (E) show gaps within the trunk muscle (arrows) , Parvalbumin staining on cross sections of siblings (D) and mutants (E) indicates reduced levels of the protein in mutants.

8 Discussion

Knörf is a zebrafish mutant, which has different degeneration phenotypes. The occurrence of gaps in the IPL and INL of the retina is one of them. My studies tested which cell types are affected. Rods and cones, bipolar, horizontal, amacrine cells and the ganglion cells are the main classes in the retina. Among those, cell bodies of amacrine cells, bipolar and Müller glia cells are localized in the IPL, and therefore potentially affected.
The location of the degenerating cells suggests that it´s most likely amacrine cells that are affected by the mutation. Different antibodies for the distinct amacrine cells were tested to identify the particular identity of the degenerating cells.

8.1 Characterization of the retina phenotype in *knörf* mutants

In my previous studies I counted all amacrine cells in siblings and mutants to identify differences in the number of cells. By using this approach it can be decided if the amacrine cells are affected in the mutants, which might explain the gaps in the mutant retinae.
The analysis shows that the siblings have on average 48.8 cells and the mutants 34.2 amacrine cells per counted sections. So the mutants have 30 % less amacrine cells than the siblings. This result confirms our thesis that one type of cells that disappear in the mutants are amacrine cells and lead to the gap phenotype we see on the histological sections. So as to further specify the type of amacrine cells that is affected, I tested for different markers such as parvalbumin, GABA, Tyrosine Hydroxylase and Serotonin. In the end, parvalbuminergic cells were identified to be affected.
The time course shows an increase of parvalbuminergic cells in siblings and a decreasing number of cells in mutants. Nevertheless, mutants are able to develop the same number of parvalbuminergic amacrine cells as wild-types, since this number is the same at day 5 of development, indicating that the mutant retina is fully developed at this stage and then it starts to degenerate. There are different possible reasons why the mutants develop normally until 5 dpf and then start to degenerate. Maybe the responsible gene is expressed at a later point of time and when it is then required, it is not functional due to the mutation. A second possibility is that the genetic material is provided maternally. When this RNA is depleted, only the mutated variation if the gene is present. So the needed protein can´t be produced and the cells start to degenerate.
After 5 dpf there is a clear decrease of parvalbuminergic cells in the mutants. This result suggests that the reason for the phenotype might not only be a defect in cell proliferation. If so, the number of mutant cells should stay nearly constant over time, while the number of cells in wild-types increases during development. But because mutant cells disappear over time, the cells also seem to die by any affect.
Parvalbumin positive amacrine cells are important components of the scotopic or rod visual pathway, and they interconnect rod bipolar cells with cone bipolar cells and ganglion cells. If they are lost in the retina, important connections do not exist and the

ability to see is impaired. Also the lateral signal transmission along the horizontal and amacrine cells is disturbed; whereby different signals maybe can´t reach the brain. If the rod visual pathway is interrupted they can´t see in dim light, which exacerbates orientation in the dark.
A restricted ability of vision can impair the survival of the animal and can be one possibility for the death of the animals after 2 weeks.

Other tested amacrine cell types are the GABAerig cells and cells positive for tyrosine hydroxylase. Siblings have 38 and mutants 35 GABAerig cells at 2 dpa, which is not significant different and also the tyrosine hydroxylase staining showed no difference with approximately 5 cells at 4 dpa. This means, that these cell types are not affected by the mutation.
Other retinal cell types like bipolar cells and Müller glia cells were tested if they are also affected. There are around 90 bipolar cells in 2 dpa larvae of mutants and siblings, but at later stages (4 dpa) siblings have around 121 and mutants 101 cells on average. Also the staining for Müller glia cells, which were tested with the Anti-glutamin synthetase antibody, showed no difference between siblings with 35 and mutants with 38 cells. In conclusion of these results, these cells types are not primarily affected by the mutation. The decreasing of cells by 4 dpa could be explained as a secondary effect caused by the loss of amacrine cell connections to the other cell types. Without the neuronal input of amacrine cells, bipolar and Müller glia cells might degenerate.

8.2 Brain

Since the retina is part of the central nervous system, we tested if cells of the brain are also affected in mutants with special focus on parvalbuminergic cells, since these are the cells that are lost in the retina. Parvalbumin is for example expressed in the zebrafish telencephalon, and analyses of siblings and mutants shows that the protein levels in the mutant telencephalic region are highly reduced. This means that because of the loss of these cells the function of the telencephalon could be impaired.
The olfactory sense is considered to be the main function of this area. For a larva which grows under laboratory conditions this would not hinder to surviving but it could involve a change of behavior. Studies on goldfish with impaired telencephalic function showed, that this has effects on the schooling, aggressivity and reproductive behavior of the fish. It is also known that forebrain less fishes are more indolence than others. This is maybe attributed to a decrease in the ability to process information [Donald, Bernstein, 1969]. So maybe the impairment of the telencephalon in the larval mutant *knörf* also leads to a decreased information processing and therefore to a reduced movement.
Another point which also affects the behavior is the short-term memory. It was shown that an ablation of the telencephalon leads to a degradation of the short-term memory function, which is necessary for the memory retention of stimuli [Ohnishi, 1997].
Taken together, there are a lot of possible changes in the brain which could impair the larval association, the memory and the behavior.
But why do the parvalbuminergic cells in the brain disappear earlier than in the retina? One possible reason could be the duration of the impact of the mutated gene. The

neurogenesis of the brain starts during late gastrulation, which is at approximately 10 hpf [Chapouton, Bally-Cuif , 2004]. In the retina it starts noticeably later at 36 hpf [Avanesov, Malicki , 2004]. So the cells in the brain are much longer susceptible to the effect of the gene. Therefore they start to disappear earlier than the cells in the eye.

8.3 Muscle

Mutants show a significant degeneration of skeletal muscle, which gets stronger in distal areas. The parvalbumin staining resulted in a significant difference between siblings and mutants. Siblings have positive cells over the whole tail, in mutants these cells disappeared nearly completely.
Parvalbumin is known as a calcium binding protein and as a regulator of muscular contraction. It is mainly located in fast skeletal muscles, like in the tail muscle of the zebrafish larva, in which the requirement of calcium binding proteins is much higher. In resting muscle, it is mainly saturated with magnesium, but when calcium concentration rises, magnesium is exchanged for calcium [Berquin, Lebacq, 1991].

In skeletal muscle cells, calcium binds to troponin C, which starts the contraction cycle by moving the tropomyosin on actin and exposing the actin binding sites. This process endures as long as calcium is present. After a contraction the calcium is sequestered and binds to proteins like parvalbumin. Then the tropomyosin returns to cover the actin binding sites and the muscle relaxes [Wareham, 2011].
Without parvalbumin there is still free calcium, so that the contraction cycle possibly never ends. That would lead to an overload of the muscle, which could finally end in a slow destruction of the muscle.
Distal areas of the mutants show a significant higher degeneration of muscle. During swimming, these distal trunk parts of the animals are moving and bending stronger than the proximal ones. Those muscles have to contract and relax much faster and more often than the more proximal muscles. The loss of parvalbumin would then lead to an overload of the distal muscles more quickly than of the proximal ones, which could result in the faster loss of distal muscles.

9 Future investigations

The zebrafish mutant *knörf* has got a degeneration phenotype, which shows an occurrence of gaps in the retina. I showed that these gaps occur due to less amacrine cells, namely parvalbuminergic amacrine cells. With the PKC staining I could show, that the number of bipolar cells decreases at later stages. Therefore the different other antibodies should be tested at 4 dpa or later to test whether these cells are also degenerating. This effect seems to be rather indirect than being a primary phenotype of the mutants since it occurs much later than the parvalbumin phenotype. However, it would be interesting to examine why these cells are degenerating.
To make sure that these cell types are secondarily affected, a double staining of the parvalbumin and the different other retinal antibodies could be performed. This would show if the axons of the parvalbuminergic cell are connected to the different types of cells in the IPL. If so, the loss of this connection would be a reason why these cells are degenerating at later stages of development.
The parvalbuminergic cells in the brain disappear earlier than those of the retina. Already at 2 dpa most of these cells are gone in the mutants. Next point would be to check much younger stages to find out if there is a time point at which siblings and mutants have the same number of cells. This would show that it needs some time before the cells get affected by the mutation.
To further characterize the mutant phenotypes, it would be interesting to know how the loss of the parvalbuminergic cells in the retina influence the vision of the animals. Parvalbuminergic cells of the retina are important for the movement perception therefore one experiment could be to test the optokinetic response [Maurer et all, 2010]. Here the larvae are immobilized and then a black and white striped paper drum circulates around the animals. The movement of the eyes is then monitored to investigate whether they can follow the stripe pattern. If not, the movement perception is impaired in the animals which will show that parvalbuminergic amacrine cells are indeed required for the zebrafish movement perception.

10 Summary

The larval zebrafish mutant *knörf* loses the ability to regenerate their caudal fin. No regenerative outgrowth of the finfold takes place indicating that they fail to initiate regeneration.
Besides, the mutation causes various degeneration phenotypes. This was discovered in the retina and skeletal muscle by histological analyses. Wild-type retinas at 7-9 dpf display nicely organized neuronal layers but mutant retinas show gaps in the INL and IPL at 7 and 8dpf. The number of gaps increases during the maturation of the larva.
I showed by pax 6 staining that amacrine cells are affected and that specific cell type of amacrine cells which are positive for parvalbumin disappear.
To examine the loss of parvalbuminergic cells in more detail the staining was performed in a time course. At 5 dpf, there is no difference between the number of parvalbuminergic amacrine cells in siblings and mutants (19 cells on average). At 2 dpa mutant retinas contain significantly fewer cells than wild-types. The number further decreases until they are nearly completely gone at 9dpf. This could lead to an impairment of the vision ability. Then the brain as another central nervous system structure was examined. In the telencephalon these cells disappear already at 2 dpa with a significant difference between siblings with 23 and mutants with 3 cells on average. Probably, this impairs the olfactory sense of the larvae which leads to a decreased perception of their environment. It could also affect the information processing and the short-term memory, which can cause changes in the behavior.
Parvalbumin is also present in the muscle. Muscle degeneration of mutants starts at 7dpf showing the strongest phenotype in the distal trunk muscles, more proximal areas are less affected.
The role of parvalbumin is mainly the binding of calcium and therefore it supports the adjustment of the resting potential after an excitation in the central nervous system. In muscles, it assists in enhancing relaxation after a contraction of a muscle. Taken together, parvalbumin is a protein which is located in many areas of the larval body. A loss of this protein can cause distinct degeneration phenotypes.

11 References

Akimenko M., Marı´-Beffa M., Becerra J. Ge´raudie J. (2003): Old Questions, New Tools, and Some Answers to the Mystery of Fin Regeneration. In: *DEVELOPMENTAL DYNAMICS* (226), S. 190–201.

Avanesov, A. Malicki J. (2004): Approaches to Study Neurogenesis in the Zebrafish Retina. In: *METHODS IN CELL BIOLOGY* (76),

BERQUIN A., LEBACQ J.: PARVALBUMIN, LABILE HEAT AND SLOWING OF RELAXATION IN MOUSE SOLEUS AND EXTENSOR DIGITORUM LONGUS MUSCLES. In: *Journal of Physiology* 1992 (445), S. 601–616,

Chapouton P., Bally-Cuif L. (2004): Neurogenesis. In: *METHODS IN CELL BIOLOGY*, 2004 (76), S. 136–206.

Dinort J. (2008): Zebrafisch als Modellorganismus. Injektion von fluoreszierenden Proteinen in das Blutgefässsystem von Zebrafischembryonen. Maturaarbeit. Gymnasium Bäumlihof, Basel.

Donald A., Bernstein (1969): Role of the Telencephalon in Performance of Conditioned Avoidance Responses by Goldfish. In: *EXPERIMENTAL NEUROLOGY* (23), S. 445–456,

Eysel, U. (2007): Signalaufnahme und Verarbeitung in der Netzhaut. In: *Ophthalmologe* (104), S. 79–92,

Fethob, J. R.: Zebrafish as a Model System for Studying Neuronal Circuits and Behaviora,

Kawakami A., Fukazawa T., Takeda H. (2004): Early Fin Primordia of Zebrafish Larvae Regenerate by a Similar Growth Control Mechanism With Adult Regeneration. In: *DEVELOPMENTAL DYNAMICS* (231), S. 693–699.

Konantz, J. (2012): unpublished data.

Larsena B. D., Rampallia S. Burnsa L. E. Brunettea S. (2009): Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. In: *PNAS* (9), S. 4230–4235,

Masland, R. H. (2001): The fundamental plan of the retina. In: nature neuroscience (9),

Maurer C., Schönthaler H., Mueller K., Neuhauss S. (2010): Distinct Retinal Deficits in a zebrafish pyruvate dehydrogenase-deficient mutant. In: The journal of Neuroscince (30). S. 11962-11972

Mueller T., Donga Z., Berberoglua M., Guoa S., (2011): The Dorsal Pallium in Zebrafish, Danio rerio (Cyprinidae, Teleostei), In: Brain Res. March 24; 1381: S: 95–105

Nüsslein, Dahm (2002): Zebrafish. A Practical Approach. Oxford: Oxford universal press.

Ohnishi, K. (1997): Effects of telencephalic ablation on short-term memory and attention in goldfish. In: *Behavioural Brain Research* (86), S. 191–199

Poss K. D., Keating M. T., Nechiporuk A. (2003): Tales of Regeneration in Zebrafish. In: *DEVELOPMENTAL DYNAMICS* (226), S. 202–210.

Süßmann C. (2007): Abnahme displatzierter Amakrinzellen in der zentralen Netzhaut von Fischen während des Wachstums. Universität Tübingen,

Wareham, A. C. (2011): Muscle. In: *Anaesthesia & Intensive Care Medicine* (12), S. 249–252,

Yamashita, M. (2008): Extensive apoptosis and abnormal morphogenesis in pro-caspase-3 transgenic zebrafish during development. Mizusawa, N., Hojo, M., Yabu, T. In: *The Journal of Experimental Biology* (211), S. 1874–1881,

[URL Nr. 1](26.11.2011) Avaron F., Smith A., Akimenko M.: Sonic Hedgehog Signaling in the Developing and Regenerating Fins of Zebrafish. URL: http://www.ncbi.nlm.nih.gov/books/NBK6368/,

[URL Nr. 2](26.11.2011) Dahm R. (2003): der Zebrafisch in der Forschung: ein kleiner Fisch schlägt hohe Wellen. MPI für Entwicklungsbiologie. URL: http://www.muenchner-wissenschaftstage.de/mwt2003/content/e5/e278/e541/filetitle/Dahm_Schoenthaler_ger.pdf,

[URL Nr. 3](22.03.12) Kolb, H. (2012): Simple Anatomy of the Retina – Webvision. URL: http://webvision.med.utah.edu/book/part-i-foundations/simple-anatomy-of-the-retina/,

[URL Nr. 4](12.04.2012) McDowall, J.: Caspases. URL: http://www.ebi.ac.uk/interpro/potm/2004_8/Page1.htm,

[URL Nr. 5](02.04.2012) U.S. National Library of Medicine: Apoptosis. URL: http://www.apoptosisinfo.com/,

[URL Nr. 6] Robinson S. (1998): Diagram of the Retinal Layers. URL: http://www.bing.com/images/search?q=retina+layer&view=detail&id=543FF9DF391766BD3C2EE11384D1FDF73406AF03&FORM=IDFRIR,

12 Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Stellen, die wörtlich oder sinngemäß aus Quellen entnommen wurden, sind als solche kenntlich gemacht.

Diese Arbeit wurde in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegt.

Dresden, den 27.08.2012

Mandy Schönbrodt-Rühl

Glossary

Alexa Fluor 488	Secondary antibody coupled to a green fluorescent protein
amacrine cells	interneurons that interact at the second synaptic level of the vertically direct pathways, used for movement vision
Apoptosis	programmed cell death
bipolar cells	Type of neuron which has two extensions. Bipolar cells are specialized sensory neurons for the transmission of special senses. As such, they are part of the sensory pathways for smell, sight, taste, hearing and vestibular functions
blastema	mass of cells capable of growth and regeneration into organs or body parts, composed of undifferentiated pluripotent cells
caspases	family of cysteine proteases that play essential roles in apoptosis
DAPI	fluorescent stain that binds strongly to A-T rich regions in DNA
epithelial cells	cells in the epithelium , one of the four basic types of animal tissue, function: secretion, selective absorption, protection, transcellular transport
ganglion cells	final output neurons of the vertebrate retina
horizontal cells	laterally interconnecting neurons in the outer plexiform layer of the retina
humid chamber	sealable box with the purpose of creating a controlled, humid environment
inner nuclear layer	area of the retina, connected by the optic nerve to the brain
inner plexiform layer	area of the retina that is made up of a dense reticulum of fibrils formed by interlaced dendrites of retinal ganglion cells and cells of the inner nuclear layer
MESAB	MESAB blocks sodium channels, used to anesthetize the larvae .
mesenchymal cell	cells in mesenchyme, a type of undifferentiated loose connective tissue

outer nuclear layer	layer of the vertebrate retina, it is host of the horizontal cells
outer plexiform layer	layer of neuronal synapses in the retina of the eye. It consists of a dense network of synapses between dendrites of horizontal cells
photopic vision	is mediated by cone cells under well-lit conditions, it allows color perception
postmitotic	a mature cell that is no longer capable of undergoing mitosis, occurring after or pertaining to the time following mitosis
rods and cones	light sensitive photoreceptor cells in the retina
scotopic vision	vision of the eye under low light conditions
sarcolemma	cell membrane of the muscle
sarcomeres	repeating units of a myofibril joined together by dense material
sarcoplasm	cytoplasm of the muscle cell
TUNEL	fluorescent staining for apoptotic cells