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Medaka as an alternative model organism in the study of transcriptional regulation

Využití medaky jako alternativního modelového organismu při studiu transkripční regulace

PhD. Thesis

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Statement/Prohlášení

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V Praze / In Prague 12/11/2024 Simona Mikula Mršťáková

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Abstract (ENG)

Model organisms are essential tools in biological research, offering insights into the biology of other organisms. This research approach is enabled by the shared evolutionary origins of all living organisms and the conservation of metabolic and developmental pathways, as well as genetic material, over time. A broad range of model organisms supports biological research, from prokaryotes like bacteria and viruses to eukaryotes, including yeast, algae, and multicellular organisms. Widely used animal models include *C. elegans*, *Drosophila*, *Xenopus*, zebrafish (*Danio rerio*), chicken, mouse, and rat, with alternative models like medaka (*Oryzias latipes*) employed for specific research needs.

In this thesis, we demonstrate the potential of medaka as an alternative model organism for studying transcriptional regulation. The studies described here provide evidence that medaka is an excellent model organism and, in some cases, may be more suitable than the commonly used zebrafish.

The first case study explores the use of medaka as a model organism for analyzing gene function, focusing on *Pax6*, a key regulator of eye development across species. *Pax6* governs numerous target genes essential for ocular formation, yet its role during embryonic eye development remains less understood outside of mouse studies. Medaka, with three pax6 genes (*Pax6.1*, *Pax6.2*, and *Pax6.3*), is a more suitable model than zebrafish for studying pax6 genes in teleost fish, as zebrafish possess two *pax6.1* genes and lack *pax6.3*. The presence of *pax6.3* in medaka offers a valuable opportunity to investigate *pax6* genes from an evolutionary perspective.

The second part of this thesis describes two independent studies that demonstrate the potential of medaka for transgenic research. The first study uses medaka to investigate the role of the *Pitx2* gene and its enhancer, known as the asymmetric enhancer (ASE), in the epithalamus. Medaka allows for observation of gene expression patterns and assessment of changes following ASE mutagenesis, even in the F0 generation. The second study employs both medaka and zebrafish to examine the role and evolutionary conservation of the novel *Pax6* enhancer IrisE, illustrating the effective use of medaka as a complementary model alongside zebrafish.

In conclusion, this thesis highlights medaka's potential as a valuable model organism, particularly for studies of gene regulation and developmental biology. By emphasizing its unique advantages and complementarity to zebrafish, the findings presented here advance our understanding of transcriptional regulation and evolutionary genetics in vertebrates.

Abstract (CZ)

Modelové organismy jsou zásadními nástroji biologického výzkumu, poskytujícími vhled do fungování jiných organismů. Tento přístup je umožněn společným evolučním původem živých organismů a konzervací metabolických a vývojových drah, stejně jako genetického materiálu. Výzkum dnes využívá široké spektrum modelových organismů, od prokaryotických bakterií a virů až po eukaryotické organismy, jako jsou kvasinky, řasy a různé mnohobuněčné organismy. Mezi běžně používané modely zvířat patří *C. elegans*, *Drosophila*, *Xenopus*, zebřička (*Danio rerio*), kuře, myš a potkan; pro specifické účely se využívají i alternativní modely, jako je medaka (*Oryzias latipes*).

V této práci ukazujeme potenciál medaky jako alternativního modelu pro studium transkripční regulace. Zde popsané studie dokládají, že medaka je vynikající modelový organismus a v některých případech může být pro daný výzkum vhodnější než běžně používaná zebřička.

První případová studie se zaměřuje na využití medaky při analýze genové funkce, zejména genu *Pax6*, který je klíčovým regulátorem vývoje oka u různých druhů. Tento gen řídí mnoho cílových genů nezbytných pro tvorbu oka, avšak jeho role během embryonálního vývoje oka mimo model myši zůstává méně prozkoumána. Medaka, která obsahuje tři geny pax6 (*Pax6.1*, *Pax6.2* a *Pax6.3*), představuje vhodnější model než zebřička pro studium těchto genů u ryb, jelikož zebřička má dva geny *pax6.1* a chybí jí gen *pax6.3*. Přítomnost genu *pax6.3* u medaky poskytuje cennou možnost zkoumat evoluční perspektivu funkce genu *Pax6*.

Druhá část této práce popisuje dvě nezávislé studie demonstrující využití medaky pro transgenní výzkum. První studie využívá medaku ke zkoumání role genu *Pitx2* a jeho enhanceru, známého jako asymetrický enhancer (ASE), v epitalamu. Medaka zde umožňuje sledování genové exprese a hodnocení změn po mutagenezi ASE již v generaci F0. Druhá studie zkoumá s využitím medaky a zebřičky roli a evoluční konzervaci nového enhanceru genu *Pax6* s názvem IrisE, a ukazuje tak efektivní využití medaky jako doplňkového modelu vedle zebřičky.

Závěrem tato práce zdůrazňuje potenciál medaky jako cenného modelového organismu, zejména pro studium genové regulace a vývojové biologie. Poukazem na její jedinečné vlastnosti a komplementaritu k zebřičce se prezentované poznatky přibližují k lepšímu porozumění transkripční regulace a evoluční genetiky obratlovců.

Table of contents

Statement/Prohlášení 3

Acknowledgement 4

Abstract (ENG) 5

Abstract (CZ) 6

List of abbreviation 10

Introduction 12

Medaka (Oryzias latypes) 12

Medaka as a model organism 15

Medaka vs. Zebrafish 16

Aims of the study 18

First Case Study: Medaka as a model organism for gene function analysis 19

The role of Pax6 genes during the embryonic eye development of medaka 19

Literature overview 20

Vertebrate eye development 20

Early eye development 20

Optic vesicle to optic cup transformation and lens induction 21

Lens formation 23

Retina development 25

Differentiation of specific retinal cell types 26

Ciliary marginal zone 29

Structure of Pax6 gene/protein 30

The role of *Pax6* gene during the eye development 33

Drosophila 33
Mouse 34
Human 35
Teleost fish 35
Aims of the study 42
Materials and methods 43
Results 50
The expression of pax6 genes during embryonic development of medaka 50
Generation of mutants 55
Morphological analysis of pax6 mutants 58
Analysis of *Pax6.1* mutant 62
Analysis of *Pax6.2* mutant 76
Analysis of *Pax6.3* mutant 78
The role of pax6 genes during the embryonic eye development of medaka 80
Do other pax6 genes compensate for the missing *Pax6.1* gene in medaka? 80
Analysis of *Pax6.1* and *Pax6.3* double mutant 84
Discussion 87
Types of mutation in *Pax6* gene in vertebrates 88
The role of *Pax6* during medaka embryonic eye development 90
The role of *Pax6* during medaka embryonic lens development 90
The role of *Pax6.1* during medaka embryonic retina development 92
Conclusion 95
Conclusion: Medaka as a model organism for gene function analysis 96
Second case study: Medaka as a model organism for transgenic studies 97

Asymmetric *Pitx2* expression in medaka epithalamus is regulated by nodal signaling through an intronic enhancer 97

Literature overview 98

Aims of the study 100

Material and methods 101

Results 104

Discussion 111

Conclusion 112

Identification of a conserved Pax6 enhancer controlling iris development 113

Overview of the study 114

Conclusion: Medaka as a model organism for transgenic studies 118

Conclusion 119

List of publications relevant for this thesis 120

Genetic analysis of medaka fish illuminates conserved and divergent roles of Pax6 in vertebrate eye development 120

Asymmetric pitx2 expression in medaka epithalamus is regulated by nodal signaling through an intronic enhancer 120

Identification of a conserved *Pax6* enhancer controlling iris development. 121

References 122

List of abbreviation

ACs	Amacrine cells
ADHD	Attention deficit hyperactivity disorder
ASE	Asymmetric enhancer
BCs	Bipolar cells
BMP	Bone morphogenic protein
CMZ	Ciliary marginal zone
DNA	Deoxyribonucleic acid
Dpf	Days post fertilisation
DRR	Downstream regulatory region
EE	Ectodermal enhancer
Ey	Eyeless
FGF	Fibroblast growth factor
GCL	Ganglion cell layer
GFP	Green fluorescent protein
HCs	Horizontal cells
HD	Homeo domain
INL	Inner nuclear layer
KO	Knock-out
LP	Lens placode
MGCs	Muller glia cells
OC	Optic cup
ONL	Outer nuclear layer

OV	Optic vesicle
PCs	Photoreceptor cells
PD	Paired domain
pNR	Presumptive neural retina
pOS	Presumptive optic stalk
pRPE	Presumptive retinal pigmented epithelium
PST	Proline-Serine-Threonine
RA	Retinoic acid
RGCs	Retinal ganglion cells
RPCs	Retinal progenitor cells
RPE	Retinal pigmented epithelium
SE	Surface ectoderm
Sey mouse	Small eye mouse
Shh	Sonic hedgehog
Sri mutant	Sunrise mutant
TGF	Transforming growth factor
Toy	Twin of eyeless
WGD	Whole genome duplications
WT	Wildtype

Introduction

Medaka (Oryzias latypes)

The medaka fish (Figure 1) was first officially described under the name `*Poecilia latipes*` in the 1850 publication `Fauna Japonica` by Phillip Franz von Siebold (reviewed in Hilgers and Schwarzer, 2019). In 1906, Jordan and Snyder reclassified the species, designating the Latin name *Oryzias latipes* (Jordan and Snyder, 1906). This nomenclature reflects the fish's natural habitat in rice fields (*Oryza sativa*), a feature that also influenced its common English name, "ricefish."

Figure 1: Picture of medaka (*Oryzias latipes*) (Ivana Dobiášovská).

Taxonomically, medaka belongs to the infraclass *Teleostei*, a highly diverse group within the class *Actinopterygii* (ray-finned fishes). *Teleostei*, derived from the Greek words *teleios* meaning "complete" and *osteon* meaning "bone," represents the largest infraclass of fishes, comprising 96% of all extant fish species. This group includes over 26,000 described species, organized into approximately 40 orders and 448 families (Miller and Harley, 2007). A key distinguishing feature of teleosts is the presence of a movable premaxilla, along with specialized jaw musculature, allowing for the protrusion of the jaw to capture prey more efficiently. Additionally, teleosts have a symmetrical caudal fin, with equal-sized upper and lower lobes, and a spine that terminates at the caudal peduncle, unlike other bony fishes in which the spine extends into the upper lobe of the tail fin. (Patterson and Rosen, 1977)

Medaka, is a small freshwater vertebrate native to East Asia, primarily found in Japan, Korea, and China. The diversity within the medaka (*Oryzias latipes*) species complex has gained increasing recognition, revealing distinct strains and species within the group. For many years, the northern and southern Japanese populations, from which the well-known Hd-rR and HNI inbred lines are derived, were considered a single species. However, accumulating evidence has led to the recognition of the northern population as a separate species, *Oryzias sakaizumii* (Asai et al., 2011). These species exhibit differences in craniofacial anatomy, body coloration, aggressiveness, and sexual dimorphism (Kimura et al., 2007;

Asai et al., 2011; Kagawa, 2014). Despite these distinctions, both species can interbreed, producing viable hybrids in laboratory settings, offering unique opportunities to study genetic and physiological diversity (Murata et al., 2012).

Although medaka is the most commonly studied species in the ricefish family (*Adrianichthyidae*), this family includes 36 described species, with 32 species in the *Oryzias* genus and four species in *Adrianichthys* (Parenti, 2008; Herder and Behrens-Chapuis, 2010; Magtoon, 2010; Asai et al., 2011; Parenti et al., 2013; Mokodongan et al., 2014; Mandagi et al., 2018) (Figure 2). This broader diversity extends beyond the laboratory strains and includes significant genetic and phenotypic variation in natural populations. The rich diversity within ricefish species underscores the potential for further research into their evolutionary and biological traits, offering valuable insights into the genetic mechanisms driving these variations.

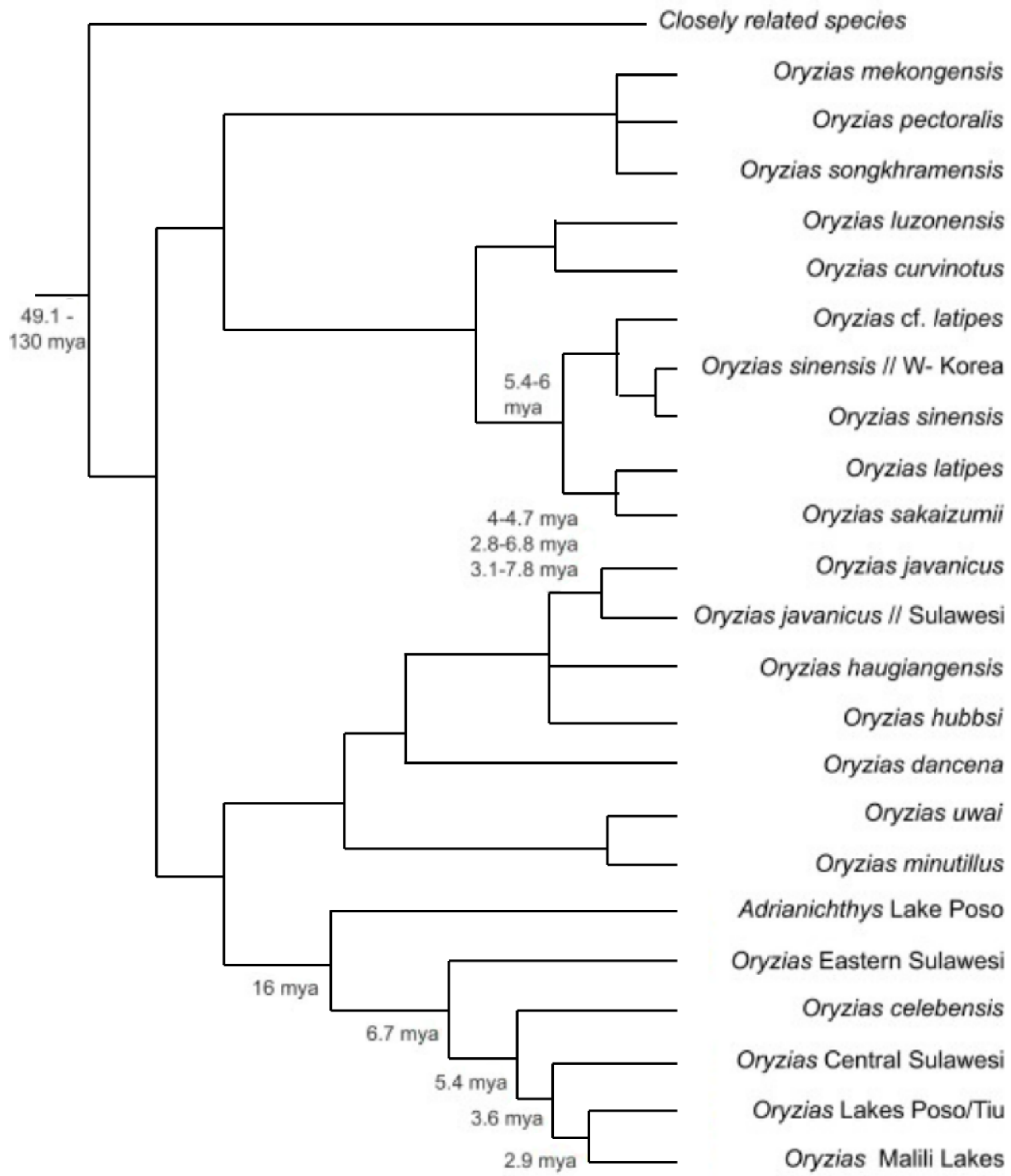

Figure 2: Phylogeny of medaka species (Modified from Hilgers and Schwarzer, 2019).

Medaka as a model organism

Medaka (*Oryzias latipes*) is a widely utilized model organism in biological research, particularly in genetics and evolutionary studies. Its high survivability in laboratory conditions and ease of maintenance make it an ideal choice for a range of experimental applications.

Medaka exhibits remarkable tolerance to variations in salinity and temperature, thriving in conditions ranging from 0 to 40°C. Under laboratory settings, medaka reaches sexual maturity within approximately two months post-hatching, with clear sexual dimorphism, allowing easy identification of males and females. The spawning process is influenced by environmental factors such as water temperature, food availability, and light conditions, with adult females laying 10-30 eggs daily (Wittbrodt et al., 2002; Shima and Mitani, 2004; Murata et al., 2019). Medaka is an oviparous fish, meaning that fertilization of eggs and embryonic development occur externally. The embryos are protected by tough, transparent membrane called the chorion, enabling direct observation of embryogenesis under a microscope (Wittbrodt et al., 2002; Shima and Mitani, 2004). The entire embryonic development process, from fertilization to hatching, is rapid, taking approximately 10 days. A detailed staging of medaka embryonic development has been published by Iwamatsu (Iwamatsu 2004).

All teleost fish, including medaka, have undergone three rounds of whole genome duplication. As a result, some genes were either lost or have acquired new functions. The medaka genome has been fully sequenced and is well-annotated (reviewed in Kobayashi and Takeda, 2008), enabling researchers to explore the roles of various genes in comparison with their orthologues in mouse and human, as well as study unique medaka-specific genes from an evolutionary perspective (Kasahara et al., 2007). Over the past decade, numerous genetic tools, such as TALENs, CRISPR-Cas9, ISH, IHC, sectioning, and fluorescence microscopy, have become accessible for research.

Medaka is an excellent model organism due to its simple husbandry requirements, short reproductive cycle, external and transparent embryonic development, fully sequenced genome, and the availability of diverse genetic tools.

Medaka vs. Zebrafish

Medaka and zebrafish, both belonging to the teleost fish group, diverged from their last common ancestor approximately 110 million years ago (Figure 3) (reviewed in Wittbrodt et al., 2002). Despite their evolutionary separation, these two model organisms share numerous similarities. Both species offer well-established systems for embryonic manipulation, genomic resources (such as high-density genetic maps and draft genome sequences), and advanced transgenic and genome editing techniques like TALENs and CRISPR-Cas9.

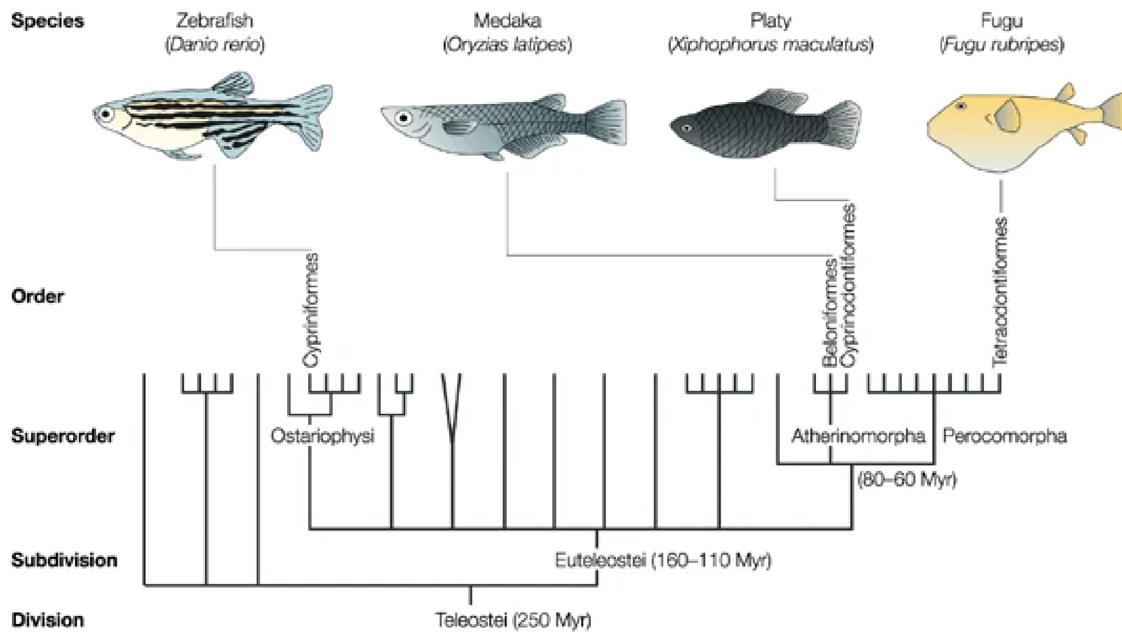

Figure 3: The evolutionary tree illustrating that the last common ancestor of medaka and zebrafish lived more than 110 million years ago. Myr: million years ago (Modified from Wittbrodt et al., 2002).

In laboratory settings, the generation time for medaka ranges from 6 to 8 weeks, while for zebrafish, it is typically between 8 and 10 weeks. Both medaka and zebrafish are egg-laying species with transparent embryos that develop within a clear chorion. Their reproduction is tightly regulated by the light cycle. Medaka females spawn between 20 and 40 eggs daily, typically within an hour of the onset of light, while zebrafish females do not spawn daily, producing several hundred eggs at intervals of about one week. Thus, the overall weekly egg production of medaka and zebrafish is comparable. A key difference lies in the fertilization process: zebrafish eggs are fertilized immediately as they are laid and fall to the ground, whereas medaka eggs remain attached to the female via filaments, facilitating easy identification of reproductively active females. Zebrafish mating pairs require more frequent setups and monitoring to prevent harm, while medaka pairs can remain together for extended periods, with females spawning daily for several months. This allows for more continuous and efficient collection of eggs in medaka, enabling faster confirmation of mutant phenotypes compared to zebrafish, where it may take weeks to accumulate

sufficient data. In terms of early development, both species develop rapidly, with zebrafish progressing slightly faster. Zebrafish larvae hatch from the thin chorion within 2 days, while medaka embryos, supported by a yolk sac, remain inside a tougher chorion for around 10 days before hatching as feeding juveniles (Wittbrodt et al., 2002; Shima and Mitani, 2004; Westerfield, 2007; Murata et al., 2019).

Medaka and zebrafish, both key model organisms, exhibit differences in chromosome count and genome size. Medaka has 24 chromosomes and a genome size of approximately 800 Mb, while zebrafish has 25 chromosomes and a larger genome of around 1700 Mb. Despite these differences, the genomes of both species have been fully sequenced (Freeman et al., 2007; Kasahara et al., 2007; Rouchka, 2010). Additionally, advanced methods for mutant generation and transgenesis are well established in both medaka and zebrafish, making them powerful tools for genetic and developmental research.

	Medaka	Zebrafish
Generation Time	6-8 weeks	8-10 weeks
Daily Egg Production	20-40 eggs/day	Hundreds/week
Egg Attachment	Eggs remain attached to female	Eggs fall to ground
Embryo Development Time	10 days before hatching	2 days before hatching
Chorion	Hard	Soft
Number of Chromosomes	24	25
Genome Size	800 Mb	1700 Mb
Genome Analysis	Finished	Finished
Transgenic Technology	Well established	Well established

Table 1: Comparison of medaka and zebrafish.

While medaka and zebrafish share many experimental advantages, their differences in reproduction, egg handling, and embryonic development provide distinct benefits depending on the research focus, making both species invaluable models for developmental and genetic studies. Medaka serves as an alternative model that is both comparable and complementary to zebrafish.

Aims of the study

The aim of this study is to establish medaka (*Oryzias latipes*) as a model organism for genetic research, demonstrating its potential as a superior or complementary alternative to the more commonly used zebrafish (*Danio rerio*) for specific experimental contexts. While zebrafish has been a central model in developmental and genetic studies, we aim to show that medaka offers unique advantages in particular research areas. Through a series of example studies, we will illustrate medaka's effectiveness in diverse applications, including gene function analysis through knock-out experiments and the study of gene regulatory networks. We will also highlight the use of medaka in transgenesis for investigating gene regulatory elements. Additionally, we will describe a comparative study where medaka and zebrafish are used side-by-side, emphasizing medaka's role as a complementary model. Together, these studies will underscore medaka's utility and value in advancing our understanding of genetics and molecular biology.

First Case Study: Medaka as a model organism for gene function analysis

The role of Pax6 genes during the embryonic eye development of medaka

Medaka (*Oryzias latipes*) is a valuable model organism for genetic studies, particularly in the exploration of gene regulatory networks. Its fully sequenced genome, coupled with the availability of advanced genetic tools such as CRISPR-Cas9 and TALENs for targeted gene knockouts, makes medaka an excellent system for investigating the role of specific genes. These methodologies enable precise manipulation of gene function, allowing researchers to elucidate the complex roles that genes play in developmental processes.

In the first study presented in this thesis, we will investigate the roles of all pax6 genes during medaka embryonic eye development. Our findings will demonstrate why medaka serves as a better model organism for this specific study, in comparison to the more commonly used zebrafish. The data obtained will provide valuable insights into the evolutionary conservation of pax6 genes across different species.

Literature overview

Vertebrate eye development

The vertebrate eye is a highly organized and specialized sensory organ that originates early in embryonic development from the neuroectoderm. A fully developed vertebrate eye comprises several distinct structures, including the optic nerve, retina, sclera, iris, cornea, lens, ciliary body, and conjunctiva.

The development of the vertebrate eye proceeds through a series of coordinated processes. The eye field, located on either side of the head neuroectoderm, gives rise to the optic primordium, which subsequently transforms into the optic vesicle (Figure 3). The optic vesicle evaginates and forms the optic cup. During this transformation, cells of the optic cup undergo migration and differentiation, ultimately leading to the formation of the mature retina. The lens develops from the lens placode, a region of surface ectoderm positioned near the optic vesicle. In both mouse and fish, lens formation occurs through invagination and delamination, respectively.

The following chapter will discuss key aspects of retina and lens development in vertebrates.

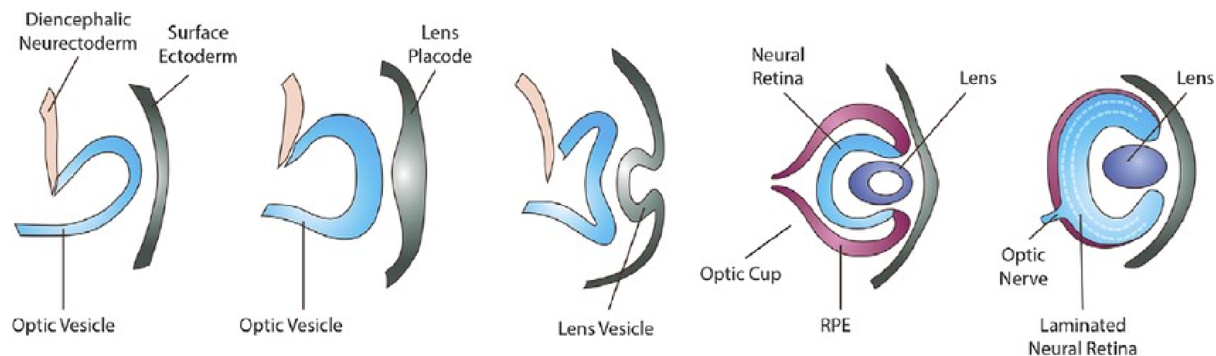

Figure 3: Scheme of the vertebrate eye development in Xenopus. RPE: Retinal pigmented epithelium (Modified from Mellough et al., 2014).

Early eye development

During the gastrula stage, a single eye field is established within the anterior neural plate, which later divides into two distinct fields. These fields subsequently give rise to the optic primordia and, eventually, to the eyes (Adelmann, 1936; Li et al., 1997). The cells within the eye field serve as progenitors for all neural-derived structures of the eye (Zaghloul et al., 2012). Disruptions in eye field formation can result in anophthalmia, a condition characterized by the absence of eyes (reviewed in Harding and Moosajee, 2019). Conversely, failure to properly split the eye field into two separate fields leads to cyclopia, a malformation where a single eye is present in the center of the head (Varga et al., 1999).

The first morphological appearance of eye development is the formation of the optic primordium (optic pit) on either side of the developing diencephalon, emerging as early as the neurula stage (Li et al., 1997; Wilson and Houart, 2004). The optic primordium subsequently transforms into the optic vesicle through cell migration and evagination (Rembold et al., 2006). The specification of the optic primordium and the formation of the optic vesicle are regulated by signaling pathways involving Wnt, BMP, FGF, and Shh molecules (reviewed in Yang, 2004). Additionally, *Pax6*, in conjunction with other eye field-specific transcription factors such as *Six3*, *Six6*, *Rx*, and *Lhx2*, plays a crucial role in these developmental processes (Zhao et al., 2001; Zuber et al., 2003; Esteve and Bovolenta, 2006; Heavner and Pevny, 2012; Zaghloul et al., 2012).

The optic vesicle is divided into three domains, which give rise to the optic stalk, neural retina, and retinal pigment epithelium (RPE). As each domain eventually develops into a specific structure, their boundaries are established by differential gene expression. Studies in various vertebrates have shown that Shh, FGF, and TGFβ signaling pathways are critical during this stage of eye development (Hyer et al., 1998; reviewed in Chow and Lang, 2001; Martinez-Morales et al., 2004; Yang, 2004). The optic stalk domain is characterized by the expression of *Vax* and *Pax2*, while the prospective neural retina expresses genes such as *Pax6*, *Chx10*, *Lhx2*, and *Rx*. In the future RPE, *Pax6*, *Mitf*, and *Otx2* are expressed (Martinez-Morales et al., 2004; reviewed in Chow and Lang, 2001) (Figure 4). In *Pax6*-deficient mice, the neural retina and RPE fail to develop, with an expanded *Pax2* expression domain, suggesting an antagonistic regulatory relationship between *Pax2* and *Pax6* during the specification of optic vesicle domains (Schwarz et al., 2000).

Optic vesicle to optic cup transformation and lens induction

The continued evagination of the optic vesicle results in its contact with the overlying surface ectoderm, a crucial step in eye development. This close interaction induces the thickening of the surface ectoderm, which subsequently gives rise to the lens placode and, ultimately, to the lens (Figure 4). Evidence suggests that reciprocal inductive signaling between the optic vesicle and surface ectoderm is essential for proper eye formation. For instance, the neural retina fails to develop correctly if the surface ectoderm is removed (Hyer et al., 1998).

Simultaneously with lens formation, the cells of the optic cup continue to evaginate, eventually folding into a double-layered optic cup (Figure 4). One of the key factors regulating the transformation of the optic vesicle into the optic cup is the retinal homeodomain transcription factor *Rx* (also known as *Rax*) (Mathers et al., 1997; Mathers and Jamrich, 2000; Loosli et al., 2003). *Rx* plays an essential role in the expression of genes critical for early eye development, such as *Pax6*, *Six3*, and *Lhx2* (Zuber et al., 2003; Yun et al., 2009). Mutations in *Rx* genes in mouse, zebrafish, and medaka result in the failure to form the optic cup and no-eye phenotype (Mathers et al., 1997; Winker et al., 2000). Additionally, retinoic acid (RA) plays a crucial role in proper optic cup formation. Disruption of RA signaling leads to arrested development of the retina and lens (Mic et al., 2004). In humans, mutations in receptors involved in the RA pathway are often associated with anophthalmia or microphthalmia (White et al., 2008).

As previously mentioned, the close contact between the developing optic cup and surface ectoderm leads to the establishment of the lens placode (Figure 4). Proper lens induction relies on multiple signaling pathways, with BMP, Wnt, and FGF being the most prominent. Experimental evidence underscores the critical role of BMP signaling, particularly in the optic vesicle/optic cup for lens induction. Disruptions in BMP activity, especially *Bmp4* and *Bmp7*, result in significant impairments in lens development, either during the activation of the lens placode or later during lens formation (Furuta and Hogan, 1998; Wawersik et al., 1999; Yun et al., 2009; Huang et al., 2015).

The precise regulation of Wnt/β-catenin signaling within the surface ectoderm and lens epithelium also appears to be essential for lens induction and subsequent development (Machon et al., 2010). Studies using mouse models with conditional β-catenin deletion in the lens placode and surrounding head ectoderm demonstrate that Wnt/β-catenin signaling is not strictly required for lens development (Smith et al., 2005; reviewed in Fujimura, 2016). However, ectopic lentoid body formation in Wnt/β-catenin-deficient mice suggests that the Wnt signaling pathway plays a key role in modulating the development of other structures by suppressing lens fate (Smith et al., 2005; Kreslova et al., 2007; reviewed in Fujimura, 2016).

Additionally, FGF signaling is involved in lens induction, mainly by regulatory interactions with key genes such as *Pax6*, *Bmp4*, and *Bmp7* (Faber et al., 2001; Gotoh et al., 2004; reviewed in Smith et al., 2009).

Lens induction is a complex process that relies on the interplay between various signaling pathways (as previously described) and specific transcription factors, particularly *Sox2*, *Six3*, and *Pax6*. Studies have demonstrated the ability of the homeobox gene *Six3* to induce lens formation when ectopically expressed in the optic placode of medaka (Oliver et al., 1996). Deletion of *Six3* results in severe disruption of the transition from surface ectoderm to lens placode, leading to either absent or abnormal lens formation (Liu et al., 2006).

Pax6 is widely recognized as a critical factor for lens induction and its subsequent development. Conditional knock-out of *Pax6* in mouse embryos leads to the absence of lens placode formation, highlighting its essential role in proper lens development (Ashery-Padan et al., 2000). Furthermore, *Pax6* activity within the optic vesicle is crucial for proper lens induction. When *Pax6* is deleted from the retina before sufficient contact between the optic vesicle and surface ectoderm occurs, lens development fails to initiate (L Klimova and Z Kozmik, 2014).

Extensive studies have also outlined the complex regulatory network between *Sox2*, *Six3*, and *Pax6*, underscoring the coordinated action of these transcription factors during lens formation (Liu et al., 2006; reviewed in Lang, 2004).

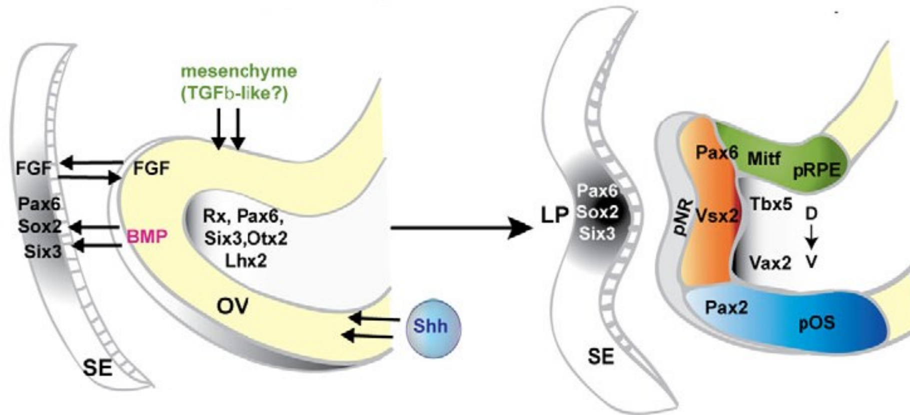

Figure 4: Optic vesicle to optic cup transition and lens induction. SE: Surface ectoderm, OV: optic vesicle, LP: Lens placode, pNR: Presumptive neural retina, pRPE: Presumptive retina pigmented epithelium, pOS: Presumptive optic stalk (Modified from Yun et al., 2009).

Lens formation

The first stage of lens development involves the formation of the lens placode through the thickening of the surface ectoderm. Following this, invagination of the lens placode leads to the creation of the lens stalk, which eventually detaches from the ectoderm to form the lens vesicle (Figure 5). At this stage, the lens vesicle is composed of two layers of cells, with a space between them. The anterior cells, situated closer to the ectoderm, will differentiate into lens epithelial cells. In contrast, the posterior cells elongate to fill the space and form primary fiber cells. Some of the epithelial cells migrate toward the equatorial region, where they elongate and differentiate into secondary fiber cells. The production of secondary fiber cells continues as the lens grows (reviewed in McAvoy et al., 1999; Graw, 2010). Additionally, studies have highlighted the critical role of environmental signals from the developing optic cup in ensuring proper lens morphology (Coulombre and Coulombre, 1963; Yamamoto, 1976).

Although the lens formation process differs in fish, its final structure and function closely resemble those of the mammalian lens. Instead of invagination, as seen in mammals, the cells of the lens placode in fish form a solid mass, within which the primary fiber cells elongate in a circular pattern (Figure 5). A lens vesicle does not form, and the connection between the lens cell mass and the ectoderm is disrupted once the primary fiber cells have differentiated. Similar to the structure of the mouse lens, a layer of epithelial cells surrounds the lens mass in fish. These epithelial cells continue to migrate, elongate, and differentiate, ultimately forming secondary fiber cells (Greiling and Clark, 2008; Graw, 2010).

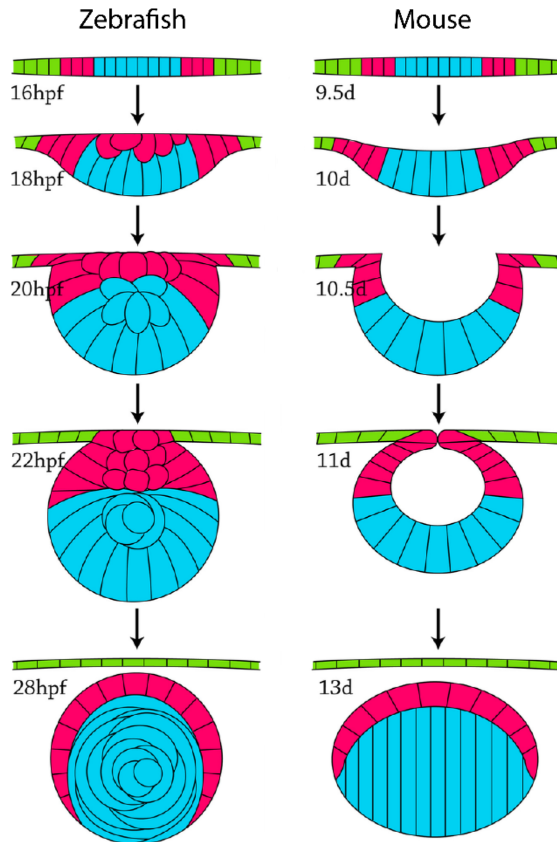

Figure 5: Comparison of lens formation in mouse and in zebrafish. Mouse lens develop by the process of invagination, while zebrafish (fish) lens form by delamination (modified from Greiling et al., 2009).

Members of the FGF signaling pathway play a crucial role in the differentiation of lens fiber cells (Chamberlain and McAvoy, 1987; Garcia et al., 2005). Studies in mouse models and rat explants demonstrate that, in addition to the presence of FGF signaling, the gradient and concentration of FGF activity are essential for inducing cell elongation and differentiation (McAvoy and Chamberlain, 1989; Robinson et al., 1995; Srinivasan and Overbeek, 1996; Lovicu and Overbeek, 1998). Furthermore, FGF signaling is important for regulating Wnt signaling, which is critical for proper lens development (Lyu and Joo, 2004). The absence of Wnt/β-catenin signaling is required for correct lens formation, as ectopic activation of canonical Wnt signaling in the retina and lens leads to severe developmental disruptions. Additionally, evidence suggests that the inhibition of the canonical Wnt pathway is dependent on the activity of the *Pax6* gene (Kreslova et al., 2007; Machon et al., 2010; Antosova et al., 2013).

Pax6 is considered the key regulator of lens development. In mice, its expression is first detected in the surface ectoderm. Following the transition from surface ectoderm to the lens placode, *Pax6* expression is downregulated in the surface ectoderm but remains active in the presumptive lens area. It continues to be expressed in the developing lens and lens epithelium during embryonic development and into early postnatal stages (Grindley et al., 1995; reviewed in McAvoy et al., 1999). *Pax6* is critical not only for lens induction (as mentioned earlier), but also for lens placode invagination, differentiation of lens fiber cells,

and lens maintenance. Several genes involved in lens placode invagination, such as *FoxE3*, *Prox1*, *Maf*, and *Mab*, are regulated by *Pax6*. Additionally, *Pax6* controls the expression of several crystallin genes during fiber cell specification (Cvekl and Piatigorsky, 1996; Cvekl and Dunkan, 2007; Wolf et al., 2009). Mutations in crystallin genes are linked to lens abnormalities and are frequently associated with the development of cataracts (Graw, 2009; Graw, 2009).

Retina development

The future neural retina region can first be identified during the optic vesicle stage. Retinal progenitor cells originating from this region will eventually differentiate into all retinal cell types (Wetts and Fraser, 1988; Turner et al., 1990). The fully developed vertebrate retina contains six types of neurons (ganglion cells, amacrine cells, bipolar cells, horizontal cells, rods, and cones) and one type of glial cell (Müller glial cells). These retinal cell types are organized into three distinct layers: the ganglion cell layer, the inner nuclear layer, and the outer nuclear layer (Figure 6). Together, these cell types form a highly specialized environment that enables the detection of light and the transmission of visual signals to the brain, allowing animals, including humans, to perceive vision.

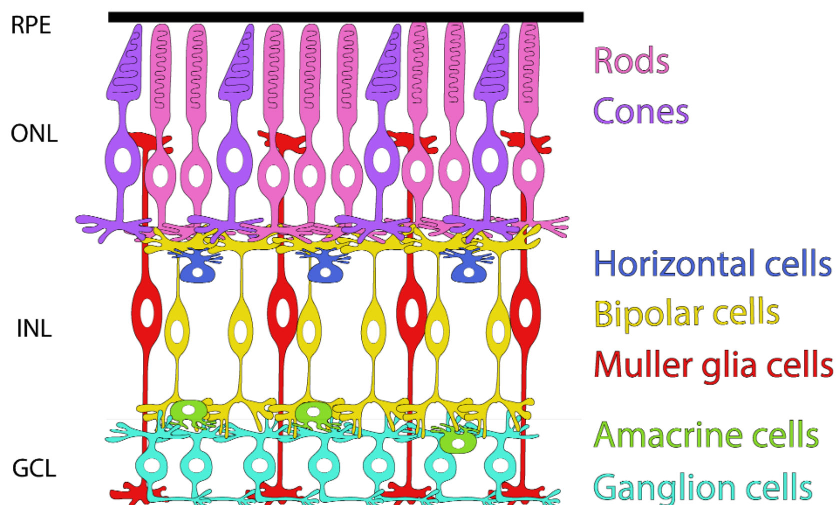

Figure 6: Schematic organisation of specific cell types in adult vertebrate retina. RPE: Retinal pigmented epithelium, ONL: Outer nuclear layer, INL: Inner nuclear layer, GCL: Ganglion cell layer

Undifferentiated retinal progenitor cells (RPCs) are characterized by the co-expression of several key transcription factors, including *Rx*, *Pax6*, *Six3*, *Six6*, *Lhx2*, and *Chx10* (Walther and Gruss, 1991; Grindley et al., 1995; Oliver et al., 1995; Mathers et al., 1997; Porter et al., 1997; Li et al., 2002; Martinez-Morales et al., 2004; reviewed in Chow and Lang, 2001). In mice, the absence of *Pax6*, *Lhx2*, or *Rx* genes results in the disruption of eye formation (Hill et al., 1991; Mathers et al., 1997; Porter et al., 1997; Winker et al.,

2000). Conversely, overexpression of *Pax6*, *Rx*, or *Six3* leads to the formation of ectopic retinal tissue (Mathers et al., 1997; Chow et al., 1999; Loosli et al., 1999). When *Pax6* is specifically deleted in retinal progenitor cells, the multipotency of the RPCs is lost, and they differentiate exclusively into amacrine interneurons (Marquardt et al., 2001).

Prior the differentiation, retinal progenitor cells undergo symmetrical divisions, where one RPC divides into two RPCs. However, once differentiation begins, asymmetrical division occurs—one RPC divides into one progenitor cell and one further differentiating cell. The Notch signaling pathway appears to play a crucial role in maintaining RPCs in their undifferentiated state. Transcription factors such as *Hes1* and *Hes5* act as repressors, and the timing of differentiation is closely linked to their downregulation or absence (Dorsky et al., 1997; Henrique et al., 1997).

The chronological order in which individual retinal cell types emerge is highly conserved across vertebrates. Retinal ganglion cells are the first to form, followed by horizontal cells and cone photoreceptors. The second wave of differentiation includes amacrine cells, rod photoreceptors, and bipolar cells, with Müller glial cells being the last to differentiate (Yang, 1985). Although retinal cell types develop in a distinct sequence, their differentiation overlaps, with multiple cell types being generated simultaneously (Figure 7).

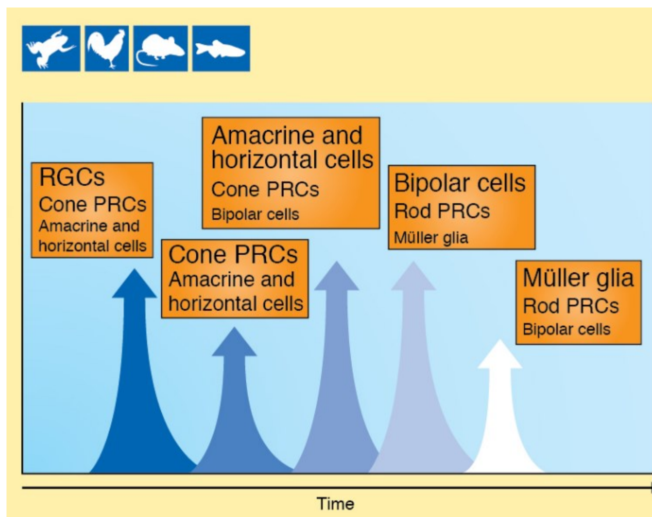

Figure 7: The sequence, in which retinal specific cell types differentiate, is highly conserved between vertebrates (Modified from Centanin and Wittbrodt, 2014).

Differentiation of specific retinal cell types

The adult retina of all vertebrates consists of the same neural and non-neural cell types. In addition to the identical chronological order of retinal cell type emergence, homologous transcription factors play crucial roles in their differentiation (Figure 8).

Retinal ganglion cells (RGCs)

Retinal ganglion cells (RGCs) are the first cell type to differentiate and are located in the innermost layer of the retina (Yang, 1985). Three key transcription factors—*Ath7* (also known as *Math5*), *Brn3*, and *Isl1*—are critical for the proper specification of RGCs (Liu et al., 2001; Yang et al., 2003; Pan et al., 2008). Studies have demonstrated that *Ath7* expression is regulated by *Pax6* (Riesenberg et al., 2009) and that *Ath7* functions upstream of *Brn3* (Wang et al., 2001). In *Ath7* mutant mice, the number of RGCs is significantly reduced (Wang et al., 2001), and in *Ath7/Brn3* double mutants, RGCs are almost entirely absent (Moshiri et al., 2008).

Amacrine cells (ACs)

Amacrine cells (ACs) are among the early cell types to differentiate during retinal development. They are primarily located in the inner nuclear layer, with some also found in the ganglion cell layer. Several transcription factors, including *Math3*, *NeuroD*, *Pax6*, *Foxn4*, *Ptf1a*, and *Six3*, play crucial roles in ACs differentiation. In *Math3/NeuroD* double knock-out mice, ACs are completely missing. Misexpression of *NeuroD* alongside *Pax6* results in an increased number of differentiated ACs, while co-misexpression of *Pax6* and *Math3* leads to an elevated number of both ACs and horizontal cells (HCs) (Inoue et al., 2002). Mutations in *Foxn4* or *Ptf1* genes cause a significant reduction in ACs and HCs in mouse (Li et al., 2004; Fujitani et al., 2006). In *Pax6* retina-specific mutants, only ACs are produced, underscoring the important role of *Pax6* in ACs differentiation (Inoue et al., 2002).

Bipolar cells (BCs)

Bipolar cells (BCs) are neuronal cells situated in the inner nuclear layer of the retina. Their differentiation is regulated by the transcription factors *Chx10*, *Mash1*, and *Mash3*. In *Mash1/Mash3* double mutant mouse, BCs fail to develop, and all potential BCs instead differentiate into Müller glial cells (MGCs) (Tomita et al., 2000). However, misexpression of *Mash1* or *Mash3* alone does not increase the number of BCs but instead promotes the development of rod photoreceptors (Tomita et al., 1996; Hatakeyama et al., 2001). In *Chx10* mutant mice, BCs are absent (Burmeister et al., 1996), though overexpression of *Chx10* alone does not significantly enhance BCs differentiation (Hatakeyama et al., 2001). Interestingly, co-misexpression of *Chx10*, *Mash1*, and *Mash3* together strongly promotes BCs specification (Hatakeyama et al., 2001).

Horizontal cells (HCs)

Horizontal cells (HCs) are interneurons located within the inner nuclear layer of the retina. The *Prox1* gene plays a critical role in the differentiation of HCs. In *Prox1* mutant mouse, no HCs are present, while misexpression of *Prox1* leads to the differentiation of HCs (Dyer et al., 2003). As previously mentioned, *Ptf1* and *Foxn4* are also important for HC specification. Mutations in *Ptf1* or *Foxn4* in mouse result in a significant reduction in the number of differentiated HCs and ACs (Li et al., 2004; Fujitani et al., 2006). Additionally, misexpression of *Pax6* and *Math3* has been shown to promote the differentiation of both HCs and ACs (Inoue et al., 2002).

Photoreceptors cells (PCs)

Two distinct populations of photoreceptors—rods and cones—reside in the outer nuclear layer of the retina. The homeobox genes *Otx2* and *Crx* play a crucial role in photoreceptor specification. Deletion of either of these genes in the mouse results in defects in photoreceptor differentiation (Furukawa et al., 1997; Furukawa et al., 1999; Nishida et al., 2003). In the mouse embryo, *Otx2* deletion leads to the promotion of amacrine cells instead of photoreceptors (Nishida et al., 2003). Overexpression of *Crx* increases the number of rod photoreceptors in the retina (Furukawa et al., 1997). Another key factor, *NeuroD*, is also critical for photoreceptor differentiation. Studies have shown that misexpression of *NeuroD* in the mouse promotes rod photoreceptor fate (Inoue et al., 2002). Additionally, the *Nrl* gene is required for the specification of rods and cones. *Nrl* directly targets *NR2E3*, which is preferentially expressed in rod cells and represses several cone-specific genes (Chen et al., 2004; Chen et al., 2005).

Muller glial cells (MGCs)

Müller glial cells are the only non-neuronal cell type present in the retina. Their primary function is to maintain retinal homeostasis by forming the neuronal scaffold, supporting neuronal survival, and regulating informational processing (reviewed in Newman and Reichenbach, 1996; Bringmann et al., 2006). The loss of MGCs results in lamination defects and retinal degeneration (Rich et al., 1995). The differentiation of MGCs is closely linked to the *Rax* genes, as well as *Hes1* and *Hes5*, which are components of the Notch signaling pathway (Furukawa et al., 2000; Jadhav et al., 2009).

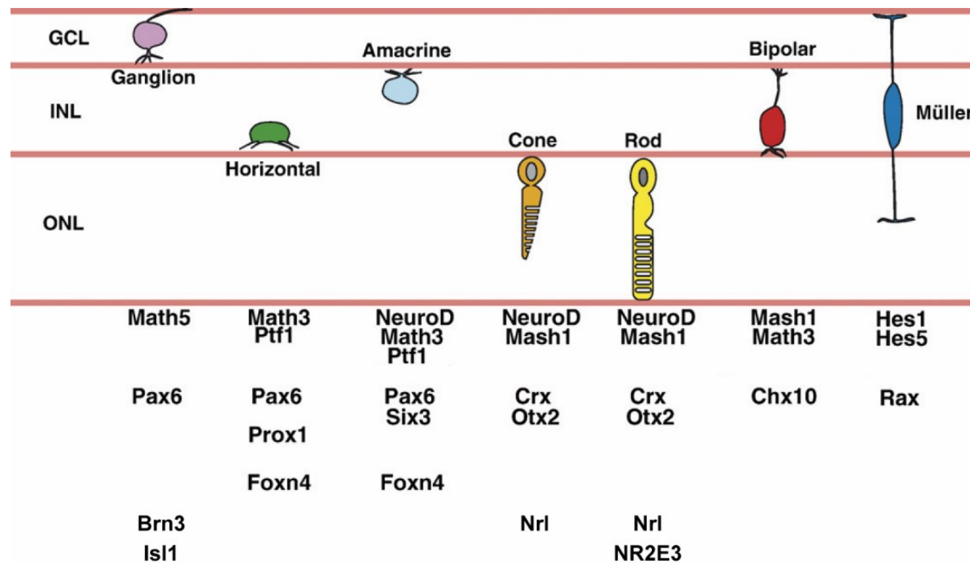

Figure 8: Transcription factors important for specific retina cell types differentiation. GCL: Ganglion cell layer, INL: Inner nuclear layer, ONL: Outer nuclear layer (Modified from Ohsawa and Kageyama, 2008).

Ciliary marginal zone

The Ciliary marginal zone (CMZ) is located at the peripheral edge of the adult retina and contains pluripotent retinal progenitor cells (Figure 9). These progenitor cells have the potential to differentiate into all retinal neural and glial cell types (reviewed in Perron and Harris, 2000). Initially, CMZ was thought to be a feature unique to amphibians and fish, whose bodies, including the retina, continue to grow throughout life. However, studies have identified the presence of CMZ or a homologous structure in chickens, mouse, and humans (Fisher and Reh, 2000; Mayer et al., 2000; Tropepe et al., 2000). Additionally, CMZ has been shown to play a significant role in retinal regeneration following injury (reviewed in Moshiri et al., 2004).

The most peripheral region of the CMZ is populated by uncommitted stem cells, which express transcription factors such as *Pax6*, *Rx1*, *Six3*, and *Chx10*. In contrast, cells located closer to the central region of the CMZ express proneural genes, indicating a more committed state toward their final differentiation (Perron et al., 1998; Raymond et al., 2006; reviewed in Amato et al., 2004). The Notch signaling pathway, which plays a critical role in maintaining the undifferentiated state of retinal progenitor cells (RPCs), shows its highest activity in the peripheral part of the CMZ, gradually diminishing toward the central region (Raymond et al., 2006).

A comprehensive study demonstrated that the retina of the teleost fish medaka also possesses a CMZ in its peripheral region, confirming that RPCs in this area are indeed pluripotent (Centanin et al., 2011).

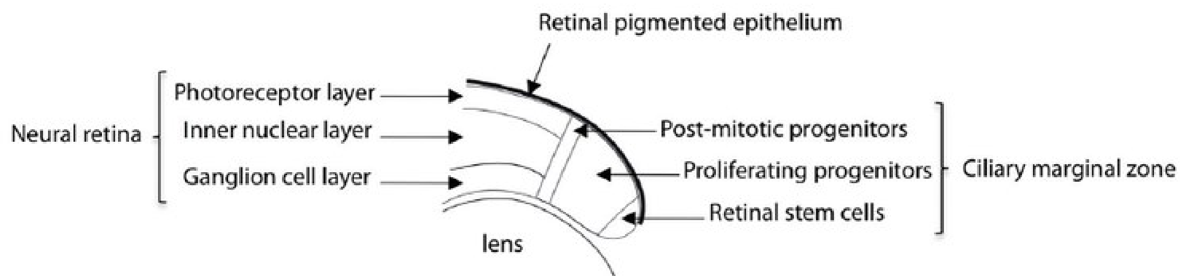

Figure 9: Schematic of a ciliary marginal zone in amphibia *Xenopus.* Retina stem cells are resided in the periphery, meanwhile proliferating and post-mitotic progenitors are located more towards the middle part of the retina (modified from Locker et al., 2010).

Structure of Pax6 gene/protein

The human *PAX6* gene is part of the pax gene family, which consists of nine highly conserved genes encoding transcription factors (reviewed in Kozmik, 2005). *PAX6* is located on chromosome 11p13, a 22kb long region and it consist of 14 exons (reviewed in Daniels et al., 2010) (Figure 10). Over the years, various upstream and downstream enhancers, as well as multiple alternative splicing variants, have been identified (Epstein et al., 1994; Williams et al., 1998; Kammandel et al., 1999; Griffin et al., 2002). Consequently, several isoforms of the Pax6 protein are present in the cell, with the "canonical PAX6" being the most abundantly expressed variant.

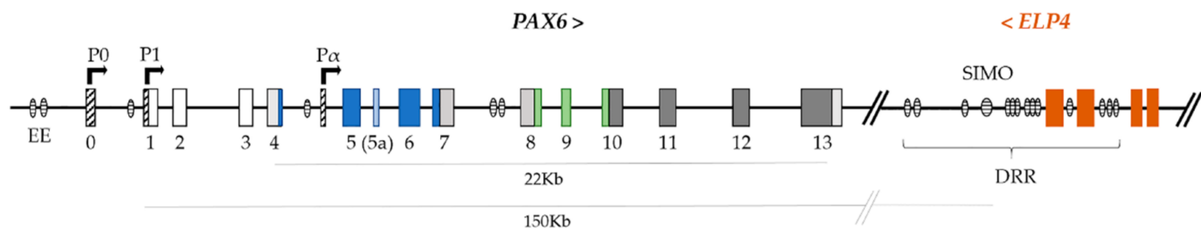

Figure 10: Structure of the human *PAX6* gene. Human *PAX6* gene contains 14 exons. Colours represent protein domains (PD - blue, HD- green, PST rich transactivation domain - dark grey). P0, P1 and Pα show the locations of promoters. Oval shapes represent regulatory elements including ectodermal enhancer (EE) and SIMO enhancer (located in the intron of the neighbour gene ELP4 – showed in orange). DRR: downstream regulatory region (Modified from Cunha et al., 2019).

The mammalian Pax6 protein consists of 422 amino acids forming two DNA-binding domains: the bipartite paired-type domain (PD) and the homeodomain (HD) (Ton et al., 1991; Glaser et al., 1992) (Figure 11). The N-terminal PD, a characteristic trait of all pax proteins, comprises 128 amino acids and is subdivided into two regions—the PAI and RED subregions (Czerny et al., 1993; Epstein et al., 1994). Each subregion consists of three α-helices linked by an extended polypeptide chain, two of which helices forming a helix-turn-helix DNA-binding motif (Czerny et al., 1993; Epstein et al., 1994; Xu et al., 1999). The PD is separated from the HD by a linker region. The C-terminal region of the HD includes a proline-serine-threonine-rich (PST) transactivation domain, which is capable of transcriptional activation (Czerny and Busslinger, 1995; Sheng et al., 1997; Tang et al., 1998). Similar to the PD, the HD is composed of three α-helices, two of which form a helix-turn-helix DNA-binding motif (Xu et al., 1999). These structural domains enable Pax6 to interact with DNA in three distinct manners: via the PD, HD, or a combination of both (Sheng et al., 1997; Xu et al., 1999).

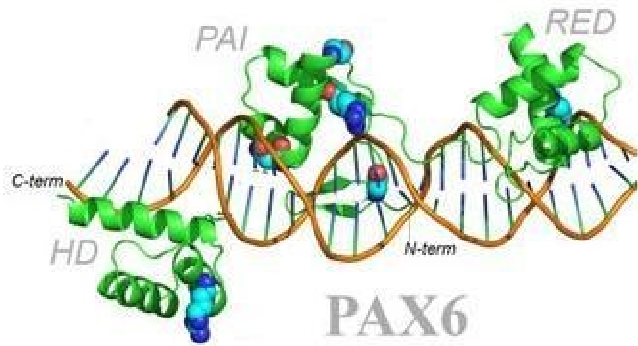

Figure 11: Model of the human PAX6 protein. Specific binding domain (PAI, RED, HD) binds to DNA. Linker between PAI and RED domain is not included in this model. (Modified from Xie et al., 2014).

Pax6 proteins exhibit an exceptionally high level of conservation at the amino acid level (Figure 12). The human and mouse Pax6 proteins are identical, while the chick and zebrafish proteins share 96% and 93% similarity, respectively. Additionally, there is over 80% homology between the human PAX6 protein and the *Drosophila* eyeless protein (reviewed in Gehring and Ikeo, 1999; van Heyningen and Williamson, 2002).

Figure 12: Comparison of amino acid sequences of Pax6 protein between human and other animals (lampetra, amphioxus, medaka -Oryzias, zebrafish – Danio, Fugu, Xenopus, Gallus and mouse) (Modified from Murakami et al., 2001).

The role of *Pax6* gene during the eye development

The crucial role of the Pax6 gene in embryonic eye development has been demonstrated repeatedly over the past decades. Research highlights its fundamental importance in eye formation across a broad range of species, from basal animals such as cnidarians (Piatigorsky and Kozmik, 2004), through invertebrates like Drosophila (Quiring et al., 1994), to more complex vertebrates including fish (Nornes et al., 1998), mouse (Hill et al., 1991), and human (Jordan et al., 1992). This evidence underscores not only the structural conservation of Pax6 across the animal kingdom but also its functional similarity in both vertebrates and invertebrates (reviewed in Callaert et al., 1997; Kozmik, 2005).

Drosophila

The Drosophila eyeless (*ey*) mutant was first described in 1915 (Hoge, 1915), but the link between the *eyeless* phenotype and the Pax6 gene was only uncovered 70 years later (Quiring et al., 1994). This finding was surprising, as it had been assumed that *Drosophila's* compound eye was not homologous to the vertebrate camera-type eye. These observations led to the proposal that Pax6 serves as a universal master control gene for eye development (Quiring et al., 1994).

The *eyeless* (*ey*) mutant is characterized by the absence of pigment and ommatidia or by a reduction in one or both eyes (Hoge, 1915) (Figure 13). In Drosophila, a second homolog of the mouse *Pax6* gene, known as *twin of eyeless* (*toy*), arose through gene duplication during insect evolution (Czerny et al., 1999). Studies show that *toy* and *ey* have nonredundant functions, with *toy* acting upstream of *ey* by directly regulating its enhancer (Czerny et al., 1999). Misexpression of either gene results in the formation of ectopic eyes (Halder et al., 1995; Czerny et al., 1999) (Figure 14). Moreover, ectopic expression of the mouse Pax6 gene in Drosophila produces similar effects (Halder et al., 1995). These findings strongly suggest that the role of Pax6 in eye development is highly conserved between the invertebrate Drosophila and vertebrate mouse.

Figure 13: Comparison of a head of WT drosophila and *eyeless* mutant drosophila. The *Pax6* KO (*eyeless*) does not develop eyes (Modified from Burgy-Roukala et al., 2013).

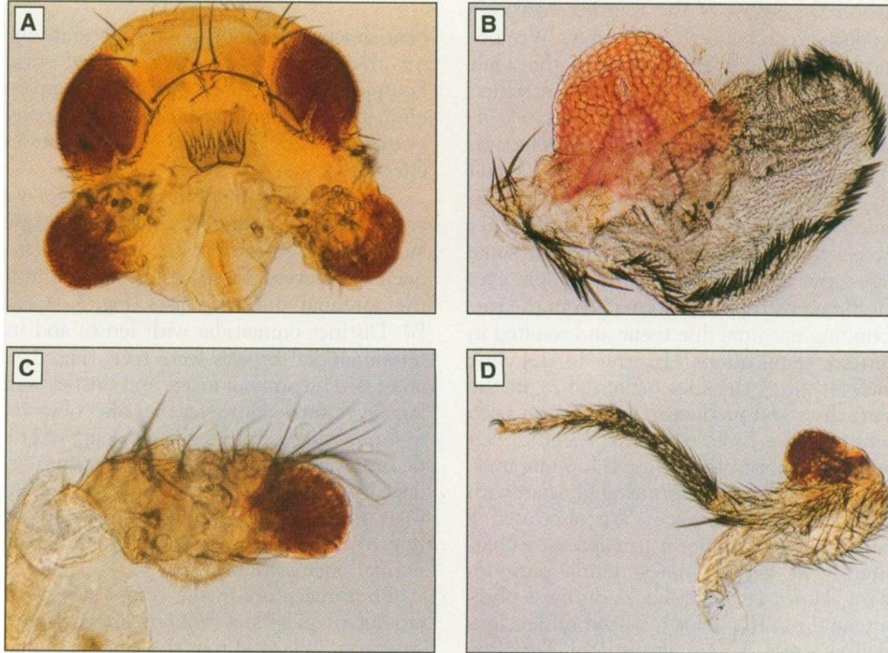

Figure 14: The ectopic expression of *eyeless* gene induces the formation of an eye structures in different tissues. A: Head, B: Wing, C: Antenna, D: Leg (Modified from Halder et al., 1995).

Mouse

The *Pax6* mutant in mouse is referred to as the "small eye" (*Sey*) mutant. In the *Sey* mutant, no eyes are present due to the arrest of eye development at the optic vesicle stage (Hill et al., 1991). Additionally, brain development is deformed, and the nasal cavities fail to develop properly (Figure 15). The *Sey* phenotype is lethal, as the pups cannot breathe while suckling (Hogan et al., 1986; Hill et al., 1991; Quinn et al., 1996). The severity of the *Pax6* mutation in mouse is dosage-dependent (Favor et al., 2008; Davis et al., 2009). Heterozygous *Pax6* mutants exhibit less severe phenotypes, which often include microphthalmia, hypoplastic iris, cataracts, corneal abnormalities, and glaucoma (Hogan et al., 1986; Lovicu et al., 2004; Kroeber et al., 2010).

Over the past decades, 43 germline mutations have been identified in the *Pax6* gene, with 190 distinct phenotypes characterized, primarily affecting the eye and neural system (The Jackson laboratory, 1996). Additionally, numerous *Pax6* knock-out alleles have been generated and analyzed using methods that allow for temporal and spatial gene deletion, such as the Cre-LoxP system (Marquardt et al., 2001; Klimova et al., 2013; L. Klimova and Z. Kozmik, 2014; Suzuki et al., 2015; Sunny et al., 2020). These findings underscore the critical role of *Pax6* in specific stages and processes of embryonic eye development.

There are numerous phenotypic similarities between *Pax6* mutants in mouse and patients carrying *PAX6* mutations (reviewed in Cunha et al., 2019). This presents a valuable opportunity to mimic and investigate PAX*6*-related human disorders, particularly aniridia, using the mouse model (Ramaesh et al., 2003; Simpson et al., 2017).

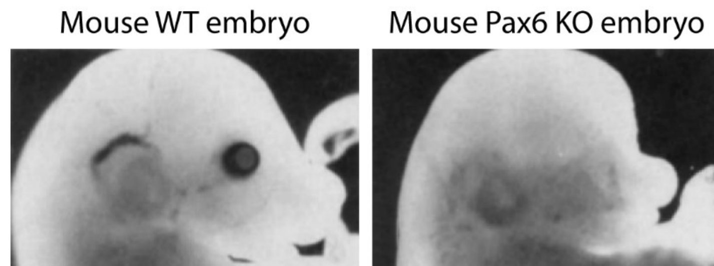

Figure 15: Comparison of wild type (WT) mouse embryo and *Pax6* mutant embryo at 15 days of development. The *Sey* homozygote embryo displays no signs of a developing eye and its snout is shortened (Modified from Hill et al., 1991).

Human

More than 500 distinct mutations have been identified in the human *PAX6* gene, according to The Human Gene Mutation Database (Cardiff, 2020). A significant number of these mutations are linked to phenotypes that negatively impact human health.

Aniridia is the most prevalent ocular disorder associated with mutations in the *PAX6* gene. This condition affects the development of the iris, lens, cornea, and/or optic nerve. Aniridia commonly manifests as partial or complete iris hypoplasia, cataracts, glaucoma, and nystagmus. In addition to aniridia, other non-aniridia phenotypes, such as isolated foveal hypoplasia, microphthalmia, anophthalmia, Gillespie syndrome, Peters anomaly, and others, have also been linked to *PAX6* gene mutations (reviewed in Cunha et al., 2019).

The *PAX6* gene is not exclusively active in the eye but also functions in other regions, including the brain, central nervous system, and pancreas. This broader expression helps explain why patients with mutations in the *PAX6* gene frequently exhibit neurodevelopmental abnormalities, such as attention deficit hyperactivity disorder (ADHD), autism, or intellectual disabilities (Chao et al., 2000; Malandrini et al., 2001; Davis et al., 2008).

Teleost fish

The genomes of vertebrates underwent two whole genome duplications (WGD) during evolution (Ohno, 1970), followed by the loss or functional modification of many newly duplicated genes. Teleost fish underwent an additional, third WGD (reviewed in Sato and Nishida, 2010; Glasauer and Neuhauss, 2014ss

2014), leading to the presence of multiple *Pax6* genes in their genomes. Both, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) belong to this group of teleost fish. Zebrafish possess two paralogues of *Pax6.1* (*Pax6.1a* and *Pax6.1b*) and one *Pax6.2* gene, whereas medaka contains one *Pax6.1*, one *Pax6.2*, and one *Pax6.3* gene (Ravi et al., 2013). The *Pax6.1* and *Pax6.3* genes exhibit the typical structure of Pax genes, encoding proteins with a paired-type domain and homeodomain. In contrast, the *Pax6.2* gene has lost the paired-type domain, leaving the protein with only the homeodomain and PST trans-regulatory domain. In both medaka and zebrafish, *Pax6.1* is considered the orthologue of the human *PAX6* gene.

Zebrafish

There are multiple *Pax6* genes present in the zebrafish genome, including two paralogues, *Pax6.1a* and *Pax6.1b*. Of these, *Pax6.1a* is considered the orthologue of the human *PAX6* gene (Krauss et al., 1991; Nornes et al., 1998; Ravi et al., 2013). Studies have shown that both *Pax6.1a* and *Pax6.1b* are capable of inducing the formation of ectopic eyes when misexpressed in the Drosophila imaginal disc, a structure that normally develops into wings or legs (Nornes et al., 1998).

The *Pax6.1b* mutant, known as *'sunrise* (*Sri*)*'* is characterized by abnormalities in lens and cornea structures, resulting in reduced eye size (Kleinjan et al., 2008; Takamiya et al., 2015) (Figure 16). However, unlike the *Pax6* mouse mutant, eyes are still present in the *Sri* mutant. The mutation in the *Sri* mutant is located in the homeodomain, and while experiments have demonstrated that the DNA-binding ability of the mutated protein is significantly reduced (Kleinjan et al., 2008), the unaffected paired-type domain may retain partial functionality. Another possible explanation for the observed phenotype could be functional redundancy between *Pax6.1a* and *Pax6.1b*. Microinjection of morpholino against *Pax6.1a* mRNA into the *Pax6.1b* germline mutant produces a range of eye-related phenotypes (Kleinjan et al., 2008), from normal eye development to an absence of eyes (Figure 17). Additionally, *Pax6.2*, which lacks a paired-type domain, is also present in the zebrafish genome. Morpholino experiments indicate that *Pax6.2* is crucial for proper eye development, as injection against *Pax6.2* mRNA results in a no-eye phenotype (Ravi et al., 2013) (Figure 18).

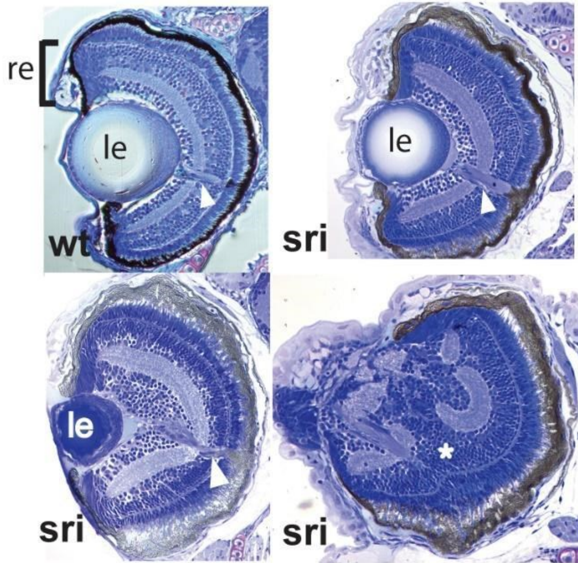

Figure 16: The section through the eye of the WT and *Pax6.1a* (*Sri*) mutant at 5 dpf. Smaller to no lens and malformed retina was observed in the mutant. Arrow is pointing at the optic nerve. re: Retina, le: lens (Modified from Kleinjan et al., 2008).

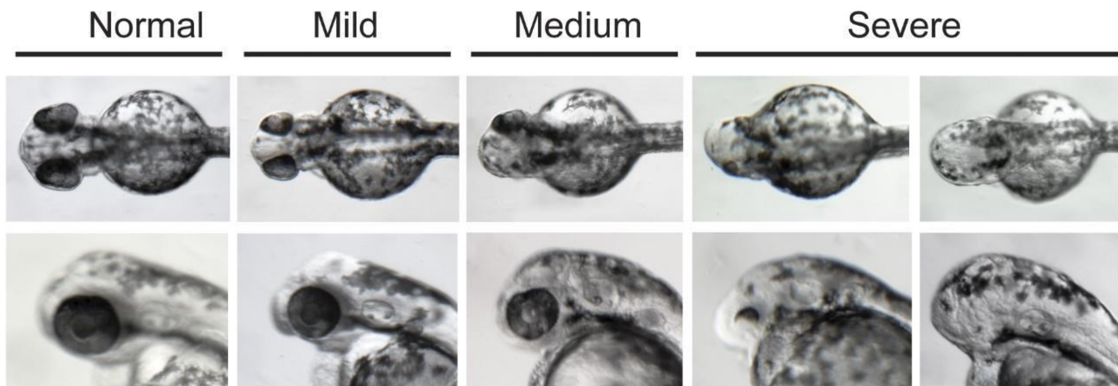

Figure 17: Morpholino injection against *Pax6.1b* mRNA in *Pax6.1a* germline mutant zebrafish embryo. Scale of phenotypes from very mild to severe – no eye phenotype can be observed (Modified from Kleinjan et al., 2008).

Figure 18: Comparison of embryo injected with non-specific morpholino and embryo after morpholino injection against *Pax6.2* mRNA at 2dpf. Embryo after morpholino knock-down is characteristic by eyes that are smaller or not developed at all (Modified from Ravi et al., 2013).

<u>Medaka</u>

Following the third fish-specific whole genome duplication (WGD), three pax6 genes are present in the medaka genome: *Pax6.1*, *Pax6.2*, and *Pax6.3*. *Pax6.1* is considered to be the orthologue of the human *PAX6* gene (Ravi et al., 2013) (Figure 19). Both *Pax6.1* and *Pax6.3* encode proteins that consist of the paired domain, homeo domain, and a C-terminal proline-serine-threonine (PST)-rich transactivation domain. However, *Pax6.2* lacks the paired domain. Comparative analysis of the amino acid structure of Pax6 proteins revealed significant similarities between the corresponding regions of these proteins (Figure 20).

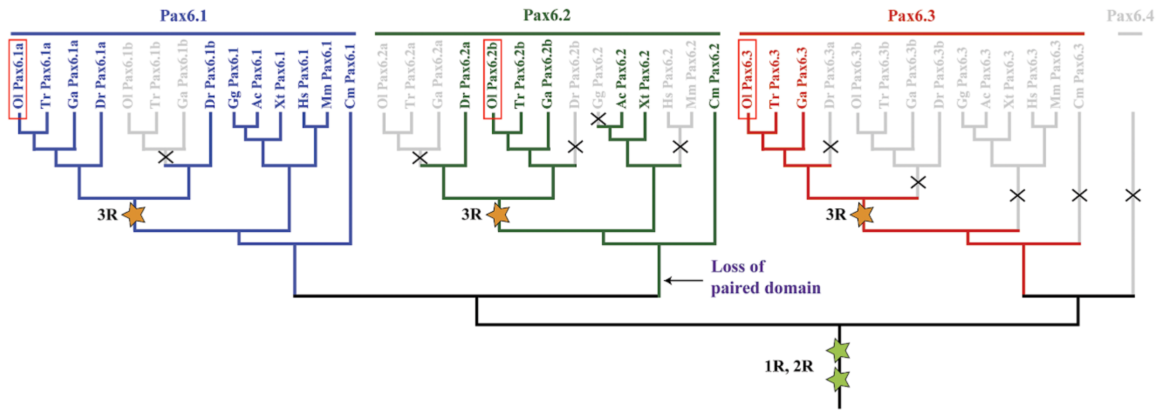

Figure 19: Two whole genome duplications (1R, 2R) at the beginning of vertebrate evolution gave rise to four pax6 genes (*Pax6.1*-*Pax6.4*). Pax6.4 as well as other pax6 genes were independently lost in different vertebrate linages. Only one *Pax6* gene is present in mammals and avian, whereas the *Pax6.2* gene is additionally present in *Xenopus*, lizard and teleost fish. After the 3th fish specific WGD the three pax6 gene were duplicated in teleost fish. As a results, one copy of each pax6 gene (*Pax6.1, Pax6.2, Pax6.3*) is present in medaka genome. The three WGD are marked by the stars. Medaka pax6 genes are highlighted in the scheme by red boxes. Hs: *Homo sapiens* (human), Mm: *Mus musculus* (mouse), Gg: *Gallus gallus* (chicken), Ac: *Anolis carolinensis* (lizard), Xt: *Xenopus tropicalis* (frog), Dr: *Danio rerio* (zebrafish), Ol: *Oryzias latipes* (medaka), Tr: *Takifugu rubripes* (fugu), Cm: *Callorhinchus milii* (elephant shark) (Modified from Ravi et al., 2013).

A

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pax6.1  MMQNTGQEVYDISDEGHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILQTHDEVQVLDSEKVSNGCVSKILG    82
pax6.2  ----------------------------------------------------------------------------------     0

pax6.1  RYYETGSIRPRAIGGSKPRVATPEVVAKIAQYKRECPSIFAWEIRDRLLSEGICTNDNIPSVSSINRVLRNLASEKQQMGAD   164
pax6.2  ----------------------------------------------------------------------------------     0

         ** ** *** * *  *     **               **                 *             ** *  ****
pax6.1  GMYDKLRMLNGQTG-TWGTRPGWYPGTSVPG---QPNQDGCQQ-----------QDGAGENTNSISSNGEDSEETQMRLQLK   231
pax6.2  -MYEKLTMLNLQSGSNWSGPNNWYHDPTGVQTQHSSVSQGCLLDTEGSMGGETGGGGSGRGGGGVPVERDEGEESQLRLQLK    81

        *********************************************************************       * *  *
pax6.1  RKLQRNRTSFTQEQIEALEKEFERTHYPDVFARERLAAKIDLPEARIQVWFSNRRAKWRREEKLRNQRRQANN---SSSHIP   310
pax6.2  RKLQRNRTSFTQEQIEALEKEFERTHYPDVFARERLANKIDLPEARIQVWFSNRRAKWRREEKLRNQRRSGGVTSCSQSQAP   163

         * *******             *****    * *   ** *     *         *   ******** **
pax6.1  ISSSFSTSVYQAIPQPTTPVSFTSGSMLGRPDSALTNTYSALPPMPSFTMANNLPMQPSQTSSYSCMLPTSPPVNGR-SYET   391
pax6.2  LSTSFSTSVYHQQHG------SSSGSMLSQAESPLPS-YSSLSV---F-SSGVQSIPPQSASSYSCMLPPSPSAPRSFDSSP   234

        *  ** *          *    **************** *** **    *******
pax6.1  YTPPHMQAHMNSQSMTTSGTTSTGLISPGVSVPVQVPGSEPD-MS----QYWSRLQ                             442
pax6.2  YSSPHLQT-------PPSANPNTGLISPGVSVPVQVPGTEPQSMSQNLGQYWARLQ                             283
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B

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                       *       ****************************************** **************
pax6.1  -------MMQNTGQEVYDISDEGHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRILKVSNGCVSKILGRYY    73
pax6.3  MPQKDYRSPASWETGVASMMQNSHSGVNQLGGVFVNGRPLPDSTRQKIVELAHSGARPCDISRIHQVSNGCVSKILGRYY    80

        ********************************************** ** * ******************* *****
pax6.1  ETGSIRPRAIGGSKPRVATPEVVAKIAQYKRECPSIFAWEIRDRLLSEGICTNDNIPSVSSINRVLRNLASEKQQMG---   150
pax6.3  ETGSIRPRAIGGSKPRVATPEVVAKIAQYKRECPSIFAWEIRDRLLAEGVCSNDNIPSVSSINRVLRNLASDKQQMGSLG   160

          ****** ***     ** *  ** **      *   ***  *      *  *  ************************
pax6.1  ADGMYDKLRMLNGQTGTWGTRPGWYPGTSVPGQPNQDGCQQQDGAGENTNSISSNGEDSEETQMRLQLKRKLQRNRTSFT   230
pax6.3  TEGMFDKLKMLNVH-TSWGGRSSWYAGTTLSSTDNKSSCQQAEGRE-NGISVNSSTEDSEETQMRLQLKRKLQRNRTSFT   238

        ***********************************************************   ************ * **
pax6.1  QEQIEALEKEFERTHYPDVFARERLAAKIDLPEARIQVWFSNRRAKWRREEKLRNQRRQANNSSSHIPISSSFSTSVYQA   310
pax6.3  QDQIEALEKEFERTHYPDVFARERLANKIDLPEARIQVWFSNRRAKWRREEKLRNQRRQISSSSSHIPISSSFSSSAYQL   318

         ***  **** ********* ** * **                                 * **** ******** *
pax6.1  IPQPTTPVSFTSGSMLGRPDSALTNTYSA-------------------------------LPPMPSFTMANNLPMQPSQT   359
pax6.3  LPQPAAPVSFSSGSMLGRSDPALSNSYSLTTSTGLISPGVSVPVQVPGSEPDMSQYWSRLQPAMPSFSMAASLPMQASSQ   398

         ********** *  ************ *  ***** * * ******************* **********
pax6.1  SSYSCMLPTSPPVNGRSYETYTPPHMQAHMNSQSMTTSGTTSTGLISPGVSVPVQVPGSEPDMSQYWSRLQ           430
pax6.3  ASYSCMLPTSPTVNARSYETYTPPHMQPHLSSQSMTSSATSSTGLISPGVSVPVQVPGSEADMSQYWPRLQ           469
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Figure 20: A: Comparison of the aminoacid structure of Pax6.1 and Pax6.2 protein. B: Comparison of the aminoacid structure of Pax6.1 and Pax6.3 proteins. High homology can be observed in the homeo domain area of all three pax6 protein as well as in the paired domain between the Pax6.1 and Pax6.3 proteins (data from ensemble 13/08/2019).

There is limited knowledge regarding the Pax6 genes in medaka. To date, neither the complete expression patterns nor the precise roles of these genes during embryonic eye development have been fully characterized. The only study published on *Pax6.1* in medaka reported a failure in lens formation (Pan et al., 2023), but this research lacked detailed molecular characterization, including the analysis of specific marker expression. Additionally, no assessment of retinal tissue development or characteristics was conducted in this study.

Aims of the study

The *Pax6* gene is considered to be a crucial factor in eye development. Its structural conservation across species, from vertebrates like humans, mouse, chickens, and zebrafish to invertebrates such as Drosophila and amphioxus, has been well-documented. Numerous studies, particularly in mouse, have demonstrated the essential role of *Pax6* in the embryonic development of both the lens and retina. Experimental data strongly suggest that the *Pax6* gene's function in eye development is highly conserved, extending from vertebrate species, like the mouse, to invertebrates, like Drosophila. This points to an ancestral role for *Pax6* across the animal kingdom. However, to fully support this hypothesis, more data from species belonging to different phylogenetic groups are needed.

In teleost fish, particularly medaka (*Oryzias latipes*), little is known about the specific roles of pax6 genes. Bioinformatic studies have revealed the existence of three pax6 genes (*Pax6.1*, *Pax6.2*, and *Pax6.3*) in the medaka genome. Partial expression analyses have indicated their presence in the developing eye, among other tissues, suggesting the involvement of all pax6 genes in eye formation. Nevertheless, the precise function of these genes during embryonic eye development in medaka remains to be fully elucidated.

The specific aims of this project were:

- To characterize the expression patterns of all pax6 genes in the eye region during medaka embryonic development.
- To generate knock-out lines for the *Pax6.1*, *Pax6.2*, and *Pax6.3* genes.
- To investigate the role of pax6 genes in lens and retina development, with a primary focus on the function of *Pax6.1*, which is considered analogous to the human *PAX6* gene.

Materials and methods

Fish stock

The medaka fish were raised under a controlled 14-hour light/10-hour dark photoperiod at a constant temperature of 28°C. Embryos were incubated in ERM solution at either 28°C or 18°C, and developmental stages were determined following the standardized staging system established by Iwamatsu (Iwamatsu, 2004).

Generation of mutants

Mutants for the *Pax6.1* and *Pax6.3* genes were generated using the TALENs technique. One-cell stage embryos were injected with TALENs specifically designed to target selected DNA regions (Table 2). For the generation of *Pax6.2* mutants, the CRISPR-Cas9 method was employed (Table 3), and mutants in the ectodermal enhancer region of the *Pax6.1* gene were similarly created using the CRISPR-Cas9 system (Table 3). All TALEN and CRISPR-Cas9 constructs were engineered by Zbyněk Kozmik based on previously established frameworks (Moore et al., 2012; Varshney et al., 2016).

To establish the *Pax6.1* ectoderm enhancer transgenic line, one-cell stage embryos were injected with a plasmid mix where GFP expression was driven by the *Pax6.1* ectoderm enhancer. For plasmid construction, the cDNA sequence corresponding to the Pax6.1 ectoderm enhancer was synthesized by annealing oligo DNAs (Table 4).

Injected embryos were raised and subsequently crossed with wild-type (WT) fish. F1 offspring were genotyped and sequenced for the desired mutations.

Gene	Sequence
Pax6.1	TTGGTGGCGTGTTTGTTAAtggaagaccgctgccGGATTCCACCAGGCAGAAAA
Pax6.3	TGGGAGACCTCTGCCCGACTccaccaggcagaagaTCGTGGAGCTGGCCCACA

Table 2: Sequences of *Pax6.1* and *Pax6.3* genes targeted by TALENs.

Gene	Sequence
Pax6.2	TTTCCAACCGGAGAGCCAAGTGG AGGAGGTGCTCAGAGGGGCTTGG
Pax6.1 ectoderm enhancer area	CGAACTGCATCTGAAAGTGCAGG TAATGTCTCGATCCAGGGCCAGG

Table 3: Sequences of *Pax6.2* gene and *Pax6.1* ectoderm enhancer area targeted by the Crispr-Cas9 assay.

	Forward primer	Reverse primer
Pax6.1 ectoderm enhancer area	ACTCGGAGTGAAGCAGGA CAC	CGCCTCTCCCTTTTGTTTCTC

Table 4: Primers used for amplification of the *Pax6.1* ectoderm enhancer area.

Genotyping

DNA was extracted from adult fish (using a small tail sample) or embryos (using either part of the tail or the whole embryo) through Proteinase K digestion (Roche). The extracted DNA served as a template for PCR amplification, which was carried out using 2xMasterMix (ThermoScientific) and the corresponding primers (Table 5). The resulting PCR products were then separated by agarose gel electrophoresis.

	Primer	
Gene	Forward	Reverse
Pax6.1	GGTCTCCTCGCAGGTCACAGT	CCCAGGAGGAAATGGTCATA
Pax6.2	CAGATGTTTTGCGAGGGAGA	GCCGTGCTGCTGATGATAAAC
Pax6.3	CTGACCCTTTGGTTTGTGTGC	GGATTCTGGAAATGTCACATGG
GFP	ACGTAAACGGCCACAAGTTC	AAGTCGTGCTGCTTCATGTG
Pax6.1 ectoderm enhancer area	ACTCGGAGTGAAGCAGGACAC	CGCCTCTCCCTTTTGTTTCTC

Table 5: Primers used for genotyping.

Molecular cloning, vectors and RNA probe synthases

The targeted gene regions were amplified by PCR using medaka cDNA as a template and cloned into the BluntII-TOPO vector (Invitrogen). RNA probes were synthesized using SP6/T7 RNA polymerase (Roche), with plasmid DNA serving as the template and DIG/FITC labeling mix (Roche) to generate digoxigenin (DIG) or fluorescein (FITC) labeled antisense riboprobes.

Cell nucleus staining

Embryos at selected developmental stages were fixed overnight at 21°C in 4% formaldehyde/PBS + 0.1% Tween, followed by manual dechorionation. Cell nuclei were stained using DAPI (Roche, 1:1000) with overnight incubation. Stained embryos were subsequently stored in 86% glycerol.

Whole-mount In situ hybridisation

Embryos at selected developmental stages were fixed overnight at 21°C in 4% formaldehyde/PBS + 0.1% Tween, followed by manual dechorionation and storage in methanol. For experimental processing, samples were rehydrated from methanol to PBS + 0.1% Tween and treated with Proteinase K to enhance probe penetration. Embryos were then processed for overnight hybridisation at 65°C with digoxigenin (DIG) or fluorescein (FITC) labelled antisense riboprobes (Table 6), followed by several washes. After hybridisation, samples were incubated with anti-DIG or anti-FITC Fab fragments (Roche), conjugated to alkaline phosphatase or peroxidase, respectively. The signal was developed using either VectorBlue (VECTOR Laboratories) or TSA™ Plus Fluorescein System (PerkinElmer). Finally, all samples were counterstained with DAPI and stored in 86% glycerol.

	Primer	
Probe	Forward	Reverse
Ath5	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Brn3C	Gift from Joachim Wittbrodt, Heidelberg, Germany	
c-Maf	GGCATCAGAGCTGGCAATGAG	CTGCATGAGCTGCGTCTTCTC
Crx	GAGGTGGCCCTCAAGATCAAC	TGTGGTCCAGGCAGTCCATAG
FoxE3	GGAGCGGTTTCCGTTTTACAG	GCATGTAGCCGCTGTTGTAGG
HuC/D	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Islet2	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Mab21l2	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Maf-B	GCCCTCTGGCTCTGGAATATG	CATCAGCTGCTGCTTCTCGTT
Meis1	GGTACGAAGACTTGCCCCACT	TCACCGTAAGGTGCTGGAAGA
Meis2	CACCGACCGTCATTCATTCAT	ATCGTGGTCTCTCCAGGAAGC
NeuroD1	Gift from Joachim Wittbrodt, Heidelberg, Germany	
NR2E3	AATGGCTGTAGCGGCTTCTTC	ACCTGCTCTGGGTCTTTGAGG

Nrl	CCAGACACCATGTCCTCTCCA	CTGAACTCAGAGGAAGTAGTC
Oc1	CGACAAATTTCCTCCTCATCA	CGCGTTCATAAAGAAGTTGCT
OcL	TTGGCACCAACTTCTACAACC	CAGGACACTGCAGAAGAGGAG
Otx1	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Otx2	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Pax6.1	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Pax6.2	ATGTATGAGAAGTTAACCATGCTGA	CCTTCTCTCCCTATCCTCTTTTCTT
Pax6.3	CTTTGCCTGGGAGATCAGAG	GACGCCAGGAGAGATCAGAC
Prox1a	ACGGATCAGACAACGACTCCA	CTTTCAGGCGGTATGGAAAGG
Rhodopsin	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Rx1	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Rx2	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Rx3	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Six3	Gift from Joachim Wittbrodt, Heidelberg, Germany	
Sox2	Gift from Joachim Wittbrodt, Heidelberg, Germany	

Vsx1	Gift from Joachim Wittbrodt, Heidelberg, Germany
Vsx2	Gift from Joachim Wittbrodt, Heidelberg, Germany

Table 6: List of primers used for the DNA amplification.

Vibratome sectioning

Embryos (either whole or isolated eyes) following whole-mount in situ hybridisation or DAPI staining were embedded in 4% low melting point agarose (Serva) and sectioned using a vibratome (Leica). Sections of 50 µm thickness were obtained and subsequently mounted on slides in 86% glycerol for further analysis.

Spur epoxy resin sectioning

Eyes from WT and Pax6.1 homozygous mutant embryos were fixed overnight in 4% formaldehyde/PBS + 0.1% Tween, followed by washing in PBS + 0.1% Tween. The samples were gradually dehydrated in increasing concentrations of ethanol (up to 100%). After dehydration, the samples were embedded in Spurr epoxy resin and polymerized at 70°C for 48 hours. The resin blocks were trimmed around the region of interest and sectioned to a thickness of 350 nm using a diamond knife on a Leica EM UC7 ultramicrotome. The sections were mounted on slides and stained with a mixture of toluidine blue and methylene blue.

Immunohistochemistry staining

Embryos were fixed overnight at 21°C in 4% formaldehyde/PBS + 0.1% Tween and manually dechorionated. Whole-mount immunohistochemistry was subsequently performed as described. To enhance permeability, embryos were treated with ice-cold acetone for 7 minutes and then washed several times with PBS + 0.1% Tween. The samples were blocked overnight at 4°C using a Phosphorylated Histone 3 (PH3) antibody (Merck) diluted in 10% BSA/PBS + 0.1% Tween (1:500). The following day, the embryos were washed with PBS + 0.1% Tween and incubated with the Alexa647 secondary antibody (ThermoFisher) for 1.5 hours. DAPI staining was performed (as described above), and samples were sectioned using a vibratome machine (see above). The sections were imaged using a high-speed confocal Dragonfly spinning disc microscope (Andor). The total number of cells (stained by DAPI) and the number of proliferating cells (stained by PH3) in the retinas of eight wild-type and eight Pax6.1 knockout embryos were manually counted using ImageJ software (Schindelin et al., 2012). The percentage of proliferating cells was calculated by dividing the number of proliferating cells by the total number of cells for each sample.

Imaging

Images of the head and eye anatomy of embryos at 10 days post-fertilization (dpf) were captured using an Axio Zoom microscope (Zeiss). Embryos subjected to cell nucleus (DAPI) staining and vibratome sectioning were mounted in 86% glycerol on slides, while embryos post-whole-mount in situ hybridization

were embedded in 1.5% low-melting-point agarose (Serva) in wells. All samples were imaged using a high-speed confocal Dragonfly spinning disc microscope (Andor). The images were processed using ImageJ software (Schindelin et al., 2012).

Results

The expression of pax6 genes during embryonic development of medaka

The *Pax6* gene is recognized as a master regulator of eye development, encoding a transcription factor that plays a crucial role in this process. Pax6 protein is highly conserved across species, with structural homology well-documented in various animals (see above, Murakami et al., 2001). In medaka, three pax6 paralogues—*Pax6.1*, *Pax6.2*, and *Pax6.3*—are present due to three rounds of whole-genome duplication (Ravi et al., 2013). The *Pax6.1* gene is considered the orthologue of the human and mouse *Pax6* gene. Although the *Pax6* gene has been extensively studied in Drosophila, mice, and other organisms, there is limited knowledge regarding the pax6 genes in medaka.

Previous studies have only partially described the expression of *Pax6.1* and *Pax6.3* during medaka embryogenesis (Ravi et al., 2013), while the expression of *Pax6.2* remains unpublished to date. To elucidate the roles of the pax6 genes during medaka embryonic eye development, we first conducted a detailed characterization of their expression patterns at all relevant developmental stages. These expression profiles were determined using *in situ hybridization*, which allowed for the visualization of mRNA transcripts of the targeted genes.

Expression pattern of *Pax6.1* gene

The complete expression pattern of the *Pax6.1* gene is shown in Figure 21. *Pax6.1* expression is first detected at stage 17, coinciding with the formation of the embryonic body. At this stage, the gene is expressed in the developing brain, particularly in the midbrain and forebrain regions, including the area where the future eyes will form. During the early stages of medaka embryogenesis (stages 18-24), *Pax6.1* continues to be expressed in the midbrain and the diencephalon, a region of the forebrain. The expression persists in the brain at all later developmental stages examined (stages 28, 32, and 36).

In addition to the brain, *Pax6.1* is expressed in the eye primordium and developing eye (retina and lens) during the early stages of development (stages 17-24, 28). Its expression remains detectable in the eye at later stages, coinciding with the differentiation of specific retinal cell types (stages 32 and 36). To determine which retinal cell types express *Pax6.1*, we performed double in situ hybridization for *Pax6.1* and genes known to be selectively expressed in distinct retinal cell types (Figure 22). Specifically, *Brn3C* was used as a ganglion cell marker (Xiang et al., 1995; Jain et al., 2012), *HuC/D* as a marker for ganglion, amacrine, and bipolar cells (Marusich et al., 1994; Ekstrom and Johansson, 2003), and *Meis1* as a marker for amacrine and bipolar cells (Heine et al., 2008). *Sox2* was used to label Müller glia cells (Lin et al., 2009), while *Rx2*

served as a marker for photoreceptors (Mathers et al., 1997). *Sox2* and *Rx2* are also expressed in the ciliary marginal zone (Reinhardt et al., 2015; Miles and Tropepe, 2021).

Double staining revealed co-expression of *Pax6.1* with *Brn3C*, *HuC/D*, and *Meis1* in the ganglion cell layer and the inner nuclear layer. Notably, *Pax6.1* was also co-expressed with *Sox2* and *Rx2* in the ciliary marginal zone. These findings collectively suggest that the *Pax6.1* gene plays a critical role in early embryonic eye development and the specification of retinal cell types.

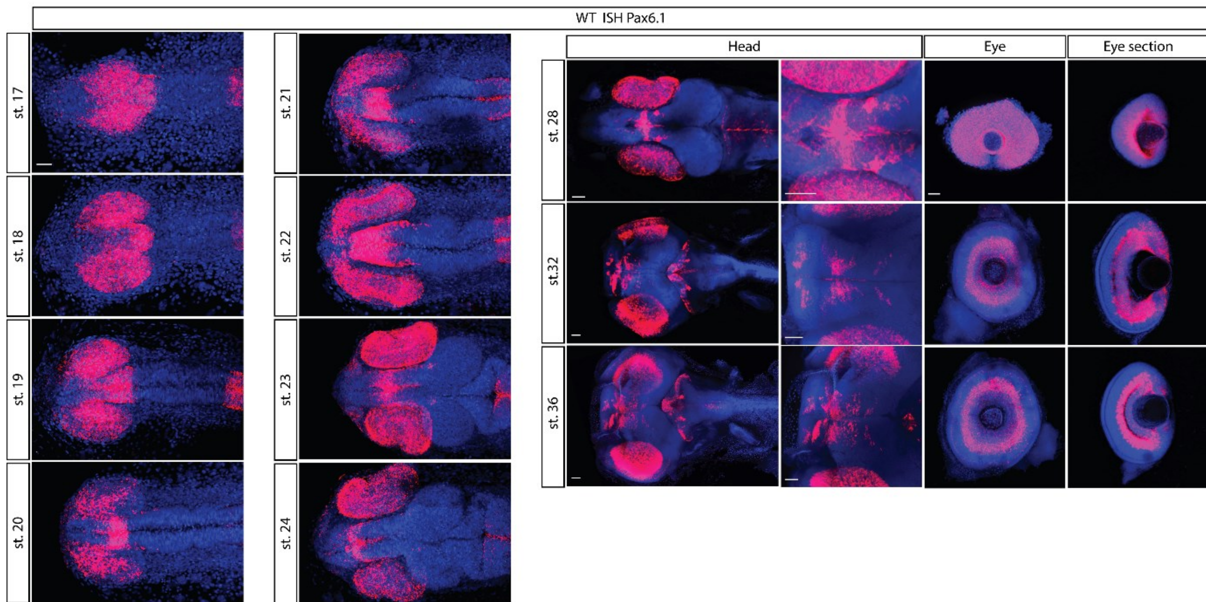


Figure 21: The expression pattern of the *Pax6.1* gene during embryonic development of medaka. Pax6.1 gene is expressed in the developing brain (midbrain and front brain) and the developing eye. Scale bar: 50µm

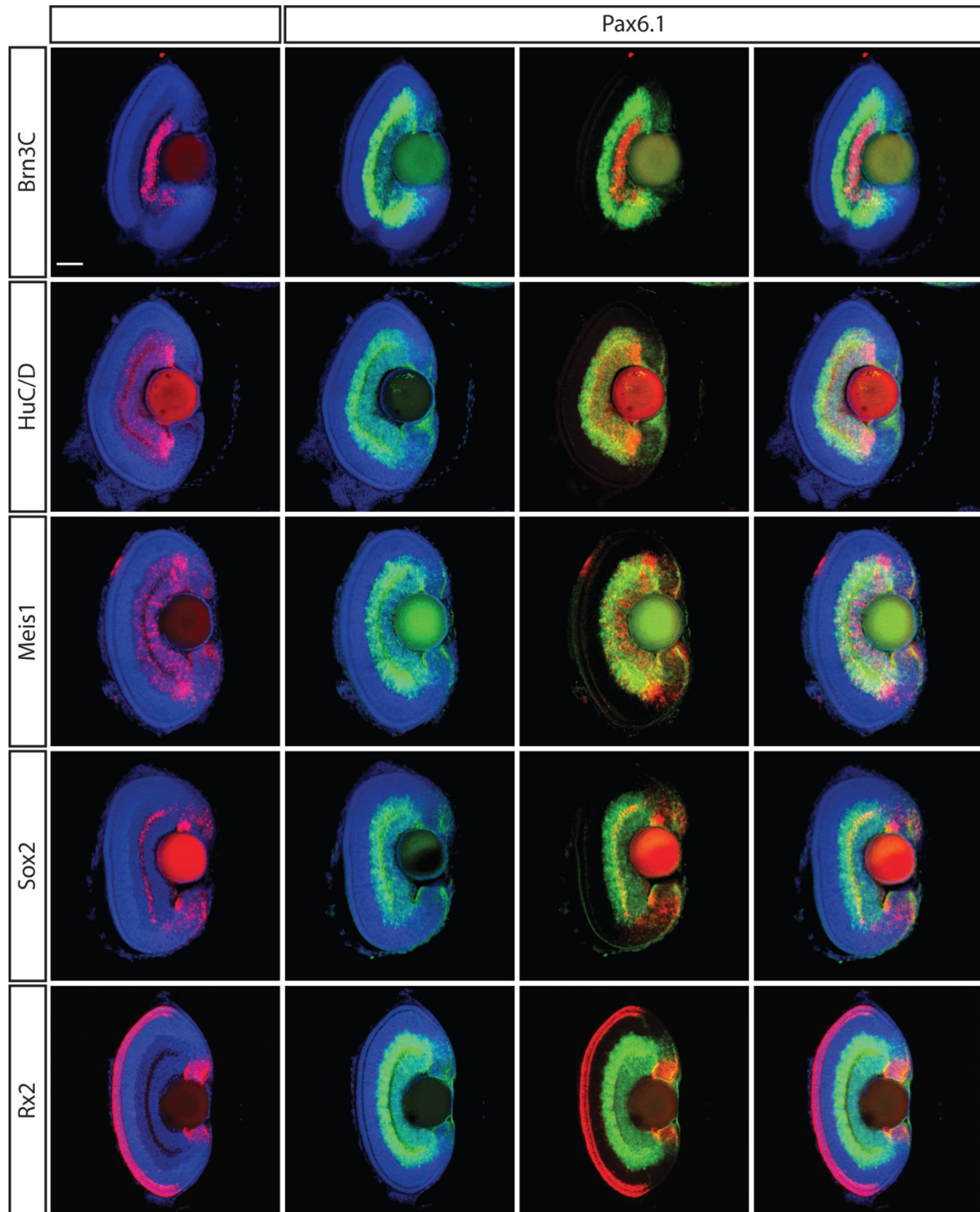


Figure 22: Double *in situ hybridisation* at stage 32 for *Pax6.1* and *Brn3C*, *HuC/D*, *Meis1*, *Sox2* or *Rx2*, respectively. Staining shows the co-expression of *Pax6.1* with *Brn3C*, *HuC/D* and *Meis1*. Partial co-expression of *Pax6.1* with *Sox2* and *Rx2* in the ciliary marginal zone was detected. 50µm thick vibratome sections after the whole mount *in situ hybridisation*. Scale bar: 50µm

Expression pattern of *Pax6.2* gene

The expression pattern of the *Pax6.2* gene is shown in Figure 23. Unlike *Pax6.1*, *Pax6.2* is not expressed during the early stages of head and eye development. Its expression becomes detectable in the eye only after the lens and retina are already partially specified (stages 22-24). At later stages (28, 32, 36), *Pax6.2* is expressed in the ganglion cell layer and the inner nuclear layer of the retina. No *Pax6.2* expression was observed in the brain at any developmental stage examined. These expression patterns suggest that *Pax6.2* may play a role in the differentiation of specific retinal cell types but is likely not involved in the early stages of eye development.

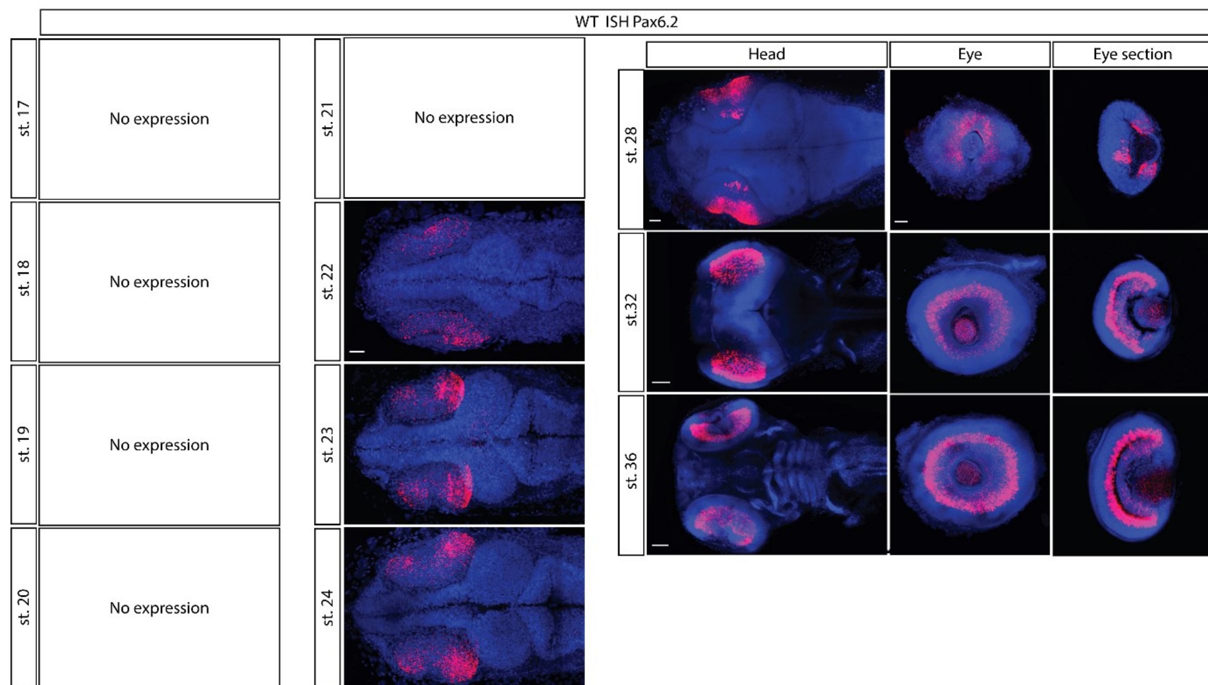


Figure 23: The expression of *Pax6.2* during the medaka embryonic development. *Pax6.2* is first expressed at stage 22 – after the eye formation was already initiated. The expression in the eye can be observed also at later stages. Scale bar: 50µm

Expression pattern of *Pax6.3* gene

The expression pattern of the *Pax6.3* gene is shown in Figure 24. *Pax6.3* is expressed from the onset of embryonic body formation (stage 17). Its signal is consistently detected in the forebrain region throughout all examined stages of development (stages 17-36). Additionally, *Pax6.3* expression is observed in the posterior domain of the developing eyes during early stages (stages 18-22). However, by the later stages

(stages 23-36), no expression is detected in the eye. This suggests that *Pax6.3* may play a role in early eye development in medaka.

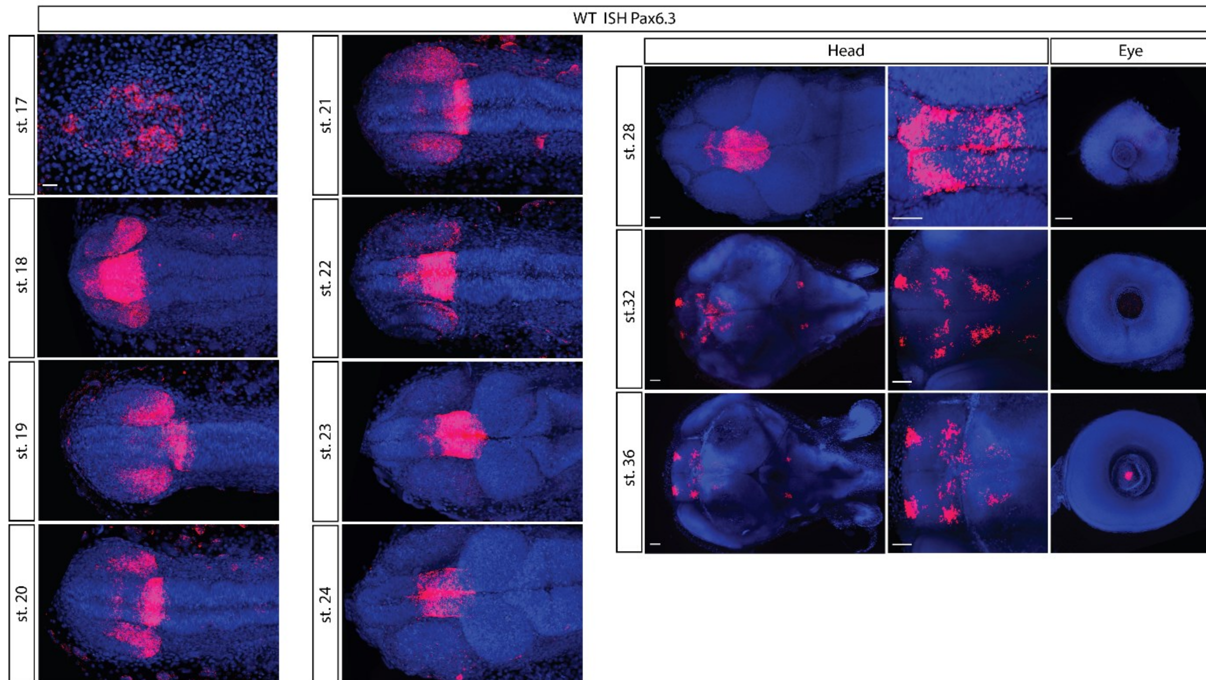


Figure 24: Expression pattern of *Pax6.3* gene during embryonic development of medaka. The expression is detected early as the embryonic body starts to develop. *Pax6.3* is expressed in the front brain and during the early stages also in the posterior domain of the eye. Scale bar: 50µm

Generation of mutants

Mutant of *Pax6.1* gene

The *Pax6.1* mutant was generated using TALENs, specifically designed to introduce a double-stranded DNA break within the region encoding the PAIRED domain (Figure 25). This approach resulted in the insertion of a 31bp sequence in exon 4. The insertion causes a frameshift in the amino acid sequence, leading to the introduction of a premature stop codon and likely resulting in a truncated, non-functional protein.

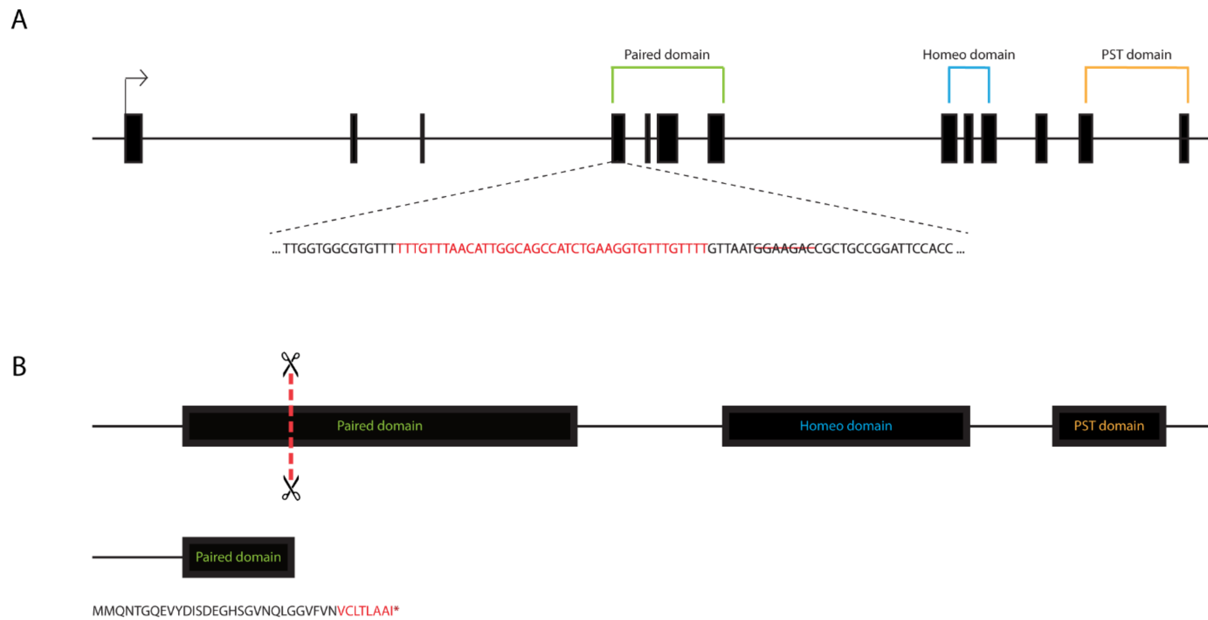
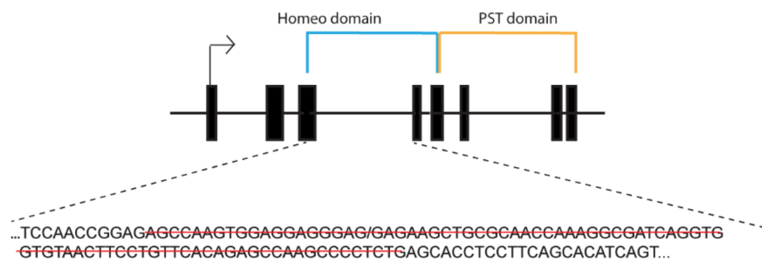


Figure 25: Schematic representation of mutation caused in *Pax6.1* gene subsequently leading to changes in the protein structure.

Mutant of *Pax6.2* gene

The *Pax6.2* mutant was generated using CRISPR-Cas9 technology (Figure 26). A targeted double-stranded break was introduced in the homeodomain region, specifically in exon 5, resulting in an out-of-frame deletion of 97bp. This mutation alters the amino acid sequence of the Pax6.2 protein, leading to the incorporation of a premature stop codon. As a consequence, the mutant protein lacks a portion of the homeodomain and the entire PST-rich transactivation domain, likely making it non-functional.

A



B

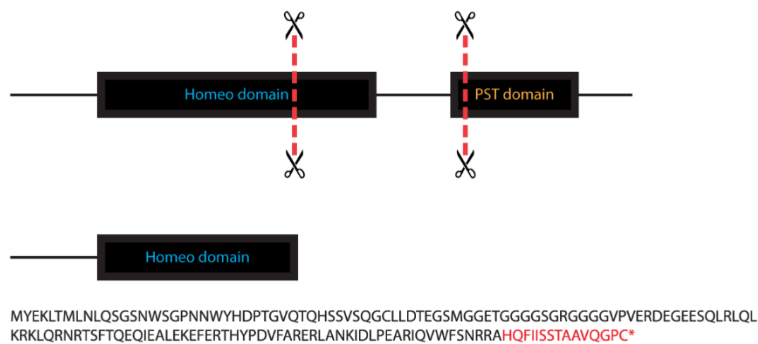


Figure 26: Schematic representation of mutation generated in *Pax6.2* gene and the structure of a protein in *Pax6.2* mutant.

Mutant of Pax6.3 gene

Similar to the *Pax6.1* mutant, the *Pax6.3* mutant was generated using the TALENs method (Figure 27). TALENs targeted exon 3, which encodes part of the PAIRED domain. A 16bp deletion was induced in the *Pax6.3* gene, resulting in an altered amino acid sequence and the introduction of a premature stop codon. Consequently, none of the functional binding domains within the PAIRED domain are present in the Pax6.3 mutant protein.

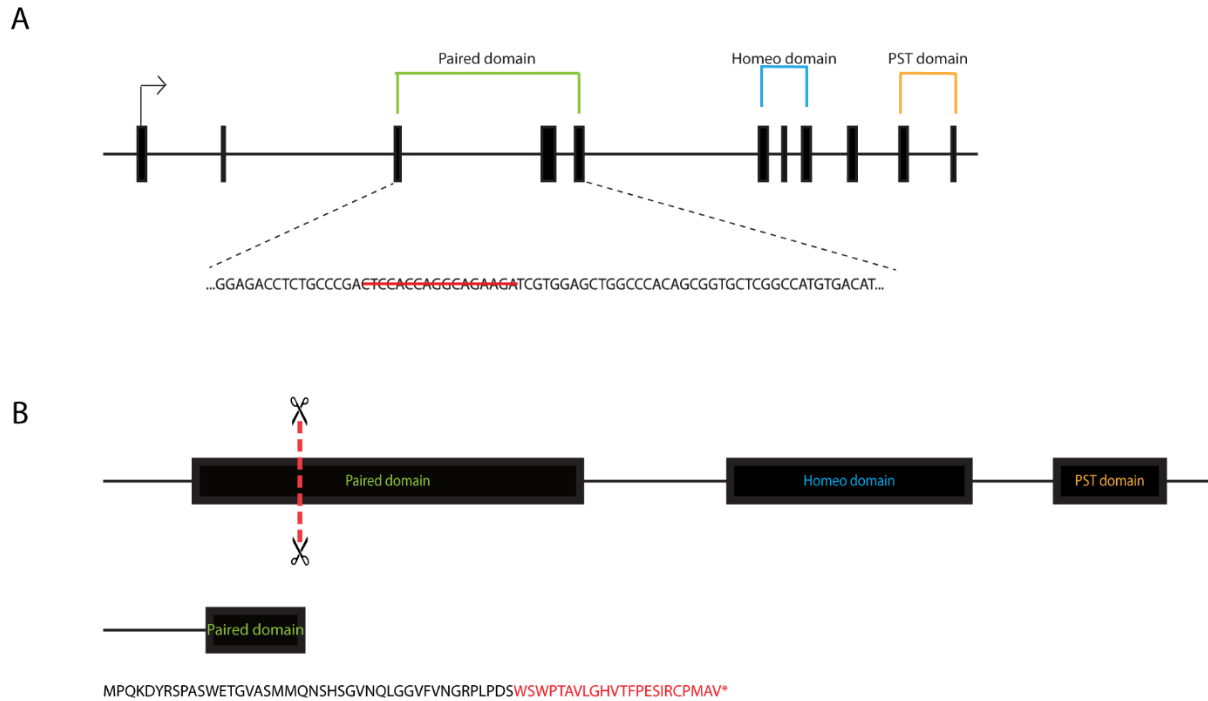


Figure 27: Schematic representation of mutation present in *Pax6.3* mutant. Deletion generated by Talens leads to changes in amino acid structure of the protein and the appearance of the early stop codon.

Morphological analysis of pax6 mutants

Comprehensive morphological analysis of Pax6 mutants was conducted following the generation of the mutants. DAPI staining was employed to enhance the visualization of morphological structures. Stage 23 (Figure 28) was chosen as a representative stage for analysis, as the eyes are already developed at this stage, although differentiation of specific retinal cell types has not yet started. Examination of *Pax6.1* mutants revealed abnormalities localized to the brain and ocular regions, with notably malformed retinas and an apparent absence of lenses. Subsequently, embryos were analyzed at a more advanced stage (stage 28), where differentiation of retinal cell types begins. The results confirmed the earlier observations, demonstrating that tissue resembling the retina was malformed, and the lenses were absent in *Pax6.1* mutants (Figure 29).

Given the early developmental findings indicating lens absence, further investigation was undertaken to determine whether lens development occurs at later stages, particularly around the hatching period (10 days post-fertilization). Anatomical assessments, complemented by histological analysis of eye sections, confirmed the persistent absence of the lens in *Pax6.1* mutants (Figure 30). The lack of lens formation also led to the structural collapse of the anterior chamber. Moreover, the mutant embryos were generally smaller, and homozygosity for the *Pax6.1* allele was lethal, resulting in embryonic death shortly before hatching.

In contrast, no discernible morphological abnormalities were observed in *Pax6.2* and *Pax6.3* mutants at any developmental stage analyzed. Both *Pax6.2* and *Pax6.3* homozygous mutants were viable and survived to adulthood."

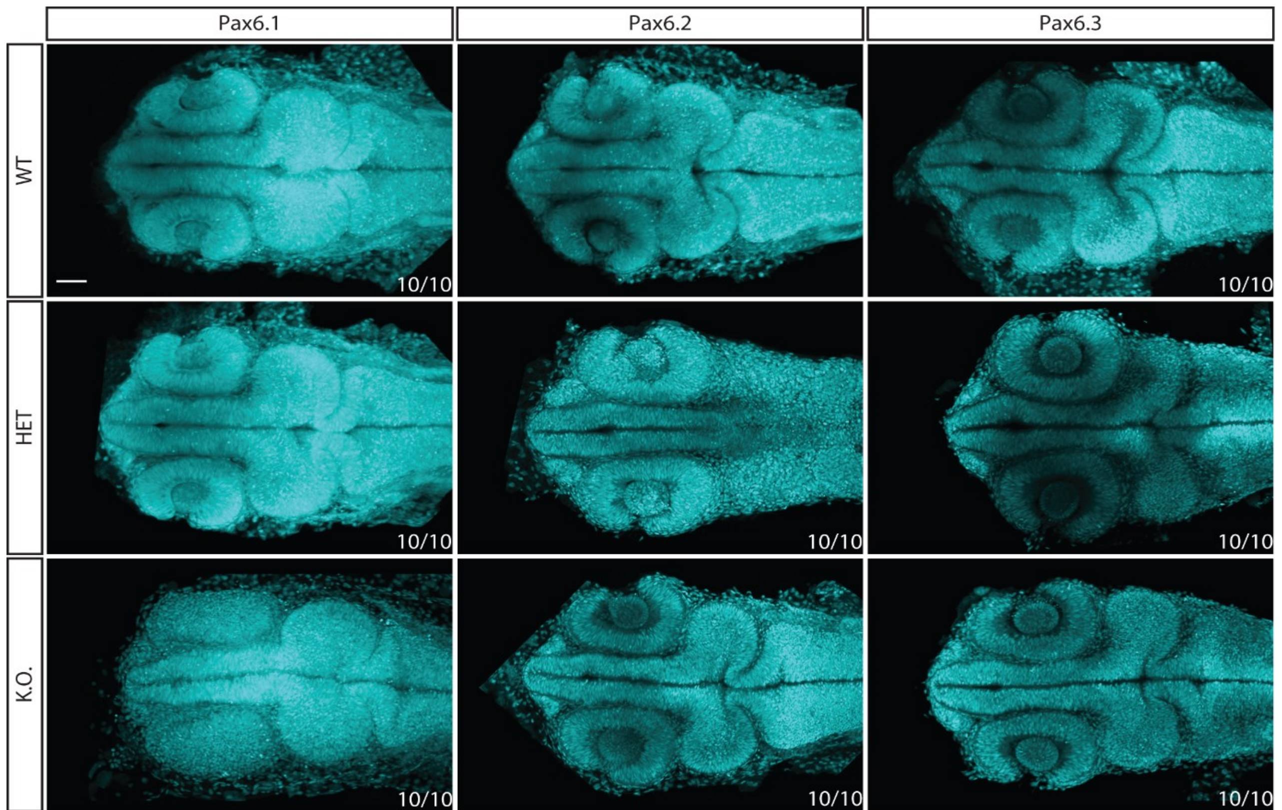


Figure 28: The morphological analysis of *Pax6.1*, *Pax6.2* and *Pax6.3* mutant at stage 23 using DAPI staining. Phenotype is present in the brain and the eye of the *Pax6.1* mutant. The retina is malformed, and lenses seem to be missing. *Pax6.2* and *Pax6.3* do not manifest any visible morphological phenotype. Scale bar: 50µm

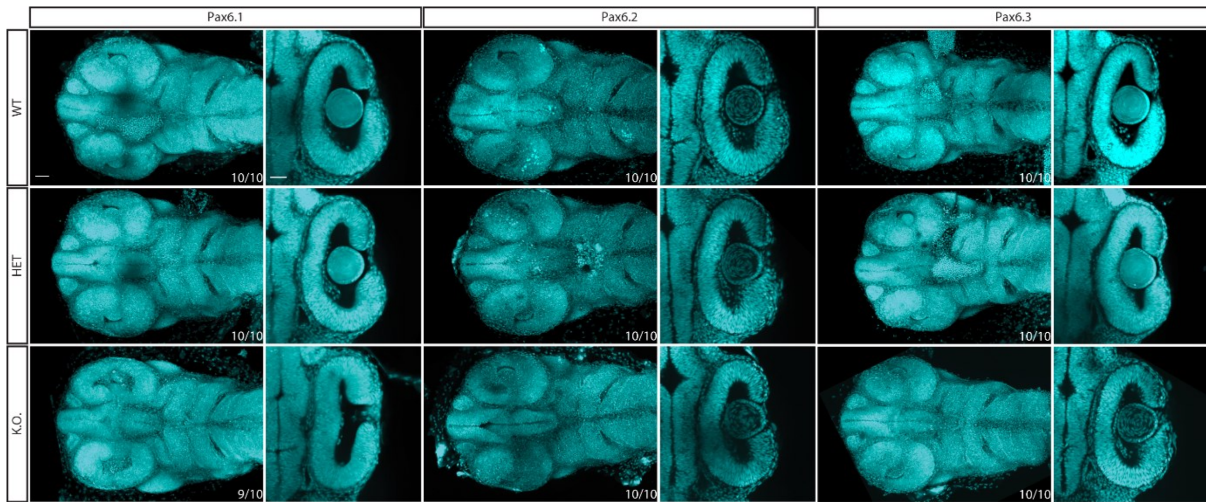
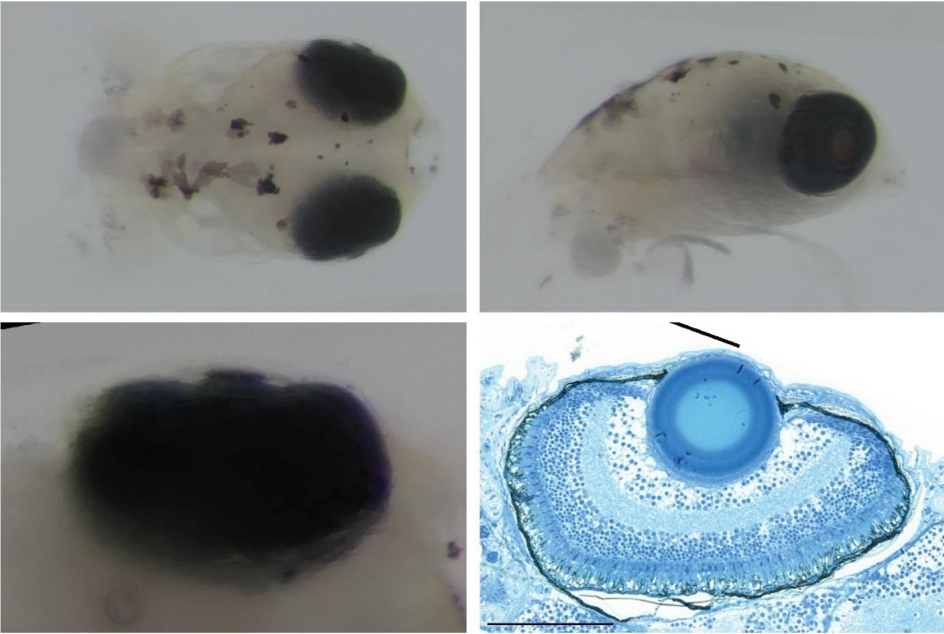


Figure 29: The morphological analysis of the *Pax6.1*, *Pax6.2* and *Pax6.3* mutant at stage 28 using DAPI staining. Phenotype can be observed in the developing eye in the *Pax6.1* mutant. The retina is present but malformed and lenses seem to be missing. No phenotype is observed in *Pax6.2* and *Pax6.3* mutants. Scale bar: 50µm



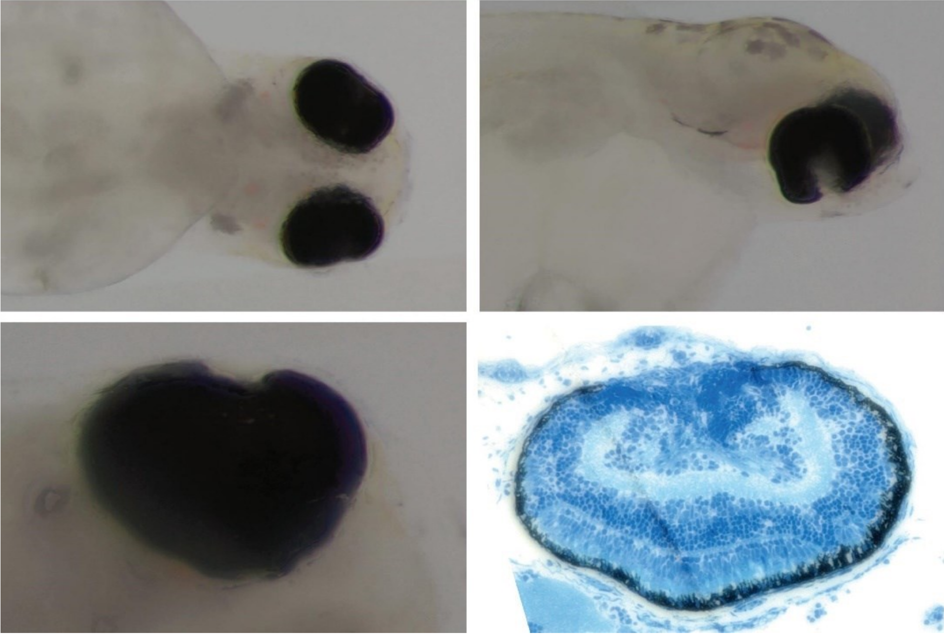


Figure 30: A comparison of WT and Pax6.1 homozygote mutant at hatching (10dpf).

Analysis of *Pax6.1* mutant

The initial phenotypic abnormalities were observed following the injection of TALENs in the F0 generation. After establishing the *Pax6.1* mutant line, basic morphological analysis using DAPI staining revealed a very prominent phenotype in the head and developing ocular region (Figures 28 and 29). This study focuses on the role of *pax6* genes during eye development in medaka; therefore, the phenotypes observed in the head region will not be discussed here."

Lens analysis

Analysis of DAPI staining revealed phenotypic abnormalities in the ocular region, with the retina exhibiting an irregular shape and the lenses appearing significantly reduced in size or entirely absent. Consequently, we aimed to investigate whether lenses were present in the *Pax6.1* mutant and, if so, in what form. Lens development initiates as cells from the surface ectoderm begin to delaminate at stages 20–21, with the lens being formed by the end of stage 23. However, there is currently no known marker for the surface ectoderm at the onset of lens development (stage 20) in medaka. Stage 21 was identified as the earliest time point at which we could visualize the developing lens.

In situ hybridization for various genes expressed during early lens development was conducted to identify lens cells in *Pax6.1* mutants. We selected *Prox1a*, *B-Maf*, *Maf-C*, and *FoxE3* as markers, given their documented expression in the lens (Glasow and Tomarev, 1998; Coolen et al., 2005; Shi et al., 2006; Swindell et al., 2008; Deguchi et al., 2009). Expression of all tested genes was detected in the developing lenses of wild-type and heterozygous animals. On the other side, no signal was observed in the lens area of *Pax6.1* homozygous embryos (Figure 31). Notably, the expression of the *Maf-B* gene in the brain was consistent across all genotypes (wild-type, heterozygous, and homozygous). These findings indicate that lens formation does not start at the appropriate developmental stage in the *Pax6.1* mutant."

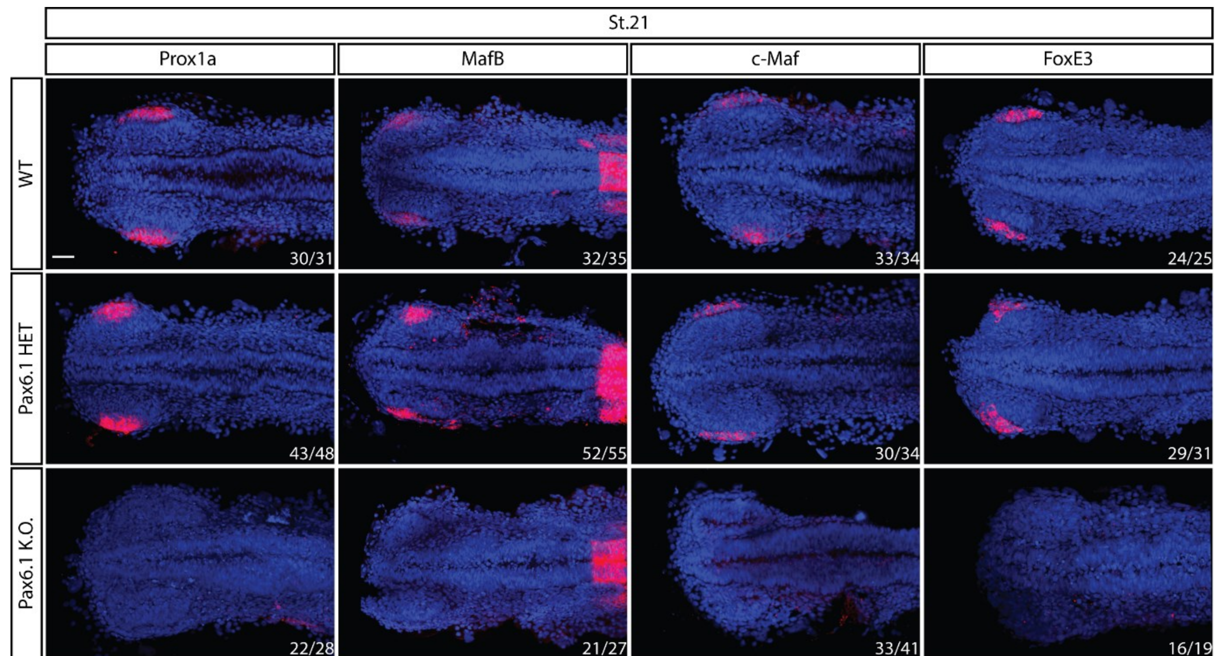


Figure 31: Comparison of the initiation of lens formation by *in situ hybridisation* for selected lens markers (*Prox1a*, *B-Maf*, *Maf-C* and *FoxE3*) at stage 21. Signal in the lens area is present in wildtype and heterozygote but no signal is visible in *Pax6.1* homozygote embryos. Scale bar: 50µm

The next step was to determine whether the initiation of lens formation in the *Pax6.1* mutant is delayed or if the lens fails to develop entirely. To address this, in situ hybridization was performed for the selected lens markers (*Prox1a*, *B-Maf*, *Maf-C*, and *FoxE3*) at later developmental stages (stages 22 and 23) (Figures 32 and 33). Additionally, the expression of the *NRL* gene (Coolen et al., 2005) was analyzed at stage 23. Results from both stages 22 and 23 showed a strong signal for all analyzed genes in the lenses of wild-type and heterozygous embryos, whereas no signal was detected in the lens region of homozygous embryos. Consistent with earlier observations at stage 21, the expression of the *Maf-B* gene in the brain remained unchanged at later stages.

These findings clearly indicate that the initiation of lens development does not occur, and the lens is not formed during the early stages of embryonic development in *Pax6.1* mutants."

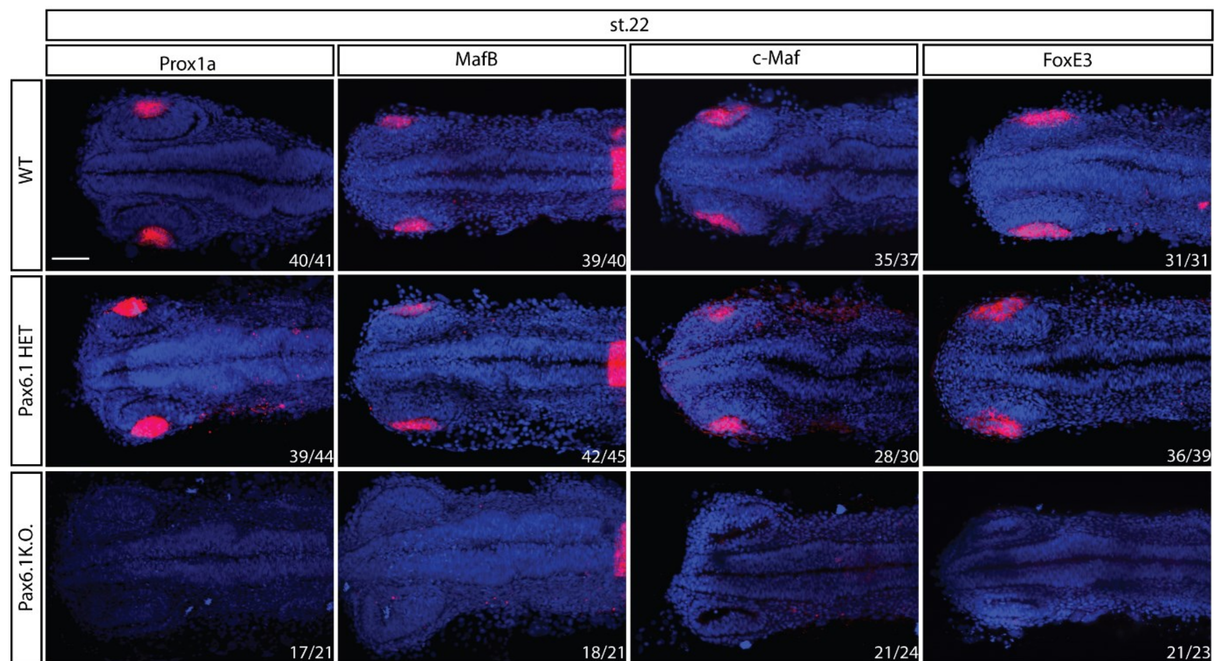


Figure 32: Comparison of the expression of selected lens markers (*Prox1a*, *B-Maf*, *Maf-C* and *FoxE3*) at the stage 22 of medaka embryonic development. The forming lens of wildtype and heterozygote embryos are stained, but no signal is present in the homozygote embryos. Scale bar: 50µm

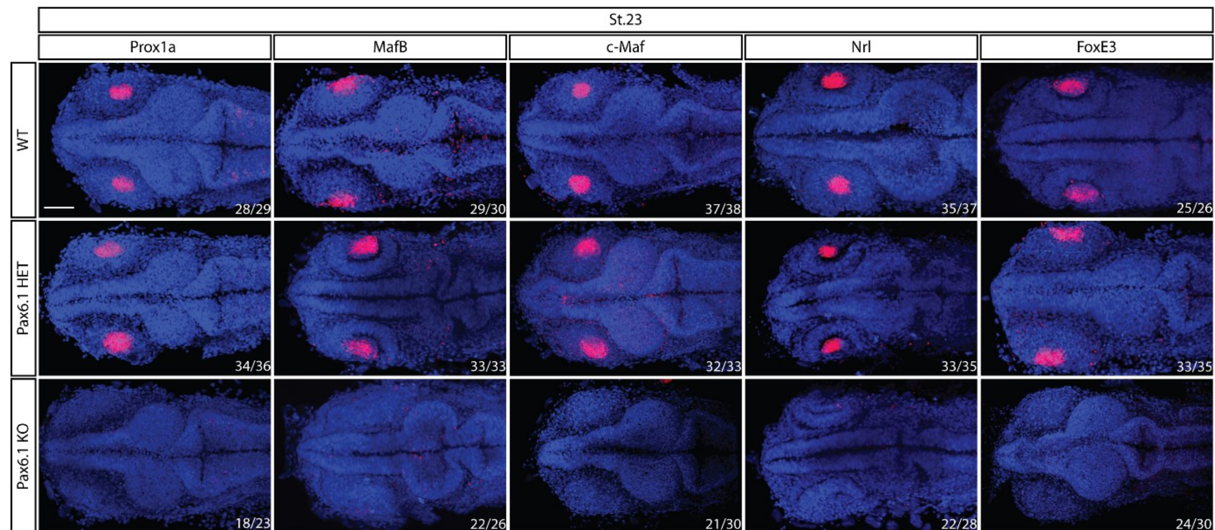


Figure 33: Comparison of the lens formation between wildtype, heterozygote and homozygote medaka embryos at the stage 23. Lens markers (*Prox1a*, *B-Maf*, *Maf-C*, *FoxE3* and *NRL*) are expressed in wildtype and heterozygote, but no expression is detected in the homozygote. Scale bar: 50µm

Retina analysis

Analysis of early retina progenitors

The region corresponding to the developing eyes becomes recognizable shortly after the embryonic body begins to form. The expression of *Pax6.1*, alongside other eye field-specific transcription factors, is crucial for proper eye development. Several of these factors play a significant role in retina development and the subsequent specification of retinal cell types. Early retinal progenitor cells, which will differentiate into specific retinal cell types, can be identified by the expression of characteristic genes from the *rx*, *meis*, *six*, *sox*, or *mab* gene families (Heavner and Pevny, 2012; Zaghloul et al., 2012; Huang et al., 2015; Marcos et al., 2015; reviewed in Zuber et al., 2003).

At stage 20, *Rx3* is expressed in early retina progenitors, followed by the expression of *Rx1* and *Rx2* in the developing retina at stage 22. Additionally, transcription factors such as *Sox2*, *Six3*, *Meis1*, *Meis2*, and *Mab21l2* are essential during vertebrate embryonic eye development (Heavner and Pevny, 2012; Zaghloul et al., 2012; Huang et al., 2015; Marcos et al., 2015; reviewed in Zuber et al., 2003).

To further investigate how early retina development proceeds in the absence of the *Pax6.1* gene, in situ hybridization was performed to detect the expression of all previously mentioned marker genes for early retinal progenitor cells at the earliest developmental stages (stages 20 and 22) (Figure 34). The expression patterns in the developing retina of *Pax6.1* homozygous and heterozygous mutants were consistent with those observed in wild-type embryos across all examined genes. The absence of differences in the expression of retina-specific genes between wild-type and knockout embryos suggests that early retina development is not affected in the *Pax6.1* mutant."

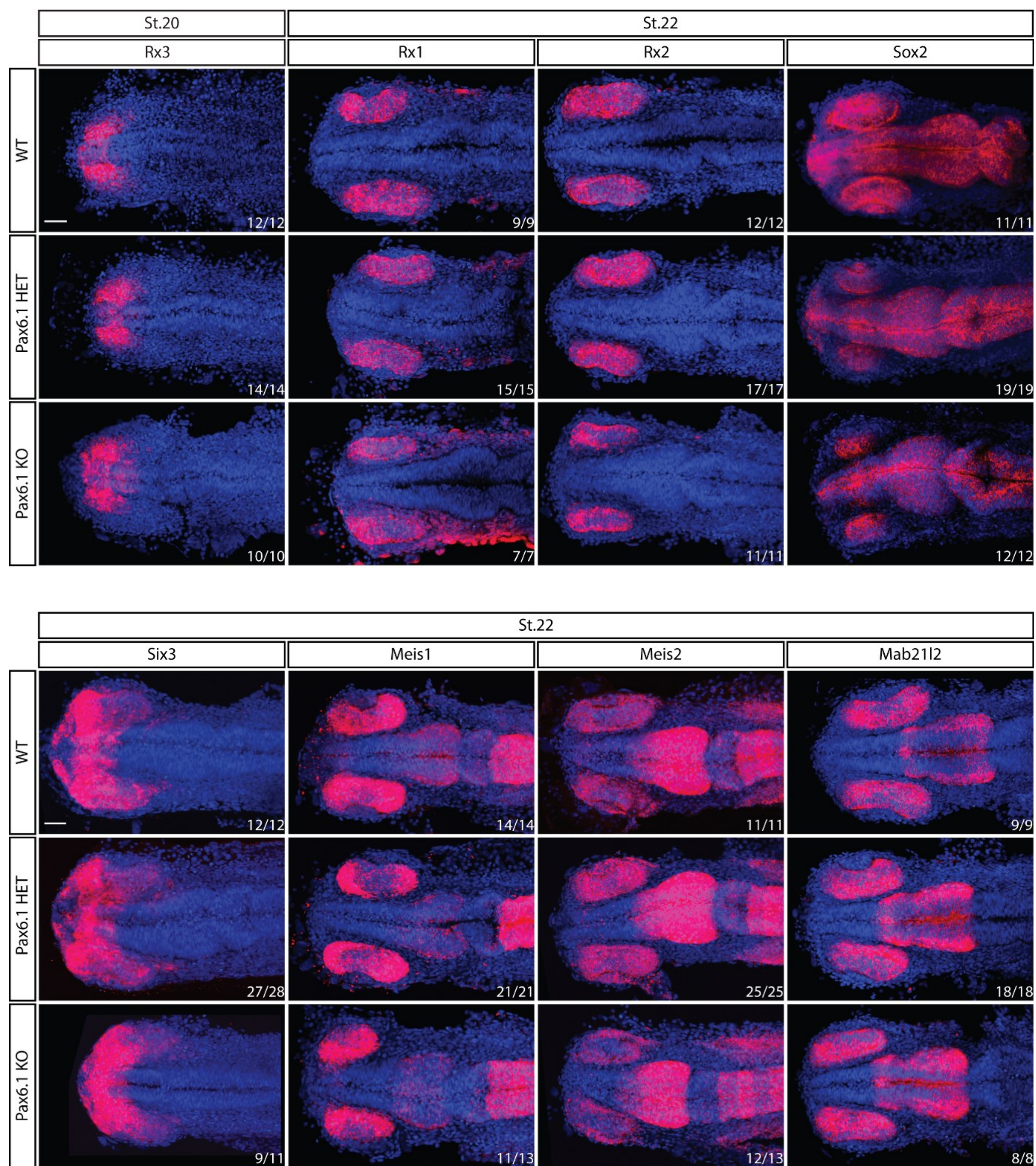


Figure 34: In situ hybridisation analysis of retina specific expressed genes, *Rx3, Rx1, Rx2, Sox2*, *Six3*, *Meis1*, *Meis2* and *Mab21l2,* during the early retina development. The expression pattern of none of these genes is changed in *Pax6.1* mutant compared to wildtype or heterozygote. Scale bar: 50µm

Differentiation of the retina specific cell types

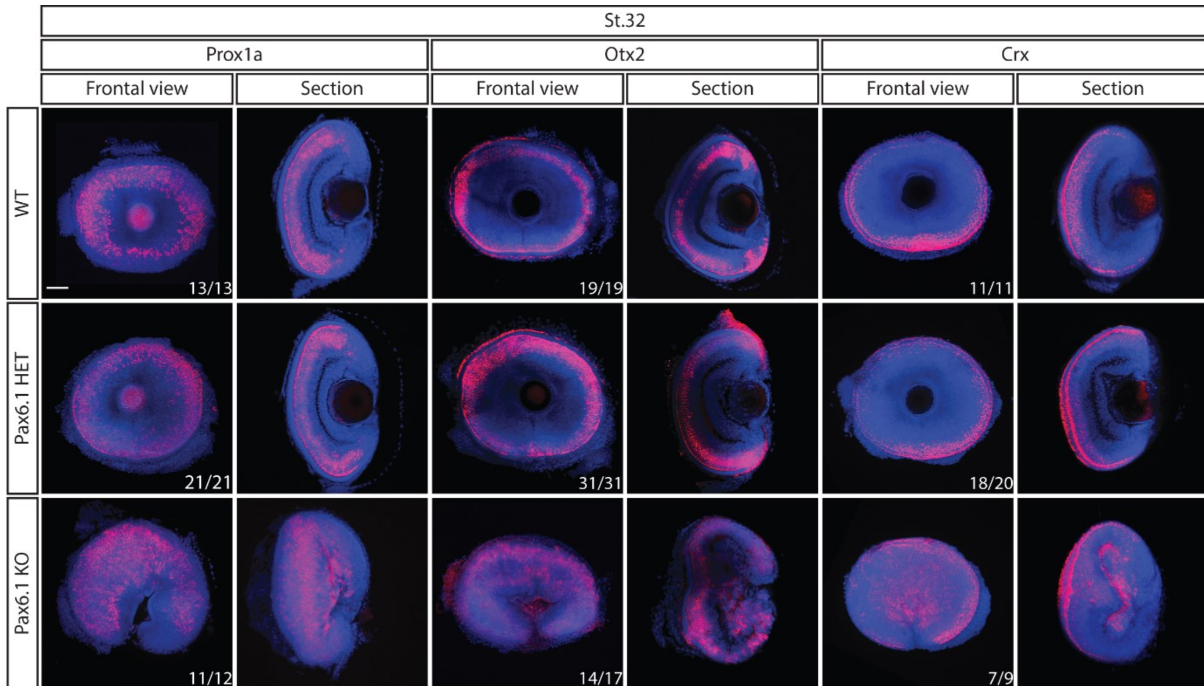
Previous experiments on retinal development indicated that the formation of the early retina remains intact in the *Pax6.1* mutant (Figure 34). Additionally, DAPI staining confirmed the presence of retina-like tissue at stage 28 (Figure 29). These findings suggest that the mutation in the *Pax6.1* gene does not result in a the arrest of embryonic retinal development. Consequently, we sought to determine whether retinal progenitor cells in the *Pax6.1* mutant are capable of differentiating into all retinal-specific cell types.

The differentiation of retinal-specific cell types begins at stage 28 and continues until stage 36. By stage 32, fully differentiated ganglion, amacrine, bipolar, horizontal, and Müller glia cells have reached their final positions. Full differentiation of rod and cone photoreceptors is completed by stage 36. For this analysis, genes previously described as specific markers for individual retinal cell types were selected, including *Ath5*, *Brn3C*, *Crx*, *HuC/D*, *Islet2*, *Meis1*, *Meis2*, *NeuroD1*, *NR2E3*, *Nrl*, *Oc1*, *OcL*, *Otx1*, *Otx2*, *Prox1*, *Rhodopsin*, *Rx2*, *Sox2*, *Vsx1*, and *Vsx2* (Nathans, 1992; Mercier et al., 1995; Xiang et al., 1995; Belecky-Adams et al., 1997; Brown et al., 1998; Passini et al., 1998; Chuang and Raymond, 2001; Liu et al., 2001; Hong et al., 2002; Ekstrom and Johansson, 2003; Pak et al., 2004; Chen et al., 2005; Coolen et al., 2005; Ochocinska and Hitchcock, 2007; Heine et al., 2008; Lin et al., 2009; Cid et al., 2010; Wu et al., 2012).

A comparative analysis was conducted between wild-type, heterozygous, and homozygous *Pax6.1* mutants to determine whether the differentiation or positioning of specific retinal cell types is affected by the *Pax6.1* mutation."

Photoreceptor cells

Genes with established expression in differentiating or differentiated photoreceptors—*Otx1*, *Otx2*, *Nrl*, *Rx2*, *Crx*, and *NeuroD1*—were selected for analysis. Additionally, *NR2E3*, which is characteristic of cones, and *Rhodopsin*, selectively expressed in rods, were examined. The data revealed that all tested genes were expressed in the outer part of the retina in the *Pax6.1* mutant (Figure 35). These findings provide evidence that photoreceptors, both rods and cones, are present in the retina of the *Pax6.1* mutant.



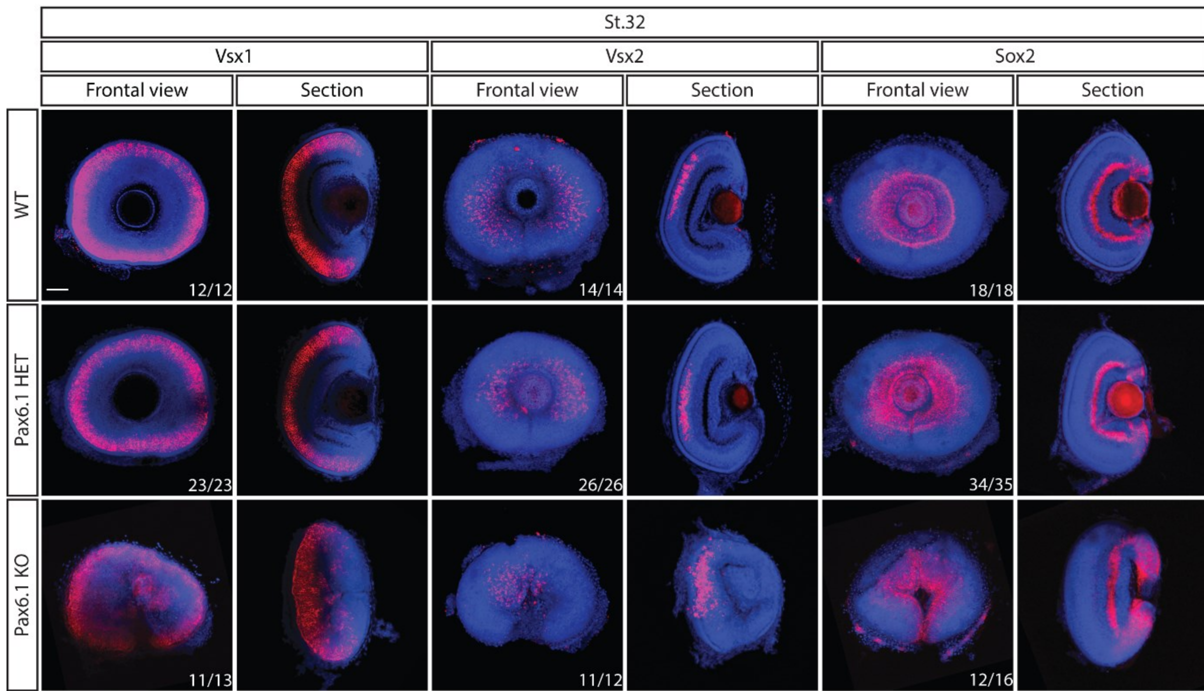


Figure 35: Expression patterns of *Otx1*, *Otx2*, *Nrl*, *Rx2*, *NeuroD1*, *Crx* and *NR2E3* genes. All of the selected genes are expressed in the retina of WT, *Pax6.1* heterozygote and homozygote. Scale bar: 50µm

Horizontal cells

HuC/D, *Prox1a*, and *OcL* were selected as markers for horizontal cells. A comparison of their expression patterns between wild-type, heterozygous, and homozygous *Pax6.1* mutants did not reveal any differences (Figure 36). These findings indicate the presence of horizontal cells in the *Pax6.1* mutant.

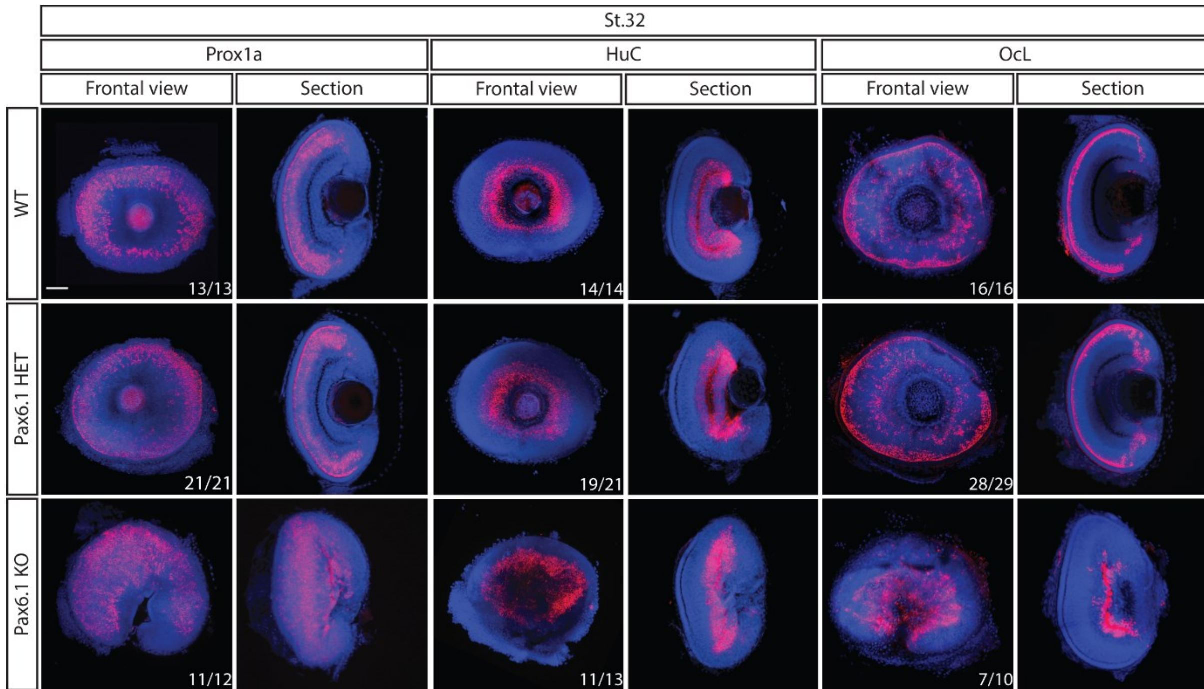
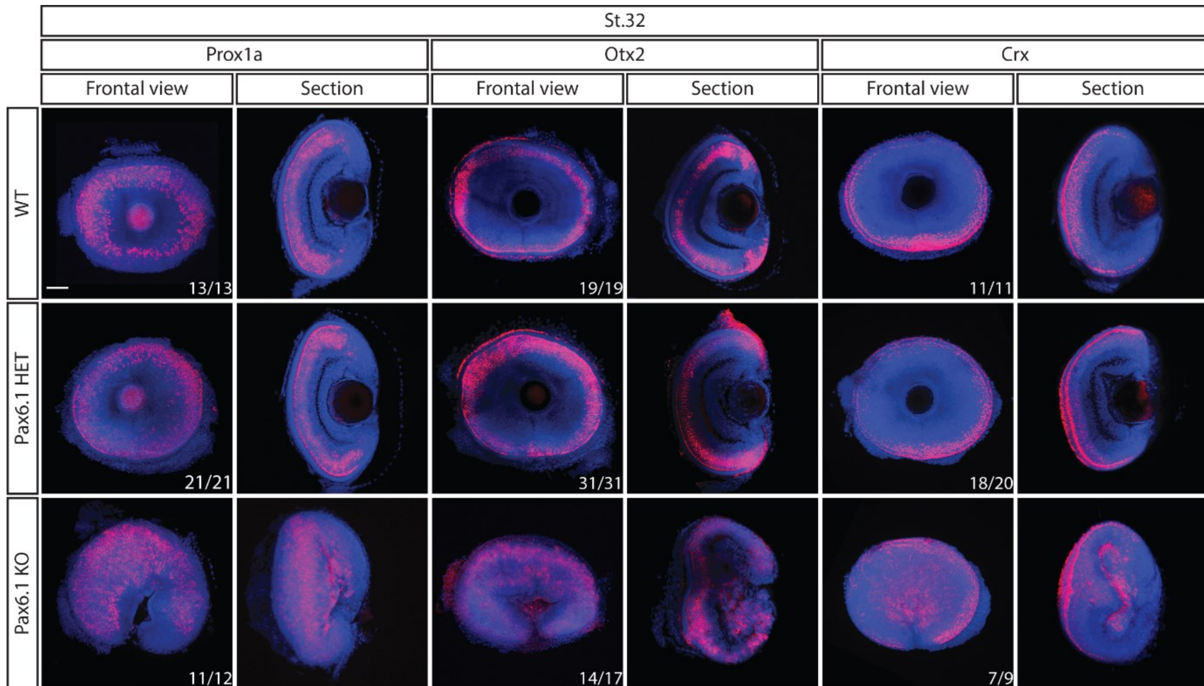


Figure 36: Comparison of expression patterns of *HuC/D*, *Prox1*a and *OcL* in the retina between WT and *Pax6.1* heterozygote/homozygote embryos. Expression was not changed in any of the examined genes. Scale bar: 50µm

Bipolar and Muller glia cells

To characterize bipolar and Müller glia cells, the expression patterns of *Crx*, *Otx2*, *Vsx1*, *Vsx2*, *Prox1a*, and *Sox2* were analyzed using in situ hybridization (Figure 37). The expression of these genes did not differ in the *Pax6.1* mutant, suggesting that the differentiation of bipolar and Müller glia cells in the *Pax6.1*-deficient retina is not affected."



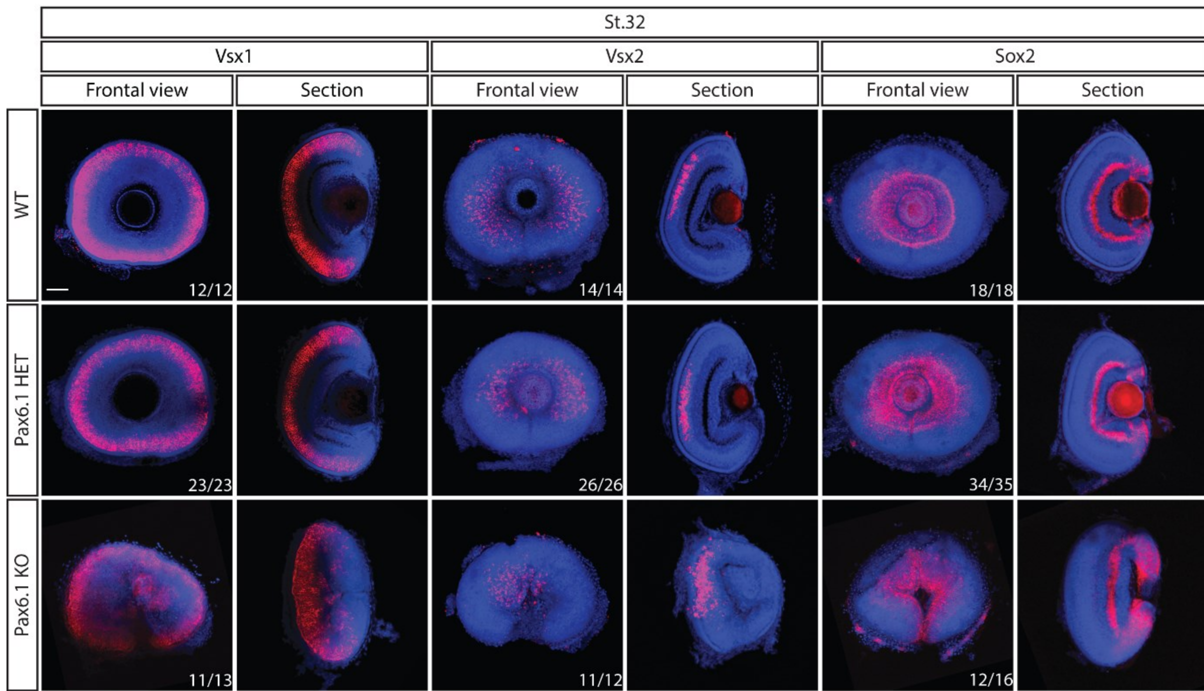


Figure 37: The expression of *Crx*, *Otx2*, *Vsx1*, *Vsx2*, *Prox1*a and *Sox2* in WT, *Pax6.1* heterozygote and homozygote. No changes in expression patterns were observed. Scale bar: 50µm

Amacrine cells

The expression of *Meis1*, *Meis2*, *NeuroD1* and *HuC/D* to detect the amacrine cells was examined in *Pax6.1* mutant (Figure 38). We did not observe any difference of the expression of selected genes in the wild type and mutant embryos. These data suggest that amacrine cells are present in the retina missing the *Pax6.1* gene.

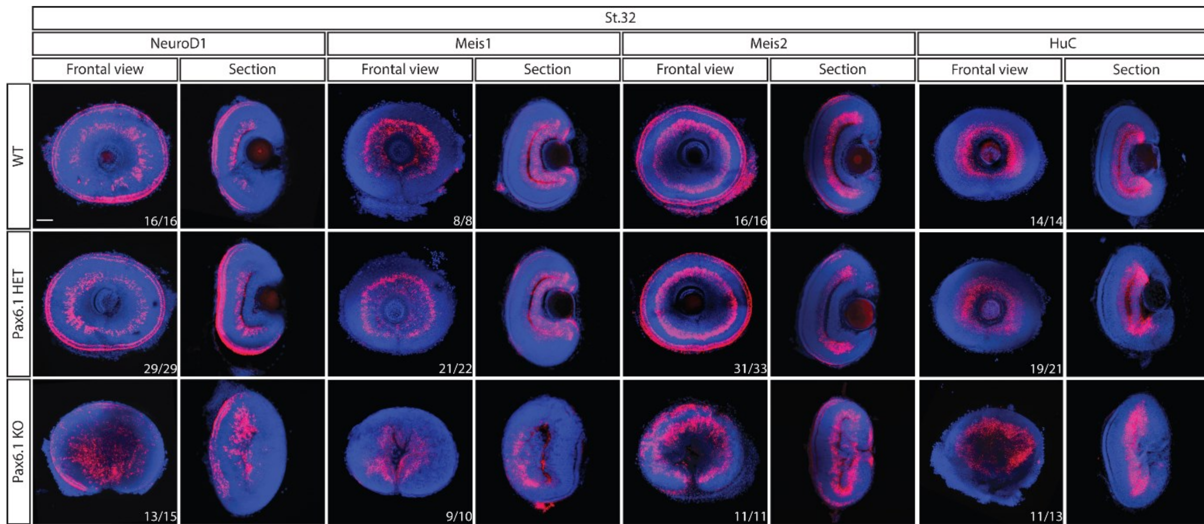
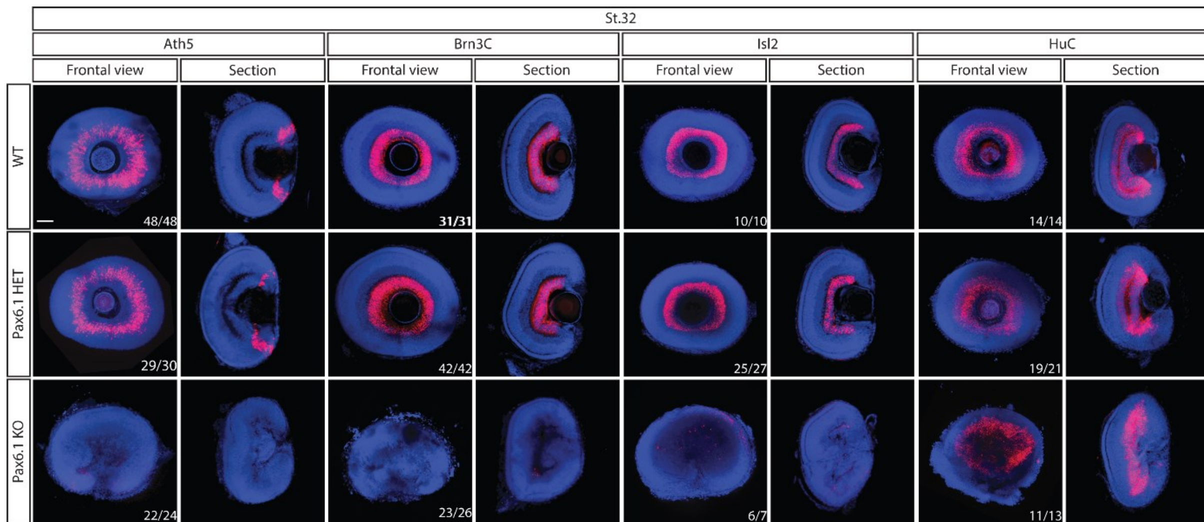


Figure 38: Comparison of *Meis1*, *Meis2*, *NeuroD1* and *HuC/D* expression in amacrine cells in WT and *Pax6.1* mutant. All tested genes are present in the mutant retina. Scale bar: 50µm

Ganglion cells

To determine whether ganglion cells differentiate properly in the *Pax6.1* mutant retina, two sets of genes were selected. The first set, *Ath5*, *Brn3C*, and *Islet2*, are selectively expressed in ganglion cells. The second set, *Oc1*, *OcL*, *Meis1*, and *HuC/D*, are expressed in ganglion cells as well as in other cell types. In *Pax6.1* homozygous mutants, *Ath5*, *Brn3C*, and *Islet2* were not detected (Figure 39). Additionally, the expression of *Oc1*, *OcL*, *Meis1*, and *HuC/D* was absent in the innermost region of the retina, where ganglion cells are typically located. These findings demonstrate that ganglion cells are not present in the *Pax6.1* homozygote.



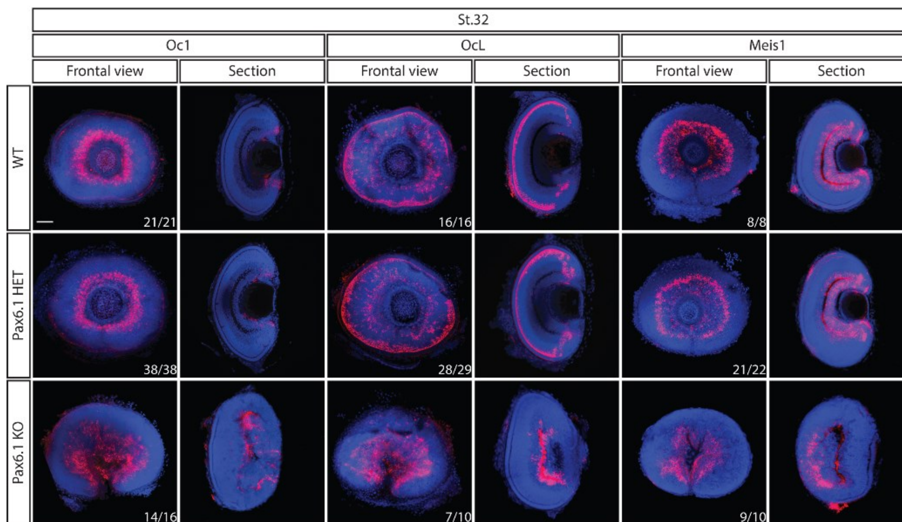
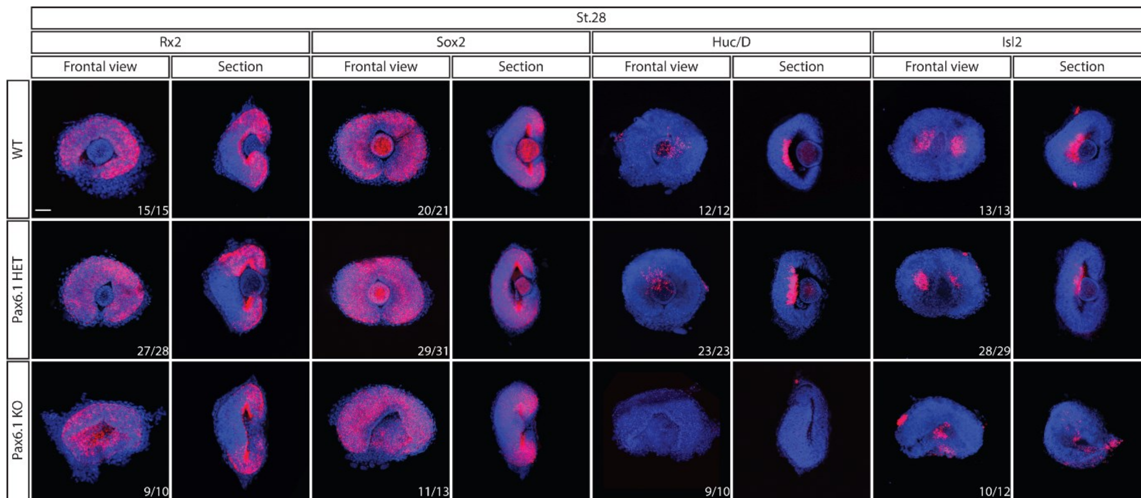


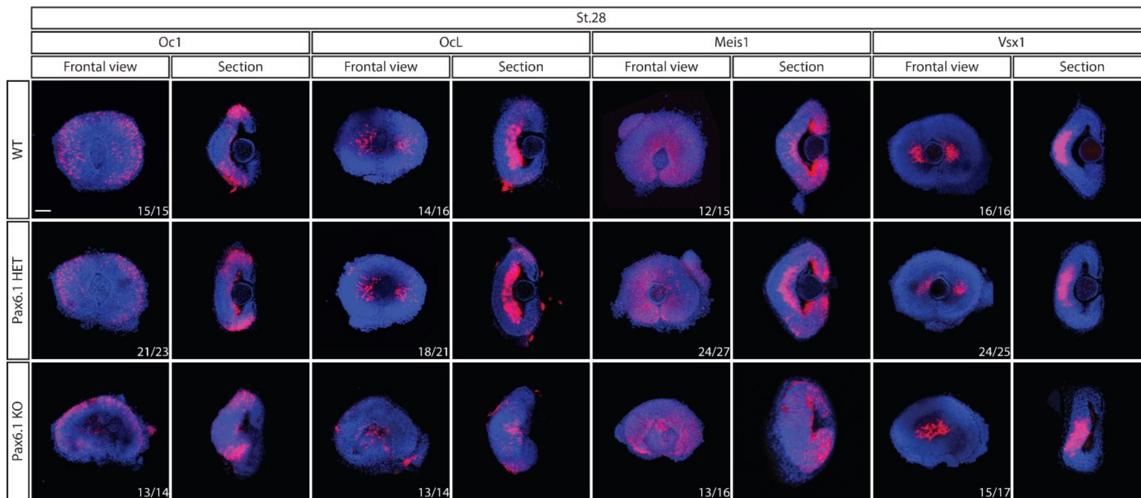
Figure 39: Comparison of the expression patterns of genes (*Ath5*, *Brn3C*, *Islet2*, *Oc1*, *OcL*, *Meis1*, *HuC/D*) expressed in the ganglion cells. None of the analysed genes is expressed in ganglion cells in *Pax6.1* homozygote. Scale bar: 50µm

<u>The role of the ciliary marginal zone for differentiation of specific retinal cell types in *Pax6.1* mutant</u>

The ciliary marginal zone (CMZ) is the most peripheral region of the retina, containing pluripotent retinal progenitor cells (RPCs). Numerous studies have demonstrated the ability of RPCs to differentiate into all specific neural and glial cell types of the retina (reviewed in Perron and Harris, 2000). During embryonic eye development and the growth of the adult eye, RPCs are actively incorporated into the retina, and they play a crucial role in retinal regeneration following injury (Moshiri et al., 2004). We investigated whether the retinal phenotype observed in the *Pax6.1* mutant medaka could be influenced by RPCs from the CMZ.

In medaka, RPCs within the CMZ become active from the end of stage 28 of embryonic development. Therefore, an in situ hybridization experiment was conducted to examine the expression of genes specifically associated with different retinal cell populations at this stage. The results indicated that all analyzed cell populations were present at stage 28 in the *Pax6.1* mutant (Figure 40). These findings suggest that RPCs originating from the CMZ are unlikely to compensate for the phenotype resulting from the absence of the *Pax6.1* gene in the developing retina.





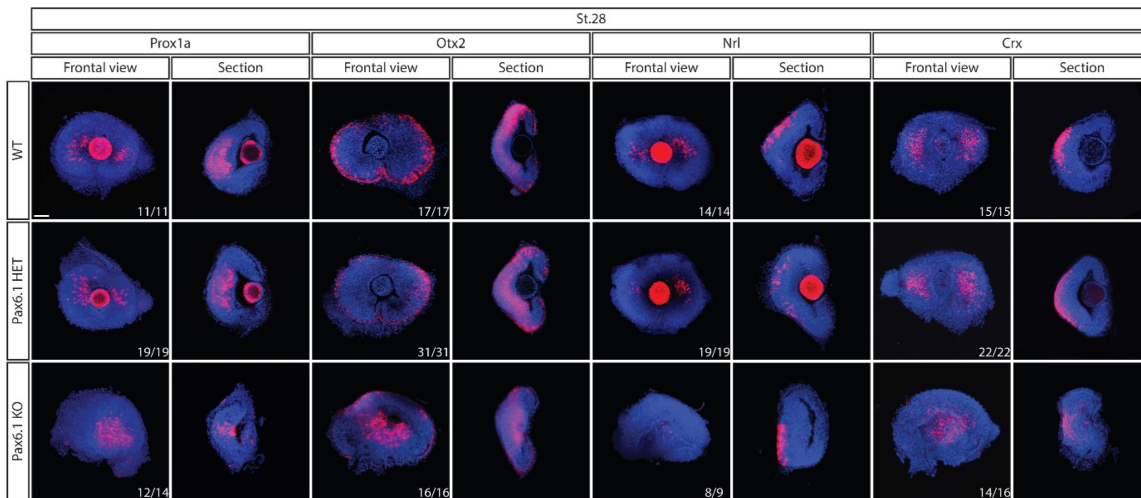


Figure 40: Comparison of the expression patterns of selected genes (*Rx2, Sox2, HuC/D*, *Islet2*, *Oc1*, *OcL*, *Meis1*, *Vsx1, Prox1, Otx2, Nrl, Crx*) in the retina between WT, *Pax6.1* heterozygote and homozygote. Expression of examined genes remained unchanged in *Pax6.1* mutant. Scale bar: 50µm

Cell proliferation in the retina of *Pax6.1* mutant

Morphological analysis indicated that *Pax6.1* mutant embryos are smaller than their wild-type littermates. We aimed to determine whether the reduced eye size was solely due to the overall smaller size of the embryo or if *Pax6.1* influences retinal cell proliferation, as observed in the mouse model. Studies in mice have shown that a *Pax6*-deficient retina is hypocellular, resulting from a decreased ability of retinal progenitor cells (RPCs) to proliferate (L. Klimova and Z. Kozmik, 2014). Therefore, we examined the rate of retinal cell proliferation in the medaka *Pax6.1* mutant.

Immunohistochemistry for phosphorylated histone 3 was used to label cells in the M-phase of the cell cycle (Figure 41 A). A comparison of the percentage of positive cells in the retinas of wild-type, heterozygous, and *Pax6.1* homozygous embryos revealed no differences in the proliferation rate of retinal cells in medaka (Figure 41 B). These results demonstrate that the *Pax6.1* gene in medaka does not affect the proliferative capacity of retinal cells.

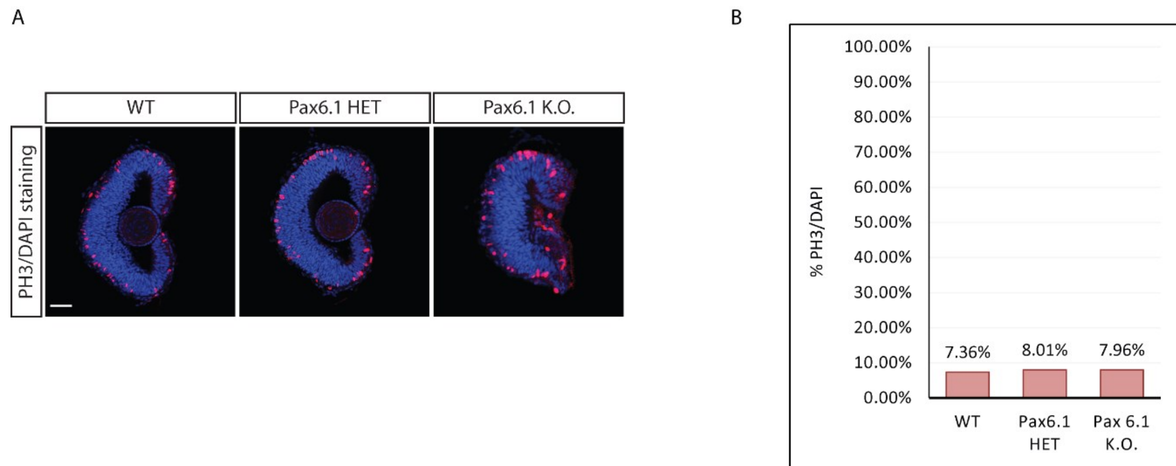
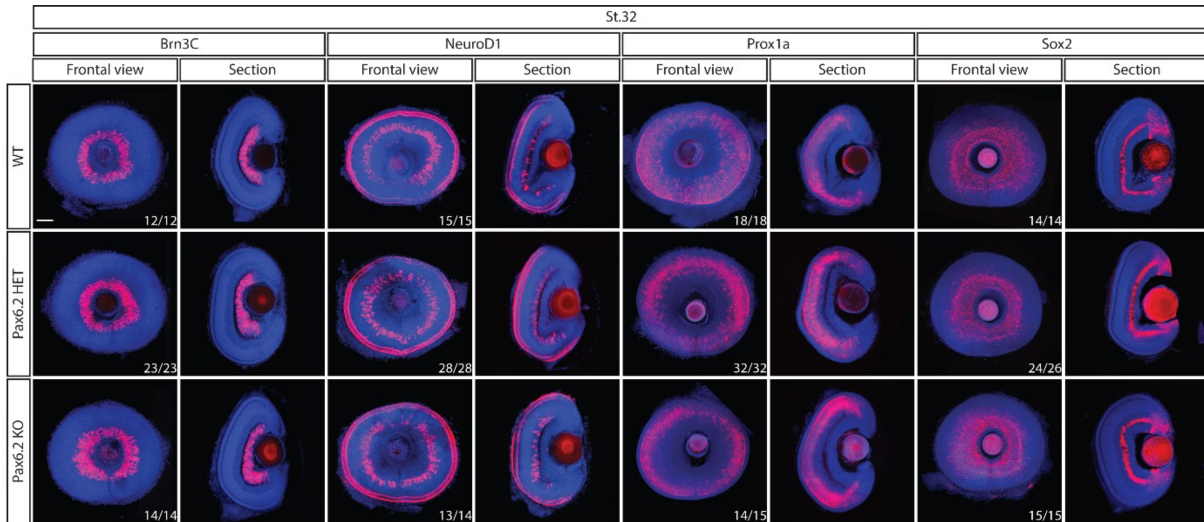


Figure 41: A: Immunohistochemistry staining for phosphorylated histone 3 in wildtype and *Pax6.1* homozygote retina. B: Quantitative analysis of the percentage of PH3 positive cells in the WT, heterozygote and homozygote retina showing no significant difference in the number of proliferating cells. Scale bar: 50µm

Analysis of *Pax6.2* mutant

The *Pax6.2* mutant was generated to investigate the role of the *Pax6.2* gene during medaka eye development. Morphological analysis using DAPI staining (Figures 28 and 29) did not reveal any visible phenotypic abnormalities; both the retina and lenses appeared normal throughout embryonic development. Consequently, we sought to determine whether the differentiation of specific retinal cell types was affected.

To analyze the presence and spatial distribution of specific cell types in the retina, various marker genes were selected. *Brn3C* was used for ganglion cells, *NeuroD1* for amacrine cells, *Sox2* for Müller glia cells, *Vsx2* for bipolar cells, *Prox1* for horizontal cells, *NR2E3* for cones, and *Rhodopsin* for rod photoreceptors (Figure 42). The data showed expression of all analyzed genes in both heterozygous and homozygous *Pax6.2* mutants. These findings indicate that the differentiation of specific retinal cell types is not affected by the absence of the *Pax6.2* gene. Overall, the study did not reveal any abnormalities in embryonic eye development when the *Pax6.2* gene was absent.



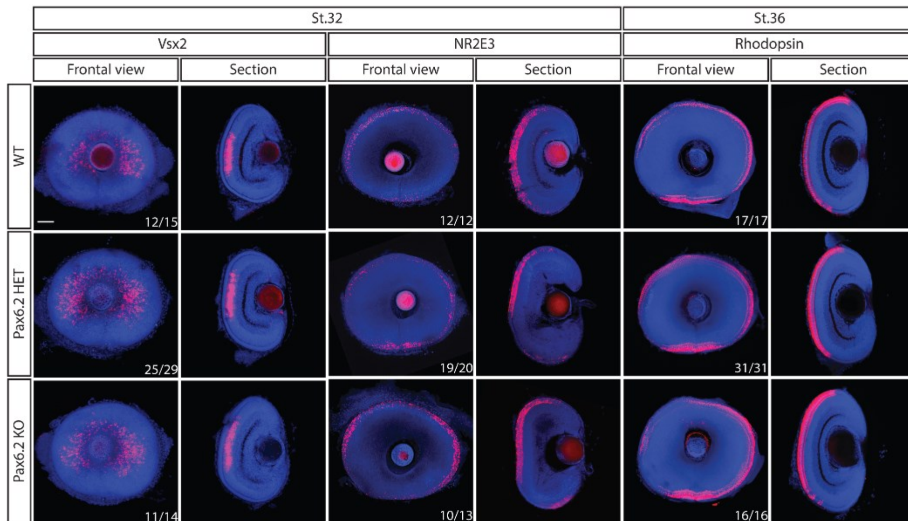
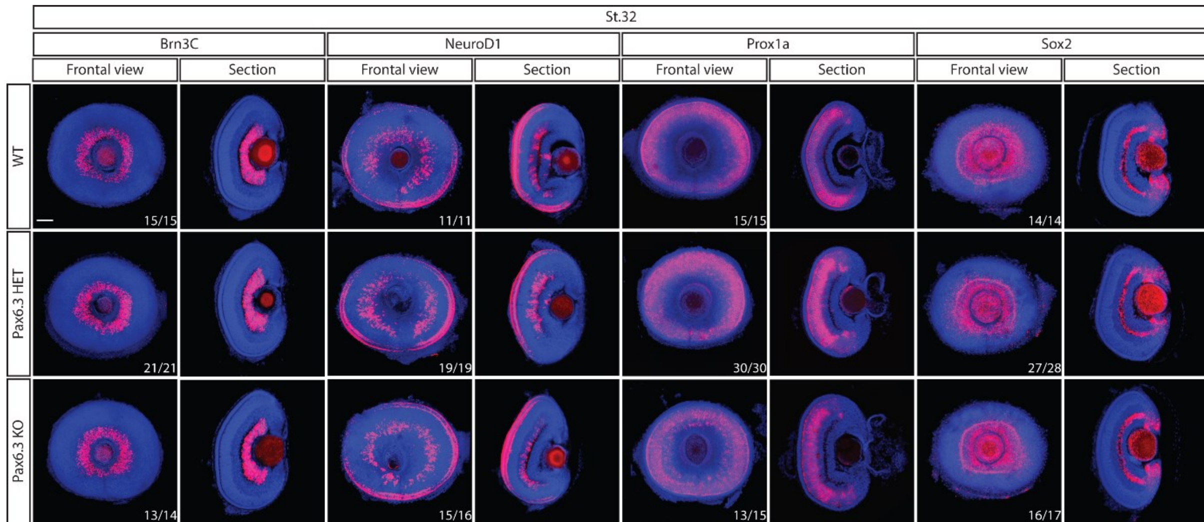


Figure 42: Comparison of the *Brn3C*, *NeuroD1*, *Sox2*, *Vsx2*, *Prox1*, *NR2E3* and *Rhodopsin* expression in wildtype, *Pax6.2* heterozygote and homozygote. All analysed genes are present in the corresponding area in the *Pax6.2* mutant retina. Scale bar: 50µm

Analysis of *Pax6.3* mutant

A mutation in the *Pax6.3* gene was generated to assess its role in eye development during medaka embryogenesis. Initial morphological analysis (Figures 28 and 29) did not reveal any abnormalities in the developing eye. Therefore, we proceeded to examine whether *Pax6.3* influences the differentiation of specific retinal cell types.

The same marker genes used in the analysis of the *Pax6.2* mutant—*Brn3C* (ganglion cells), *NeuroD1* (amacrine cells), *Sox2* (Müller glia cells), *Vsx2* (bipolar cells), *Prox1* (horizontal cells), *NR2E3* (cones), and *Rhodopsin* (rod photoreceptors)—were selected. The results showed that all investigated markers were expressed in the corresponding regions of both *Pax6.3* heterozygous and homozygous mutants (Figure 43). These findings confirm that specific retinal cell types differentiate properly in the *Pax6.3* mutant. Collectively, no discrepancies were identified in the eyes lacking the *Pax6.3* gene.



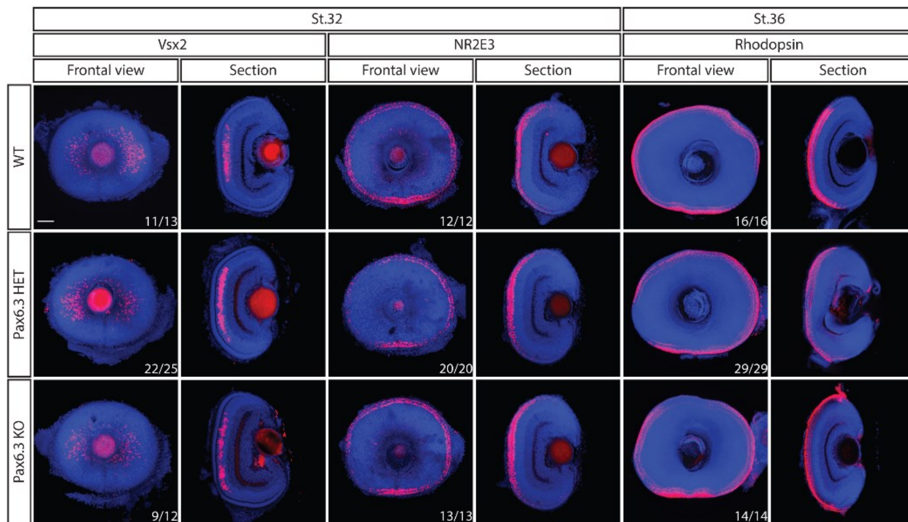


Figure 43: Comparison of the *Brn3C*, *NeuroD1*, *Sox2*, *Vsx2*, *Prox1*, *NR2E3* and *Rhodopsin* expression between wildtype, *Pax6.3* heterozygote and homozygote. All analysed genes are present in the corresponding area in the *Pax6.3* mutant retina. Scale bar: 50µm

The role of pax6 genes during the embryonic eye development of medaka

The significance of the *Pax6* gene in eye development across various animal species, including humans, is undeniable. Numerous studies have demonstrated that normal *Pax6* expression is essential for proper eye development in both invertebrates and vertebrates (Hill et al., 1991; Quiring et al., 1994; Nornes et al., 1998; Piatigorsky and Kozmik, 2004). Our experiments similarly underscore the relevance of pax6 genes for eye formation during embryonic development in medaka.

A detailed analysis of mutants for all three *pax6* genes revealed that the *Pax6.1* gene (the orthologue to the human and mouse *Pax6*) plays a critical role in this process. The eyes of *Pax6.1* mutants are characterized by the absence of lenses and a malformed retina lacking ganglion cells. In contrast, mutations in *Pax6.2* and *Pax6.3* did not impact embryonic eye development.

Do other pax6 genes compensate for the missing *Pax6.1* gene in medaka?

Previous experiments have demonstrated that *Pax6.1* is crucial for proper eye development. In *Pax6.1* mutants, lenses are absent, the retina is malformed, and ganglion cells fail to differentiate. Unlike the *Sey* mouse, which exhibits a complete absence of eye structures, medaka eyes are partially developed even in the absence of *Pax6.1*. The presence of three pax6 genes in the medaka genome (Ravi et al., 2013) and the partial development of eyes in *Pax6.1* mutants suggest the possibility of phenotypic compensation by other pax6 genes.

The vertebrate *Pax6* protein contains two DNA-binding domains—the paired-type domain (PD) and the homeodomain (HD)—along with a PST trans-regulatory domain located at the C-terminus (Ton et al., 1991; Glaser et al., 1992; Czerny and Busslinger, 1995) (Figure 10). Structural analysis of *pax6* genes and proteins in medaka (Figure 19) revealed a high degree of homology between the Pax6.1 and Pax6.3 proteins. The possibility of compensation for the loss of *Pax6.1* by *Pax6.3* was proposed due to the similarity observed in both the PD and HD regions. In contrast, the Pax6.2 protein lacks the PD, suggesting that it is unlikely to compensate for the loss of *Pax6.1* function in our mutant model.

Analysis of the expression patterns of *Pax6.1* and *Pax6.3* (Figures 21 and 24) indicates that both genes are active in the eye during the early stages of embryonic development. Double in situ hybridization was performed to precisely determine the regions where these two genes are co-expressed (Figure 44). The experiment revealed co-expression of *Pax6.1* and *Pax6.3* in the brain and the posterior domain of the eye during early development. The structural similarities, along with the temporal and partial tissue-specific co-expression of *Pax6.1* and *Pax6.3*, suggest that *Pax6.3* is a strong candidate to compensate for the loss of *Pax6.1*.

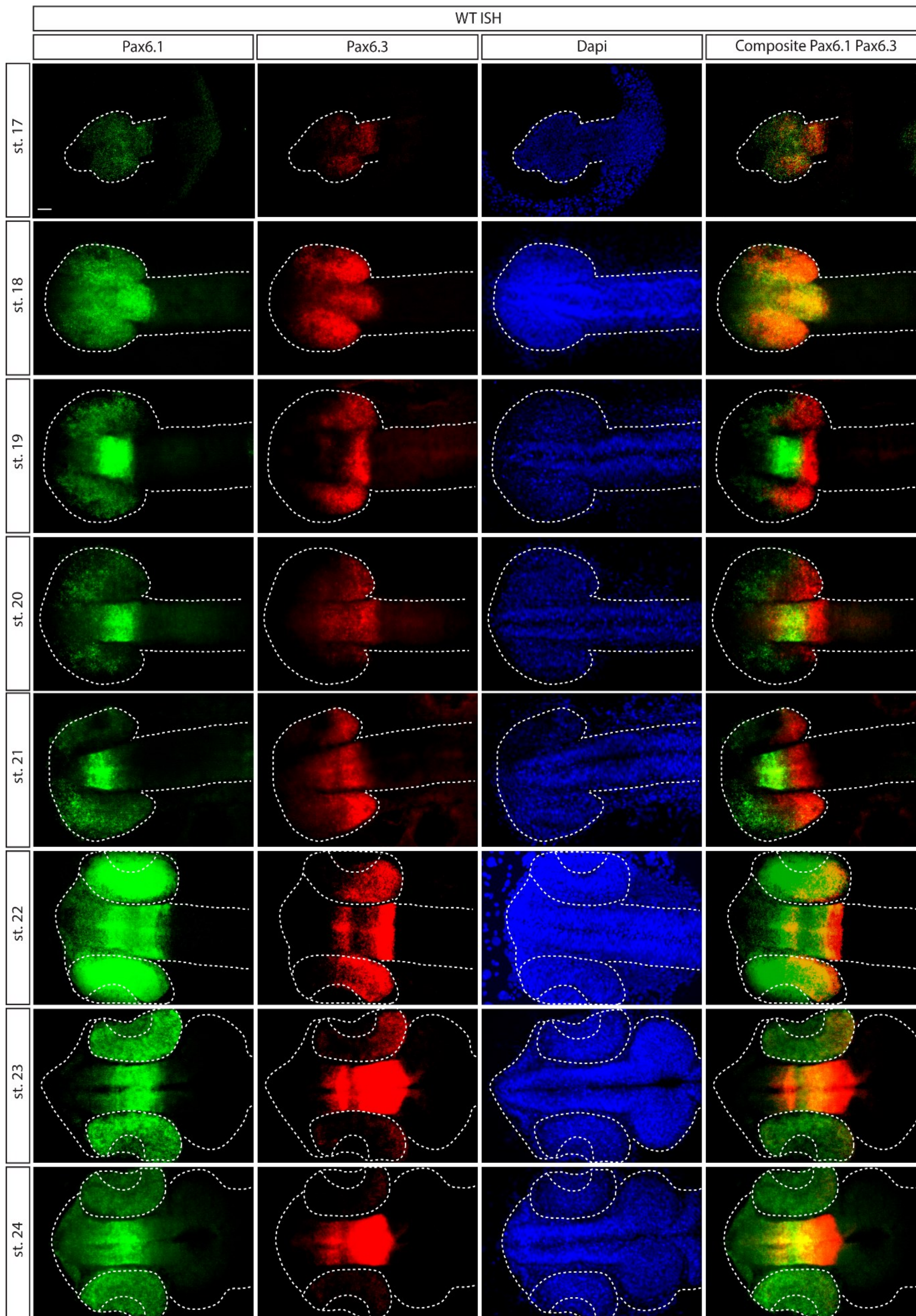
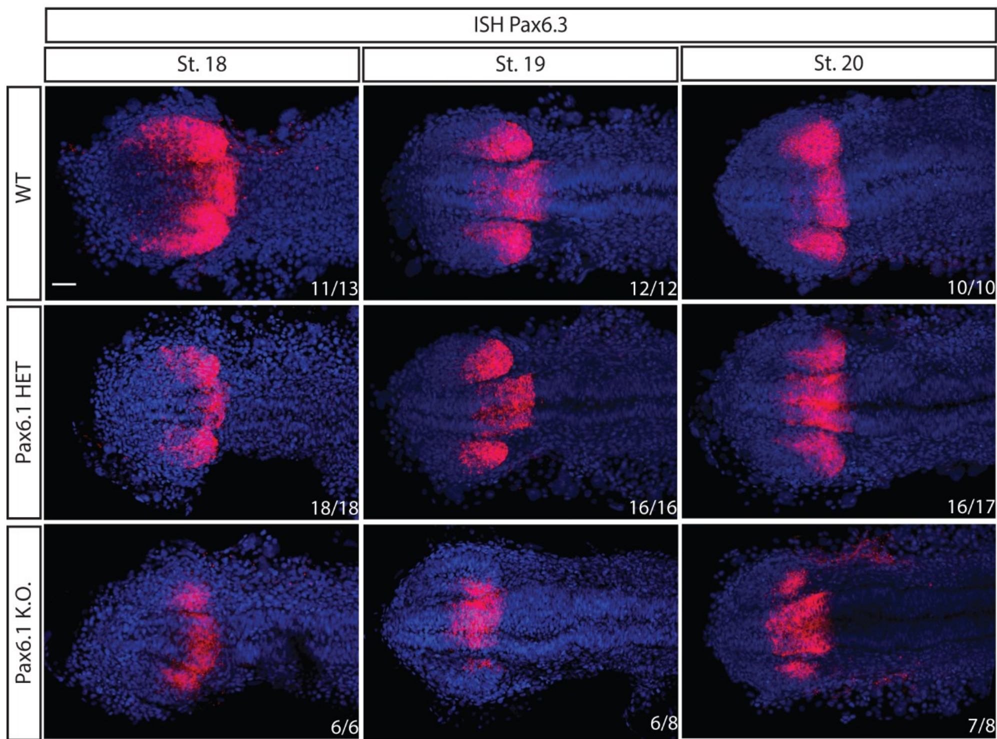


Figure 44: Double *in situ hybridisation* for *Pax6.1* and *Pax6.3* genes during the early stages of medaka eye development. *Pax6.1* and *Pax6.3* are co-expressed in the brain and the developing eye area. Scale bar: 50μm

Both *Pax6.1* and *Pax6.3* genes are expressed in the posterior domain of the developing eye; however, *Pax6.1* expression extends throughout the entire eye, including the ventral region. We sought to determine whether the expression domain of *Pax6.3* is altered in the absence of *Pax6.1*. To address this, in situ hybridization for *Pax6.3* was performed on *Pax6.1* mutant embryos (Figure 45). The results showed that the expression pattern of *Pax6.3* in developing eyes remained unchanged in the *Pax6.1* knock-out.

These findings raised an important question: Can *Pax6.3* compensate for the loss of *Pax6.1* if it is not expressed throughout the entire developing eye? To explore this possibility, a double knock-out for *Pax6.1* and *Pax6.3* was generated.



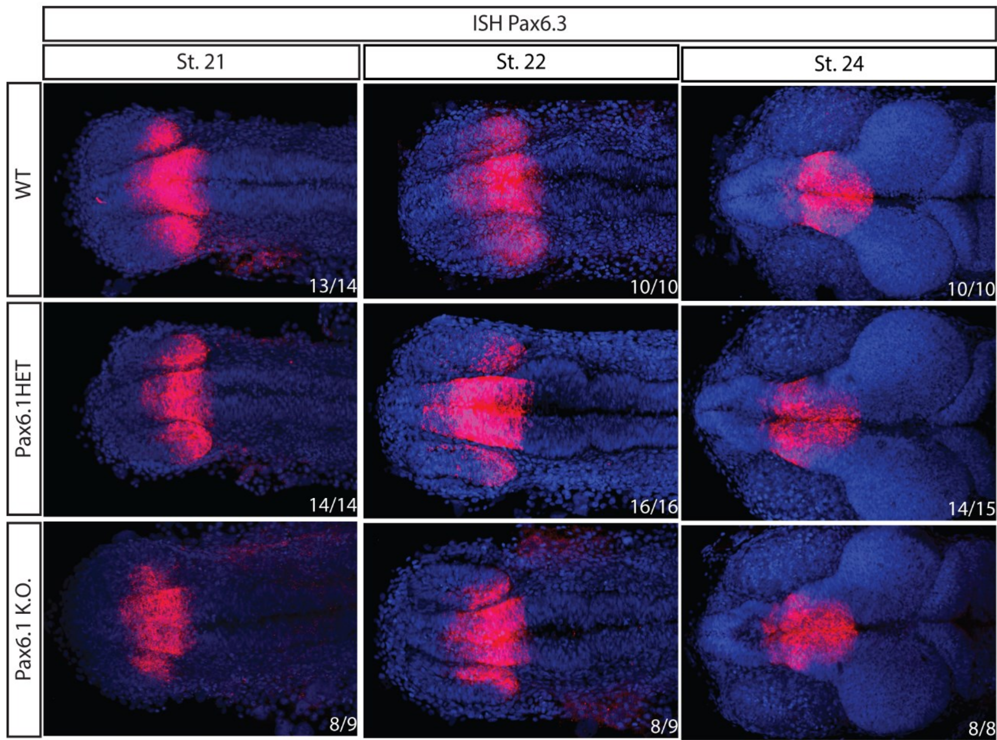


Figure 45: *Pax6.3* expression pattern analysis in the *Pax6.1* mutant. The eye expression domain of *Pax6.3* is not changed in *Pax6.1* knock-out.

Analysis of *Pax6.1* and *Pax6.3* double mutant

If *Pax6.3* were compensating for the loss of *Pax6.1* in *Pax6.1* mutants, we would expect a more severe phenotype, potentially resulting in the complete absence of eyes, following the deletion of both *Pax6.1* and *Pax6.3* genes. To test this hypothesis, we analyzed the morphological structures of developing embryos stained with DAPI (Figures 46 and 47).

Compared to wild-type embryos, the *Pax6.1/Pax6.3* double mutants exhibited the same structural abnormalities observed in *Pax6.1* mutants, including brain defects, retinal malformation, and the absence of lenses. These findings suggest that *Pax6.3* does not compensate for the loss of *Pax6.1* during early eye development in *Pax6.1* mutants.

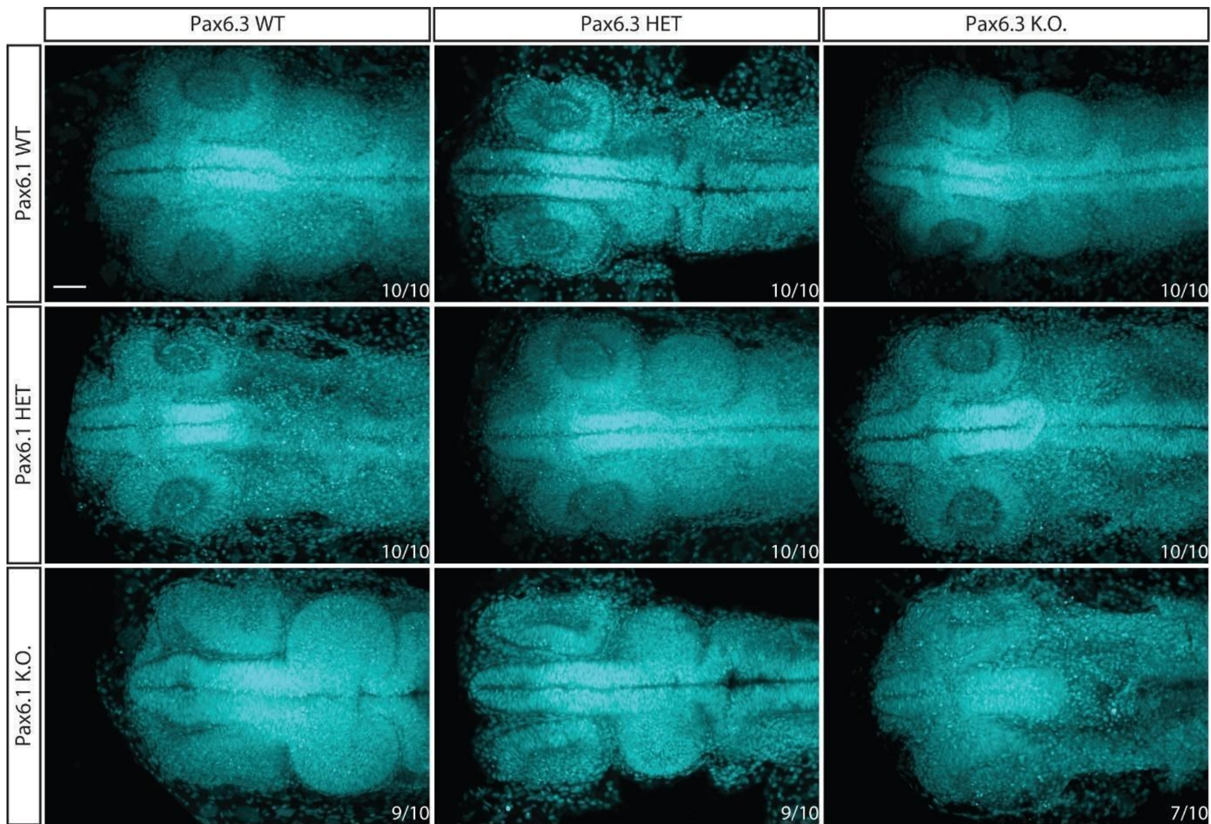


Figure 46: The morphological analysis of *Pax6.1* and *Pax6.3* double mutant at the stage 23 using DAPI staining. Phenotype is present in the brain and the eye of the *Pax6.1/Pax6.3* double mutant. The retina is malformed and lenses seem to be missing. Scale bar: 50µm

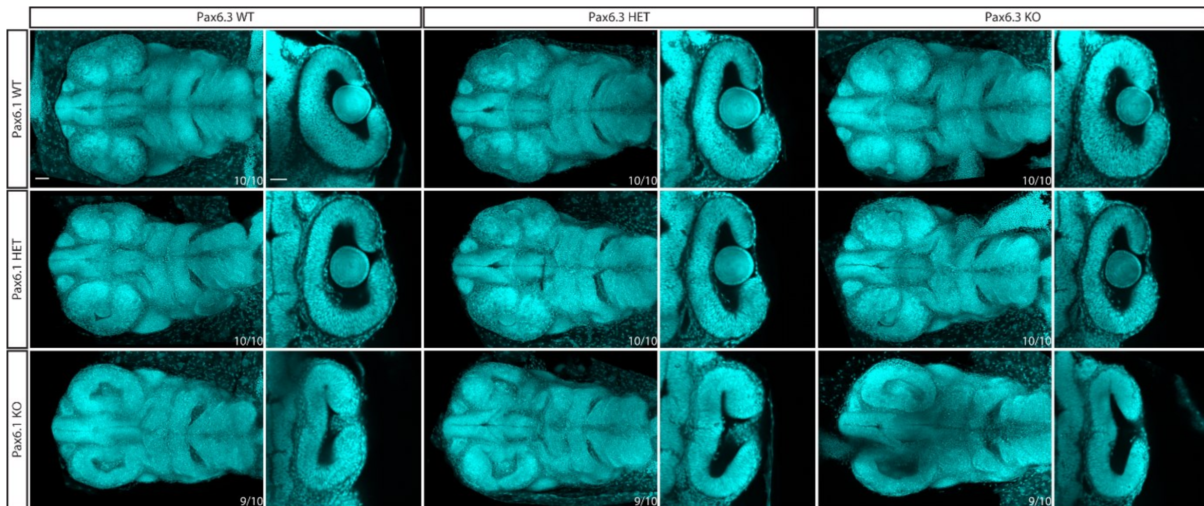


Figure 47: The morphological analysis of the *Pax6.1/Pax6.3* double mutant at the stage 28 using DAPI staining. Retina of the double mutant is present but malformed and lenses seem to be missing. Scale bar: 50µm

Morphological analysis of *Pax6.1/Pax6.3* double mutants revealed the presence of a retina at later stages of eye development. To assess the ability of retinal cells to differentiate into specific cell types, marker genes (*Brn3C*, *NeuroD1*, *Prox1a*, *Sox2*, *Vsx2*, *NR2E3*, *Rhodopsin*) were detected in the developing retinas of double mutant embryos (Figure 48).

The analysis showed that all investigated genes, except for *Brn3C*, were expressed in the appropriate regions of the retina in *Pax6.1/Pax6.3* double mutants. These results indicate the presence of all specific retinal cell types (photoreceptors, horizontal cells, bipolar cells, Müller glial cells, amacrine cells), except for ganglion cells. The observed phenotype in the eyes of the double mutants is consistent with that described in *Pax6.1* mutants. Collectively, these data confirm that the *Pax6.3* gene does not compensate for the loss of *Pax6.1* in *Pax6.1* mutants.

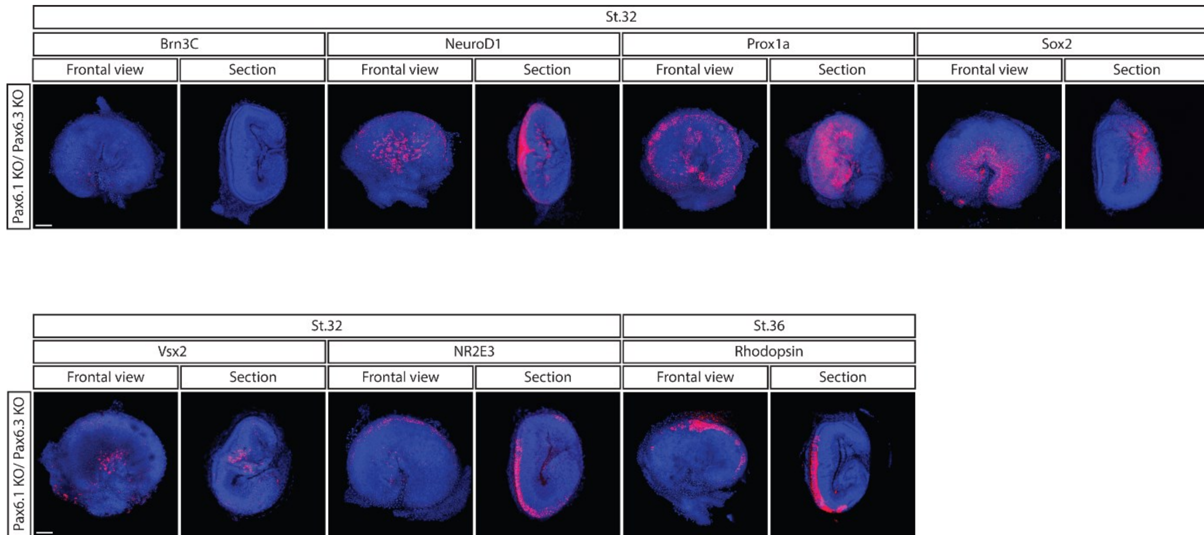


Figure 48: Expression of *Brn3C*, *NeuroD1*, *Prox1a*, *Sox2*, *Vsx2*, *NR2E3* and *Rhodopsin* in *Pax6.1*/*Pax6.3* double mutant. All genes with the exception of the *Brn3C* are expressed appropriately suggesting the presence of all retina specific cell types except for the ganglion cells.

Discussion

The *Pax6* gene is essential for proper eye development across various organisms, including *Drosophila*, chicken, *Xenopus*, fish, and mouse (Hill et al., 1991; Jordan et al., 1992; Quiring et al., 1994; Nornes et al., 1998; reviewed in Kozmik, 2005). The Pax6 protein consists of two DNA-binding domains: the bipartite paired-type domain (PD) and the homeodomain (HD), as well as a proline/serine/threonine-rich (PST) transactivation domain located at the C-terminus (Ton et al., 1991; Glaser et al., 1992; Czerny and Busslinger, 1995) (Figure 11). A significant amount of evidence demonstrates that not only is the structure of the *Pax6* gene conserved across diverse animal species, but its function in eye development is also highly conserved between vertebrates and invertebrates (reviewed in Callaert et al., 1997; Kozmik, 2005).

This PhD thesis investigates the role of the *Pax6* gene during individual stages of embryonic eye development in medaka. The data obtained provide novel insights into the function of *Pax6* in medaka embryonic eye development and its evolutionary significance in vertebrate eye morphogenesis.

Types of mutation in *Pax6* gene in vertebrates

In humans, mutations in the *PAX6* gene are associated with a range of neurodevelopmental and ocular diseases, including mental retardation, attention deficit hyperactivity disorder (ADHD), autism, aniridia, cataract, microphthalmia, Gillespie syndrome, and Peters anomaly (Chao et al., 2000; Malandrini et al., 2001; Davis et al., 2008; reviewed in Cunha et al., 2019). Approximately 71% of mutations in the *PAX6* gene are frameshift, nonsense, or splice-site mutations, all of which are predicted to result in the formation of a premature stop codon. In-frame insertions or deletions constitute 11% of *PAX6* mutations. Missense mutations, which result in a full-length PAX6 protein with a single amino acid substitution, account for 18% of all reported mutations (Figure 49). The majority of missense mutations are localized within the paired domain, whereas only a few have been identified in the homeodomain (reviewed in Hanson, 2003). These findings underscore that most mutations in the human *PAX6* gene primarily affect the paired domain of the PAX6 protein. Several studies suggest that alterations in the paired domain structure may impact the DNA-binding capacity of the homeodomain (Singh et al., 2000; Mishra et al., 2002). Notably, both missense mutations and those causing premature stop codons are linked to various diseases associated with structural abnormalities in the *PAX6* gene.

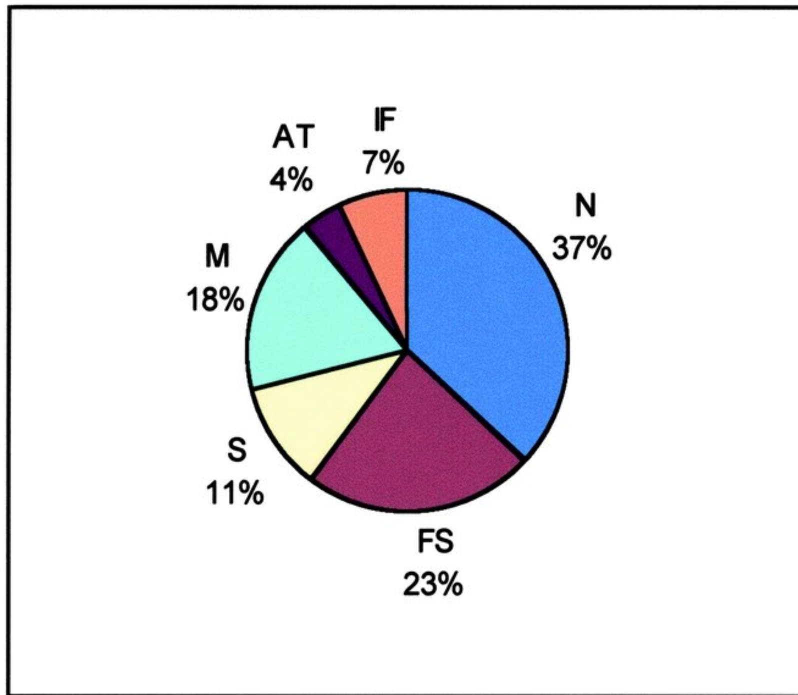


Figure 49: The chart of different mutation types among 275 mutations in the coding area of the human *PAX6* gene. AT: anti-termination mutation, IF: in-frame insertion or deletion, FS: frame-shifting insertion or deletion, M: missense mutation, N: nonsense mutation, S: splice mutation (Hanson, 2003).

The first extensively studied *Pax6* mutant in mouse, known as the *Sey* mutant, was described by Robert Hill (Hill et al., 1991). The *Sey* mutant carries a mutation in the paired domain region of the *Pax6* gene (Hill et al., 1991). Since then, numerous germline and conditional mutations of the *Pax6* gene have been identified (Singh et al., 2000; Marquardt et al., 2001; Klimova et al., 2013; L. Klimova and Z. Kozmik, 2014; Suzuki et al., 2015; Sunny et al., 2020), with the majority of these mutations located within the paired domain. Notably, most of these *Pax6* mutants exhibit eye-related phenotypes.

In a similar manner to the human and mouse mutants, the *Pax6.1* medaka mutant examined in this thesis harbors a mutation in exon 4, a region encoding the paired domain of the protein. This mutant carries a 31 bp insertion, resulting in the formation of a premature stop codon, consistent with the nature of many *Pax6* mutations observed in both mouse and human.

The role of *Pax6* during medaka embryonic eye development

The first *Pax6* mutant, known as *eyeless*, was described in Drosophila in 1915 (Hoge, 1915). Since then, the critical role of the *Pax6* gene in eye development has been observed across a wide range of species, from basal animals such as cnidarians (Piatigorsky and Kozmik, 2004) to highly complex vertebrates, including fish (Nornes et al., 1998), mouse (Hill et al., 1991), and humans (Jordan et al., 1992). A common characteristic of *Pax6* mutants across different species is the manifestation of an eye-related phenotype (Hill et al., 1991; Kleinjan et al., 2008; Takamiya et al., 2015). In both zebrafish and mouse, previous studies have demonstrated that *Pax6* mutants exhibit lens abnormalities or absence, as well as malformations in retinal structure, observed in both germline and conditional mutants, as well as in knock-down experiments (Zinkevich et al., 2006; Tittle et al., 2011; Audette et al., 2016; Vetrivel et al., 2019). The sole study examining the role of *Pax6.1* in medaka showed that lens development is similarly disrupted (Pan et al., 2023).

The role of *Pax6* during medaka embryonic lens development

Lens development is a complex, multi-step process, progressing from lens induction through invagination/delamination, to the eventual detachment of the lens from the ectoderm. Following these stages, secondary lens fibers are formed. Various studies have highlighted the crucial role of signaling from the developing retina in the early stages of lens formation and in ensuring proper fiber morphology during later stages of lens development (Coulombre and Coulombre, 1963; Yamamoto, 1976; Mathers et al., 1997; Yun et al., 2009).

The essential role of the *Pax6* gene during eye development in vertebrates has been well established across multiple species (Hill et al., 1991; Jordan et al., 1992; Quiring et al., 1994; Nornes et al., 1998; Piatigorsky and Kozmik, 2004). In mouse, homozygous *Pax6 Sey* mutants fail to develop eyes entirely (Hill et al., 1991. To further elucidate the function of the *Pax6* gene at specific stages and in distinct regions during embryonic eye development, several *Pax6* conditional mutants were generated using the Cre-LoxP system (Marquardt et al., 2001; Klimova et al., 2013; L. Klimova and Z. Kozmik, 2014; Suzuki et al., 2015; Sunny et al., 2020). Analysis of various *Pax6* chimeric mouse mutants has demonstrated that *Pax6* expression in the surface ectoderm is critical for lens development (Quinn et al., 1996; Collinson et al., 2000). Similarly, conditional inactivation of the *Pax6* gene in the surface ectoderm results in the absence of lens formation, while retinal development proceeds normally (Ashery-Padan et al., 2000). Conversely, *Pax6* expression in the optic vesicle has been shown to be equally crucial for proper lens development (L. Klimova and Z. Kozmik, 2014). If *Pax6* expression is ablated in the optic vesicle prior to its interaction with the surface ectoderm, the expression of lens-specific factors is not initiated, leading to the failure of lens formation (L. Klimova and Z. Kozmik, 2014). Collectively, these findings in mouse clearly demonstrate that *Pax6* expression is required in both the developing retina and surface ectoderm to ensure proper lens development.

DAPI staining of the *Pax6.1* mutant medaka reveals morphological abnormalities in the developing eye region, indicating the absence of a lens. However, the retina remains visible. Further detailed analysis across various stages of embryonic development confirmed the absence of lens. The expression of genes known to be active during the early stages of lens development was absent, leading to the conclusion that lens

formation is not initiated in the *Pax6.1* medaka mutant. During early embryonic eye development in medaka, *Pax6.1* expression is detectable in both the retina and the lens (Figure 21). In our mutant, *Pax6.1* expression is eliminated throughout the entire embryo, preventing us from determining whether the failure of lens formation is due to the absence of *Pax6.1* expression in the retina, the surface ectoderm, or both.

Considering previous data obtained from mouse studies (L. Klimova and Z. Kozmik, 2014), we aimed to investigate the role of *Pax6.1* expression in the surface ectoderm for lens development. In both mouse and zebrafish, two enhancers—Ectoderm enhancer (EE) and SIMO—have been identified to drive *Pax6* activity in the ectoderm (Williams et al., 1998; Dimanlig et al., 2001; Antosova et al., 2016). However, only the EE is present in the medaka genome. We generated a transgenic line containing the medaka EE fused with a sequence encoding green fluorescent protein (GFP). Unexpectedly, no GFP signal was detected in the eye during early embryonic development (Figure 50). Despite this, the EE is highly conserved across species (Williams et al., 1998; Kammandel et al., 1999; Rowan et al., 2010), and it is likely that GFP expression was too weak to be detected by standard in situ hybridisation methods.

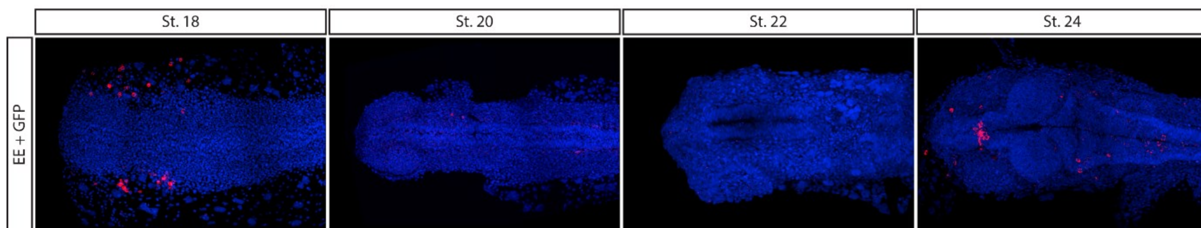


Figure 50: In situ hybridisation analysis for GFP in the transgenic line containing the medaka EE fused with GFP during early stages of embryonic development. No signal in the eye was detected.

To further explore the role of *Pax6.1* in the surface ectoderm during lens development, we generated a mutant line in which the EE was deleted from the medaka genome. Given that EE is the only known ectoderm enhancer of *Pax6* in medaka, this mutant most likely represents a tissue-specific deletion of the *Pax6.1* gene in the surface ectoderm. Previous studies in mouse have shown that conditional knock-out of *Pax6* in the surface ectoderm leads to the absence of lens formation (Ashery-Padan et al., 2000). However, in the medaka EE germline knock-out, a fully developed eye containing both the retina and lens was observed (Figure 51). Due to the one-cell layer structure of the surface ectoderm in early-stage medaka, we were unable to confirm the absence of *Pax6.1* expression in the EE mutant.

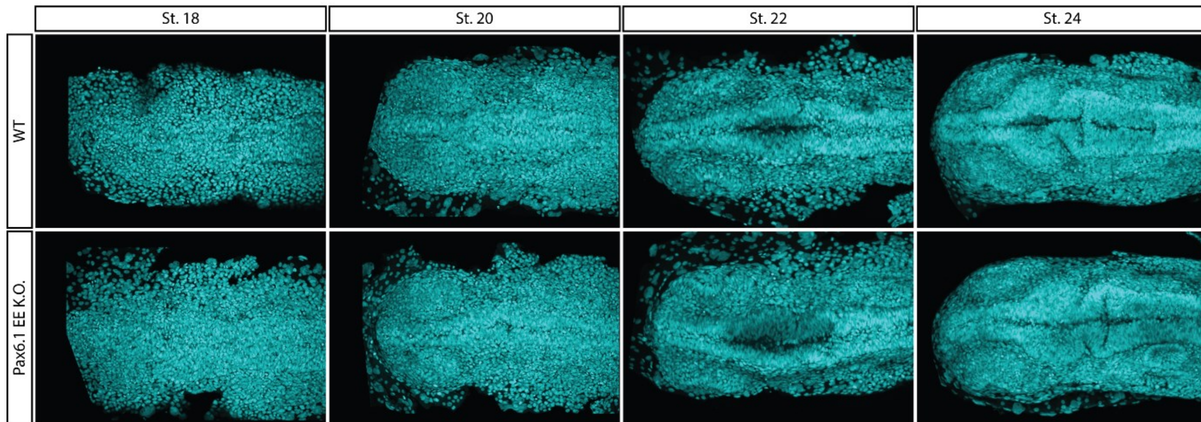


Figure 51: Morphological analysis of *Pax6.1* EE mutant using the DAPI staining. The mutation does not influence the development of the eye. Both retina and lens are present in EE mutant.

These findings suggest the existence of either a previously unidentified *Pax6.1* enhancer responsible for surface ectoderm expression, or that *Pax6.1* expression in the surface ectoderm is not essential for lens development. Overall, our results indicate that retinal signaling may play a critical role in embryonic lens development. Consequently, the lens phenotype observed in our mutant is most likely a result of *Pax6.1* absence in the retina, which is consistent with findings in the mRx-Cre transgenic mouse, where lens development is impaired when *Pax6* is deleted from the retina during early eye development (L. Klimova and Z. Kozmik, 2014).

The role of *Pax6.1* during medaka embryonic retina development

Morphological analysis of the *Pax6.1* mutant medaka reveals the presence of tissue in the presumptive eye region that likely corresponds to the retina. Subsequent experiments confirmed the presence of retinal tissue, though it is malformed, with a lack of differentiation in ganglion cells compared to the retina of wild-type individuals. The malformation of the retinal tissue is likely due to insufficient structural support in the absence of a lens. A similar phenotype of retinal malformation was observed in *Pax6* conditional knock-out mouse models when lens development was affected (L. Klimova and Z. Kozmik, 2014).

Additionally, previous studies in mouse have highlighted the role of the *Pax6* gene in both the proliferation of retinal progenitors and the differentiation of specific retinal cell types (Marquardt et al., 2001; Ohsawa and Kageyama, 2008; L. Klimova and Z. Kozmik, 2014; Klimova et al., 2015). These findings suggest that the retinal abnormalities observed in the *Pax6.1* medaka mutant may be linked to disruptions in these processes, further supporting the critical role of *Pax6* in retinal development.

The role of *Pax6* for proliferation of the retina progenitor cells

Previous studies have highlighted the crucial role of the *Pax6* gene in regulating cell proliferation in the embryonic retina (L. Klimova and Z. Kozmik, 2014). In *Pax6* knock-out (KO) mice, the ratio of retinal progenitors to differentiated neurons in the early developing eye is shifted towards an increase in differentiated neurons when compared to WT (Philips et al., 2005). Conditional inactivation of *Pax6* in the mouse retina leads to a significant reduction in the number of retinal progenitor cells. Similarly, when *Pax6* is conditionally inactivated in retinal progenitor cells, the developing retina becomes hypocellular (Marquardt et al., 2001; L. Klimova and Z. Kozmik, 2014). This hypocellularity in *Pax6*-deficient mouse retinas has been shown to result from *Pax6*'s effect on the proliferative capacity of retinal cells (L. Klimova and Z. Kozmik, 2014). However, in the *Pax6.1* mutant medaka, we did not observe any difference in the proliferation rate of retinal cells between mutants and WT. This finding suggests that the *Pax6.1* gene in medaka does not influence retinal cell proliferation in the same way it does in mouse, indicating species-specific differences in the role of *Pax6* during retinal development.

The role of *Pax6* for differentiation the retina specific cell types

The adult retina of all vertebrates contains six neural cell types—ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptors (rods and cones)—along with one non-neural cell type, Müller glia cells (Centanin and Wittbrodt, 2014). In mouse, the differentiation of specific retinal cell types begins around embryonic day 9 (E9) (reviewed in Bassett and Wallace, 2012; Centanin and Wittbrodt, 2014). However, in the *Pax6 Sey* mutant, eye development is arrested at the optic vesicle stage. As a result, the optic cup containing retinal progenitor cells fails to form, and none of the retinal cell types differentiate at later stages of development (Hogan et al., 1986; Hill et al., 1991).

In contrast, our experiments show that *Pax6.1* in medaka does not impair the formation of retinal progenitor cells or their ability to initiate the differentiation process. Despite this, several conditional mouse mutants have been generated to investigate the specific role of the *Pax6* gene in retinal progenitor cells and their differentiation into distinct retinal cell types (L. Klimova and Z. Kozmik, 2014; Klimova et al., 2015). Experiments in mouse have demonstrated that the generation of retinal-specific cell types is regulated by several transcription factors, including *Pax6* (reviewed in Ohsawa and Kageyama, 2008). Key factors involved in retinal ganglion cell (RGC) differentiation include *Ath7*, *Brn3*, and *Isl1* (Liu et al., 2001; Yang et al., 2003; Pan et al., 2008). Previous studies have shown that *Ath7* is regulated by *Pax6*, and that *Ath7* acts upstream of *Brn3* and *Isl1* (Wang et al., 2001; Riesenberg et al., 2009). In *Ath7* mouse mutants, the number of RGCs is significantly reduced (Wang et al., 2001), and almost no RGCs are present in *Ath7/Brn3* double mutants (Moshiri et al., 2008).

Similarly, in the *Pax6.1* mutant medaka, expression of *Ath7*, *Brn3*, and *Isl1* is absent in the retina. The fact that *Pax6* is positioned upstream in the regulatory network governing retinal ganglion cell differentiation in both medaka and mouse suggests that the absence of *Pax6.1* in the medaka retina likely contributes to the failure of retinal ganglion cell differentiation. This parallels the regulatory role of *Pax6* observed in mouse retinal development.

Studies in mouse have highlighted the importance of the *Pax6* gene as an upstream regulator in the differentiation of specific retinal cell types. It plays a key role in the differentiation of amacrine and bipolar cells by regulating the *Math3*, *NeuroD*, and *Mash1* genes (Hatakeyama et al., 2001; Marquardt et al., 2001; Inoue et al., 2002; L. Klimova and Z. Kozmik, 2014). Previous research has shown that amacrine cells are absent in *Math3/NeuroD* double mutants (Inoue et al., 2002), while *Math3/Mash1* double mutants fail to generate bipolar cells (Tomita et al., 1996). In addition, *Pax6* influences the differentiation of horizontal cells by regulating the expression of *Oc1* and *Oc2* genes. In *Oc1/Oc2* deficient mouse retinas, horizontal cells are completely absent (Klimova et al., 2015). *Pax6* is also important for photoreceptor generation, partially through its regulation of *Crx* expression (Oron-Karni et al., 2008; L. Klimova and Z. Kozmik, 2014). These findings demonstrate the extensive regulatory influence of *Pax6* on the development of diverse retinal cell types.

The involvement of *Pax6* in the regulation of genes critical for the differentiation of specific retinal cell types is likely the reason why none of these cell types are present in the *Pax6*-deficient mouse retina. However, in the *Pax6.1* mutant medaka, all retinal cell types, except for ganglion cells, successfully differentiate. These findings suggest a distinct role of the *Pax6* gene in the retina of teleost fish, such as medaka, compared to that in mouse.

Historically, the *Pax6* gene has been considered a master control gene for eye development in both vertebrates and invertebrates. However, our observations in medaka indicate that embryonic eye formation is not entirely dependent on *Pax6*. This insight challenges the prevailing understanding of the evolutionary role of *Pax6* in eye development across the animal kingdom.

Conclusion

This case study focused on investigating the function of pax6 genes during medaka embryonic eye development. We generated and analyzed mutants of all three pax6 genes present in the medaka genome, providing novel insights into the role of *Pax6.1* in medaka eye formation. The findings presented in this study extend current knowledge of medaka embryonic eye development and contribute to a deeper understanding of the role of pax6 genes in this process.

Key Conclusions**:**

- All three *Pax6* genes are expressed in the eye during medaka embryonic development.
- The *Pax6.1* gene, the orthologue of the mouse *Pax6* gene, plays a crucial role in proper embryonic eye development in medaka. The *Pax6 Sey* mutant in mouse is characterized by an early arrest in eye formation, with heterozygotes displaying smaller eyes and homozygotes failing to develop eyes entirely. In contrast, no visible eye phenotype is observed in medaka *Pax6.1* heterozygotes, while the eyes of *Pax6.1* homozygotes exhibit partial development.
- In the *Pax6.1* mutant medaka, the eyes do not form properly. Lens development is not initiated, resulting in the absence of a lens at later developmental stages. Although the retina is present, it is malformed, and ganglion cells fail to differentiate.
- Differences in phenotypic outcomes between the *Pax6 Sey* mutant in mouse and the *Pax6.1* mutant in medaka suggest that the role of the *Pax6* gene in embryonic eye development is not entirely conserved across species.
- The functions of *Pax6.2* and *Pax6.3* during medaka embryonic eye development remain unclear. Eyes develop normally in *Pax6.2* and *Pax6.3* mutants, indicating that these genes may not be critical for eye formation in medaka.

These conclusions highlight species-specific differences in the role of *Pax6* during vertebrate eye development and open new options for further research into the functional diversification of pax6 genes.

Conclusion: Medaka as a model organism for gene function analysis

Medaka (*Oryzias latipes*) is a small freshwater fish native to East Asia, used as a model organism in various fields of biological research. Due to its relatively small size, transparent embryos, and well-characterized genome, medaka has become an essential tool in studies of developmental biology, genetics, and toxicology. Its ability to thrive in laboratory conditions and the availability of diverse wild-type strains make it a versatile organism for experimental investigations.

Several additional advantages of medaka have been identified, making it a valuable model organism. Compared to zebrafish, medaka exhibits a shorter generation time, approximately two months versus six months, which accelerates genetic studies and breeding programs. Medaka also produces 20-40 eggs daily, and its embryos develop at a slower rate, facilitating a more detailed analysis of intricate developmental processes, such as eye formation. Moreover, medaka possesses a well-annotated genome with fewer repetitive elements, which simplifies genomic studies and gene editing compared to zebrafish. Furthermore, medaka is more adaptable to a broader range of temperature and light conditions, providing greater experimental flexibility. These characteristics collectively position medaka as a powerful and versatile model organism for developmental biology and genetic research (Wittbrodt et al., 2002; Shima and Mitani, 2004; Kasahara et al., 2007; Kobayashi and Takeda, 2008; Murata et al., 2019).

Over the past 350 million years, nearly 25,000 different fish species have emerged. A significant part of this remarkable diversity has been attributed to whole-genome duplications that occurred at the base of the teleost radiation (Vandepoele et al., 2004, reviewed in Furutani-Seiki and Wittbrodt, 2004). Three rounds of whole-genome duplication in teleost fish have resulted in the presence of multiple pax6 genes in both the medaka and zebrafish genomes. In zebrafish, two paralogues of the *Pax6.1* gene are involved in eye development (Kleinjan et al., 2008; Ravi et al., 2013; Takamiya et al., 2015). Due to the redundancy between these paralogues, creating a complete knock-out of both genes would be technologically challenging. To date, a full knock-out of both pax6.1 genes in zebrafish has not been reported. In contrast, the medaka genome contains only one *Pax6.1* gene, which is considered an orthologue of the human and mouse *Pax6* gene (Ravi et al., 2013), making it a simpler system for genetic manipulation.

In addition to *Pax6.1*, both medaka and zebrafish possess a *Pax6.2* gene, which arose from the same whole-genome duplications. Interestingly, medaka also possess a *Pax6.3* gene, which was lost from zebrafish genome. This unique preservation of *Pax6.3* in medaka, along with the differential duplication and loss of pax6 genes between medaka and zebrafish, makes medaka a particularly valuable model for studying the evolutionary dynamics of gene duplication and functional divergence in vertebrate eye development. This evolutionary perspective further enhances medaka's significance as a model organism, offering insights that are not as readily available in zebrafish.

Medaka is a valuable model organism for studying transcriptional regulation. The slower developmental pace of medaka embryos allows for a more precise analysis of gene expression dynamics over time. In certain cases, such as when single-gene orthologues are of interest, medaka can offer distinct advantages over zebrafish, making it a preferable choice for specific transcriptional studies.

Second case study: Medaka as a model organism for transgenic studies

Asymmetric *Pitx2* expression in medaka epithalamus is regulated by nodal signaling through an intronic enhancer

Medaka (*Oryzias latipes*) is a valuable model organism for genetic research, particularly in transgenic studies. Several methods for generating transgenic medaka have been described (Chou et al., 2001; Grabher and Wittbrodt, 2007; Nakamura et al., 2008; Watakabe et al., 2018). The species' slower developmental rate provides additional time for the expression of reporter genes, leading to more accurate and reliable expression pattern. This extended developmental window allows for more precise monitoring of gene expression, even for highly dynamic genes. These factors enable researchers to better elucidate the complex roles of genes in developmental processes.

In the second study presented in this thesis, we investigate the asymmetric *Pitx2* expression in the medaka epithalamus and its responsiveness to the nodal signaling pathway. Our findings will highlight why medaka serves as a better model organism for this specific study compared to the more commonly used zebrafish. The data obtained will offer valuable insights into the role of the *Pitx2* gene in the medaka epithalamus and the mechanisms regulating its expression.

Literature overview

Left-right asymmetry is a phenomenon that attracts interest across multiple fields, including anatomy, developmental biology, and evolutionary biology. This morphological characteristic is observed across a range of scales and organizational levels, encompassing everything from unicellular protists to vertebrate organs. Vertebrates exhibit left right asymmetry, characterized by the asymmetric arrangement of internal organs along the left-right axis. This asymmetry is observed not only in body structures, such as limbs or positioning of internal organs, but also within the organs themselves, including the heart and brain, and even in specific brain regions. The establishment of these asymmetries occurs early in embryonic development. Previous research has identified a critical set of gene involved in the establishment of left-right asymmetry. Notably, genes encoding TGF-β factors, such as nodal and lefty, are essential for ensuring proper asymmetrical development. These genes are expressed in the left part of lateral plate mesoderm during early embryonic development, and their expression is regulated by transcription factor foxh1, with acts as a nuclear mediator of nodal signaling (Saijoh et al., 2000; Shiratori et al., 2001; Norris et al., 2002). The execution of left- right asymmetry is governed by *Pitx2*. Misexpression of *Pitx2* disrupts the normal positioning of internal organs and alters the direction of body rotation in chick and *Xenopus* embryos. Abnormal *Pitx2* expression has also been observed in mouse mutants displaying laterality defects (Logan et al., 1998; Ryan et al., 1998; Lu et al., 1999).

Research has demonstrated that the expression of *Pitx2* in the left part of lateral plate mesoderm is regulated by an asymmetric enhancer (ASE) located within the last intron of the gene. In various vertebrates, including mouse, human, chicken, frog and zebrafish, the ASE contains two or three foxh1 binding sites and one nkx2 binding site (Figure 52) (Shiratori et al., 2001; Shiratori et al., 2006). The foxh1 binding sites are essential for the initiation of *Pitx2* expression, whereas the nkx2 binding site appears to be crucial for sustaining the expression during the later developmental stages (Shiratori et al., 2001). Studies in mouse show that construct lacking ASE are unable to drive expression of the reporter gene in the left part of the lateral plate (Shiratori et al., 2001). On the other hand, the ASEs from other vertebrates possess the capability to drive reporter gene expression in the left lateral plate mesoderm, indicating their significant conservation across species (Shiratori et al., 2006) (Figure 53).

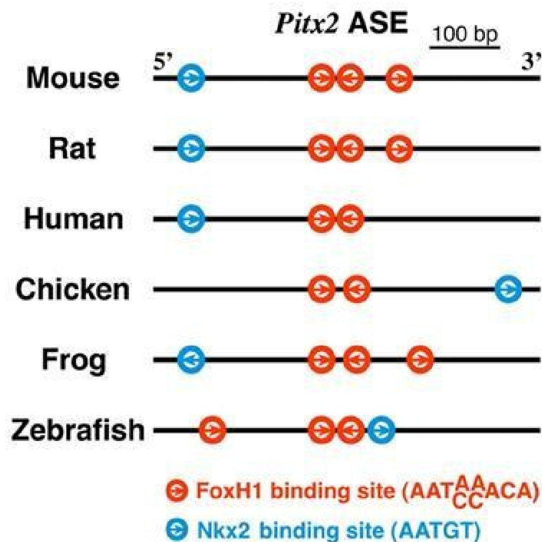


Figure 52: Structures of the *Pitx2* asymmetric enhancer region from various vertebrates. Red and blue circles indicate the binding sites for foxh1 and nkx2, respectively, with arrows showing their orientation (Adapted from Shiratori et al., 2006).

Figure 53: The reporter constructs driven by the *Pitx2* asymmetric enhancer (ASE) from various vertebrates were evaluated in mouse embryos at E8.5. All examined constructs induced reporter expression specifically in the left side of the lateral plate mesoderm (Adapted from Shiratori et al., 2006).

The vertebrate brain also demonstrates left-right asymmetry. Within the dorsal forebrain, the epithalamus exhibits asymmetries characterized by differences in size and neuronal organization of the Habenular nuclei as well as the left-sided positioning of the pineal gland (Roussigne et al., 2012; Ishikawa et al., 2015). Previous research in zebrafish have identified several factors that regulate the left-right asymmetry of *Pitx2* expression, with the nodal signaling pathway being particularly significant. Disruption of nodal signaling pathway through the knock-out of various genes involved results in the loss of asymmetric expression or the emergence of bilaterally symmetrical expression of *Pitx2* in epithalamus,, as well as the randomization of neuroanatomical asymmetries in the zebrafish forebrain Concha et al., 2000. Furthermore, pharmacological inhibition of the nodal signaling pathway result in loss of *Pitx2* expression in the zebrafish brain (Concha et al., 2000; Long et al., 2003; Roussigné et al., 2009). These findings suggest that the nodal signaling pathway is essential for the regulation of *Pitx2* expression and, consequently, for the establishment of proper left-right asymmetry in the zebrafish brain.

Aims of the study

The establishment of left-right asymmetry represents a fundamental process during embryonic development, and its accurate formation is essential for the proper development of an organism. While numerous studies have been conducted on left-right asymmetry in vertebrates, the regulatory mechanisms governing this asymmetry in specific body parts (organs) remain poorly understood. It is established that the asymmetric expression of *Pitx2* in the lateral plate mesoderm is modulated by an asymmetric enhancer (ASE) and is dependent on nodal signaling. However, the mechanisms underlying the asymmetric expression of *Pitx2* in the epithalamus, as well as the potential similarities in regulatory pathways, remain to be clarified. This study aims to investigate whether the asymmetric expression of *Pitx2* in the epithalamus is regulated by the asymmetric enhancer (ASE) and to determine if its expression is also dependent on nodal signaling.

Material and methods

Fish husbandry

The medaka fish were kept under a photoperiod of 14 hours of light and 10 hours of darkness at a temperature of 28°C. The embryos were maintained in ERM solution at either 28°C or 18°C and staged according to Iwamatsu (Iwamatsu, 2004).

Generation of mutants

One cell stage medaka embryos were injected with a mix containing plasmid in which EGFP is driven by 1Kb promotor and intron 2 of *Pitx2* gene. Injected embryos were screened for EGFP signal corresponding with known expression of *Pitx2* and subsequently raised until the adulthood. Pitx2 transgenic line was obtain by crossing these fish with wild type medaka fish.

To assess the responsiveness of the generated transgenic line to the combinatorial presence or absence of Foxh1 binding sites at three positions within the second intron of the *Pitx2* gene, individual foxh1 binding sites in the construct were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). A three-nucleotide alteration (5` AAT(A/C)(A/C)ACA 3` to 5` AAT(A/C)(A/C)CAC 3`) was subsequently introduced into each of the individual putative Foxh1 binding sites within the construct (Figure 54).

```
            10        20        30        40        mut1      60        70        80        90        100
        *    *    *    *    *    *    *    *    *    GTG  *    *    *    *    *    *    *    *    *    *
  1 GTGAGCTGGTCCCACCGGTACCGACGCTCCTAAATGAAAGAGGGAAATGTGTGGATTTAGGCCCGAATTCAGTCCAAGTAATCCCGACGGATTTATTTAT 100
                                                    <<<<<<<<
                                                    foxh1 binding site 1

            110       120       130       140       150       160       170       180       190       200
        *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
101 AATTATAGTTTTTAGCTGAAAGACAAATAATCTGATTCTTGTTTAAATAGACACTGAAGGAAAATAAATATTTTTTTCTGCTGCTGGAATGTTGATCTGC 200

            210       220       230       240       250       260       270       280       290       300
        *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
201 AGTCCACAAAATCCCAGCTCCAAGCACATCATGTTACAGCGGTTCAAAGTTTTCCACGCGGATTTTCCGAACAGGATGGTGTTATTTCTGTCCGTCAGCA 300

            310       320       330       340       350       360       370       380       390       400
        *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
301 GCCCGTTAACAATAGAAGCTGCTGTCATCGCTGACAGACCCACGCGCGCTCTGTGTCCGCGTGAAAGAGGCGCAAAGCTGGAATTTTGGCCATTGTCACT 400

            410       420       430            mut2           460       470       480            mut3
        *    *    *    *    *    *    *    *    CAC  *    *    *    *    *    *    *    *    *    GTG  *
401 GCGGGCTGTGAGATTAGGGTCAGAGTGTTTGTGGGGGAATACACATCCACTCCACGTCAAGATGCTCCTGAGAGGGCTGGGGGTGGGGGGCAATGTGGAT 500
                                         >>>>>>>>                                                     <<<<<<<
                                         foxh1 binding site 2                                         foxh1 binding site 3

            510       520       530       540       550       560       570       580       590       600
        *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
501 TCTGCTCCTATCTCATTAAAAGAGGATTCTCTGCTCAGACGGCCAGAATCTAGCCCGACCCTGCAGCGGGAAAGACGGCTTTATTTAGGGTTTTAATCCT 600
    <
    foxh1 binding site 3

            610
        *    *    *
601 TTCCTTTCTTTCTTTCAG 618
```

Figure 54: Nucleotide sequence of the intron 2 of the medaka pitx2 gene with an indication of mutations introduced into the putative foxh1-binding sites.

Pharmacological treatment

Wild type and transgenic embryos were exposed to the nodal signaling pathway inhibitor SB505124 (Sigma). The inhibitor was initially dissolved in DMSO to a stock concentration of 30 µM, and further diluted to a working concentration of 100 µM. Embryos were treated from developmental stage 21 to stage 24, after which they were fixed in 4% paraformaldehyde/PBS + 0.1% Tween.

Whole-mount In situ hybridization

Embryos at the selected stages were fixed overnight at 4°C with 4% paraformaldehyde/PBS + 0.1% Tween, subsequently dechorionated manually and stored in methanol at -20°C. During the experiment, samples were transferred from Met-OH to PBS + 0.1% Tween and treated with Proteinase K to increase penetration. Embryos were further processed for overnight hybridization with digoxigenin (DIG) labelled antisense riboprobes (Table 7) on 65°C, followed by several washes and incubation with anti-DIG fragments (Roche) conjugated with alkaline phosphatase. The colouring reaction was carried out by BM purple (Roche). All samples were afterwards fixed in 4% paraformaldehyde/PBS + 0.1% Tween and stored in 86% glycerol.

	Primer	
Probe	Forward	Reverse
Pitx2	GTTTGGTTCAAGAACAGGCG	SACCGGYCTRTCCACKGCGTA

Table 7: List of primers used for the DNA amplification.

Spur epoxy resin sectioning

Embryonic axes were excised from the yolk balls and prepared for sectioning. The samples were then gradually dehydrated in increasing concentration of ethanol (up to 100%). Following dehydration, the samples were embedded in Spurr epoxy resin and polymerized at 70°C for 48 hours. The resin blocks were trimmed around the region of interest and sectioned to 400 nm thickness using a diamond knife on a Leica EM UC7 ultramicrotome. The sections were subsequently mounted on slides and photographed using DIC optics.

Imaging

All samples were photographed using an Olympus SZX9 microscope (Olympus). Pictures were processed by ImageJ software (Schindelin et al., 2012).

Results

Asymmetrical expression of *Pitx2* has been previously recognized as a crucial determinant for proper left – right asymmetry in tissues and organs (Logan et al., 1998; Ryan et al., 1998; Lu et al., 1999). The regulation of *Pitx2* expression by the asymmetric enhancer (ASE) within lateral plate mesoderm during early embryonic development has been demonstrated in various vertebrates (Shiratori et al., 2001; Shiratori et al., 2006). However, the role of *Pitx2* and specifically the involvement of the ASE in epithalamus remains unclear. To investigate this, we created several transgenic lines of medaka fish with reporter constructs. We generated three reporter constructs containing reporter gene EGFP driven by either *Pitx2* promoter (line #115-17), *Pitx2* promoter and the ASE sequence (intron 2) (line #116-17) or minimal promoter with the ASE sequence (line #86-4) (Figure 55a). The stable lines carrying only the *Pitx2* promotor linked with EGFP (line #115-17) showed no detectable signal, while the line with the minimal promoter and ASE sequence (line #86-4) exhibited EGFP expression in the lens. Notably, the line containing both the *Pitx2* promoter and ASE sequence (line #115-17) displayed EGFP signal in the lens, ventral diencephalon and left epithalamus (Figure 55 b, c, d). All experiments carried further in this study will be on the line #116-17. These findings confirm that the ASE located in intron 2 carries essential regulatory information for *Pitx2* gene expression in the left epithalamus in medaka.

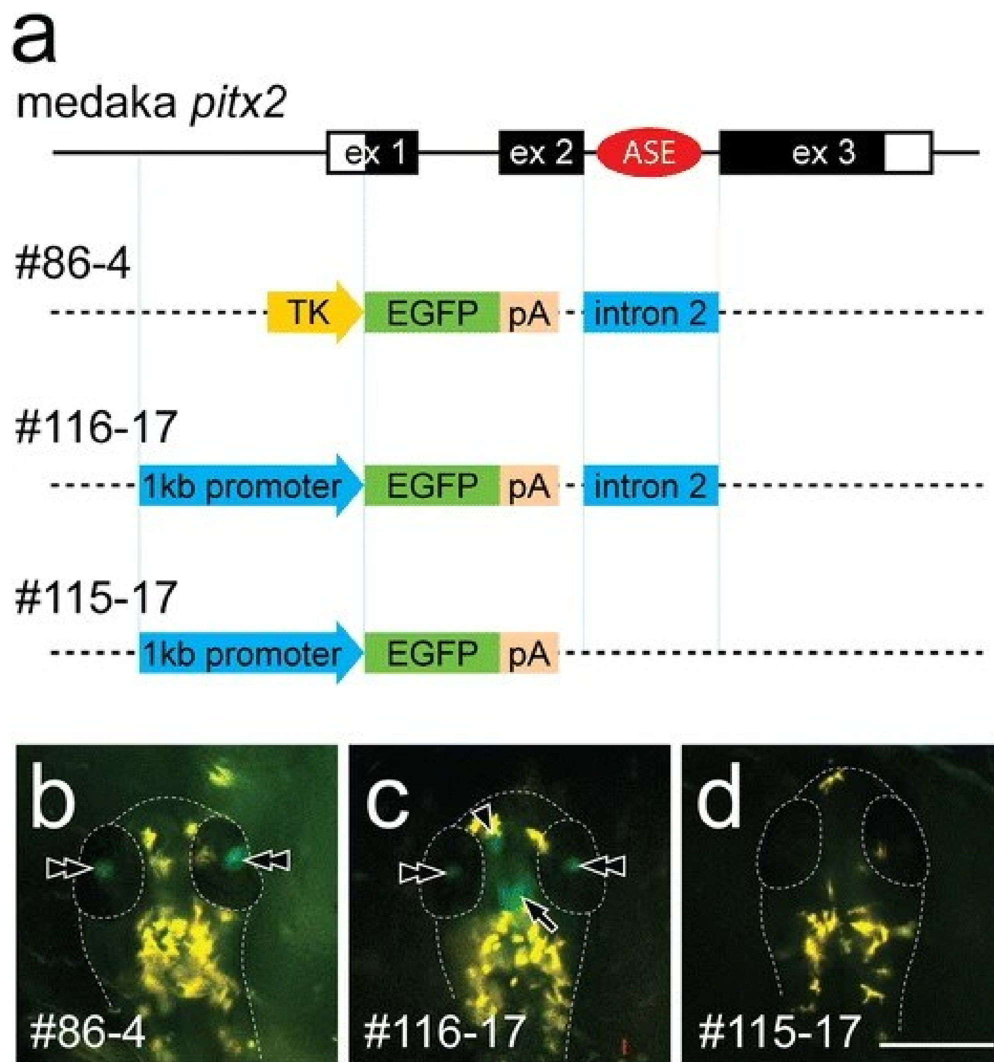


Figure 55: Design of the reporter constructs for generated transgenic medaka lines and their expression in stable lines. A: Structure of medaka *Pitx2* gene. Structure of the generated constructs containing either minimal promoter and ASE (line #86-4), *Pitx2* promoter and ASE (line #116-17) or *Pitx2* promoter (line #115-17) linked with EGFP; B: Line #86-4 shows EGFP expression in the lens (double arrowheads); C: Line #116-17 exhibits EGFP expression in the lens (double arrowheads), ventral diencephalon (arrow) and in the left epithalamus (single arrowheads); D: EGFP signal was not detectable in the line #115-17. Scale bar: 500 µm.

In subsequent analysis, we compared the expression patterns of the endogenous *Pitx2* gene with those observed in our transgenic line (line #115-17) at selected developmental stages (Figure 56). At stage 21, endogenous *Pitx2* is expressed in the diencephalic region and in the left lateral plate mesoderm, while the transgenic line displays EGFP expression in the ventral diencephalon. At stage 23, the endogenous *Pitx2* expression in the diencephalic region is reduced but persists, with continued expression in lateral plate

mesoderm. At this stage, expression in the left epithalamus also becomes evident. Correspondingly, the transgenic line exhibits EGFP expression in both the ventral diencephalon and left epithalamus. During later stages of development (stage 25, 28 or 32), asymmetric endogenous *Pitx2* expression persist in the lateral plate mesoderm and epithalamus. Importantly, the transgenic line continues to express EGFP in the ventral diencephalon, left epithalamus and lens throughout these later stages. In general, our transgenic line replicates the endogenous expression of *Pitx2* to some extent. While it does not recapitulate the expression pattern in the lateral plate mesoderm and differs in ventral diencephalic expression, the transgene faithfully recapitulates *Pitx2* expression in the epithalamus. Additionally, the transgene exhibits ectopic expression in the lens, which can serve as a marker to identify transgenic embryos. These findings indicate that our transgenic line holds potential as a valuable tool for investigating the regulation of epithalamic asymmetry.

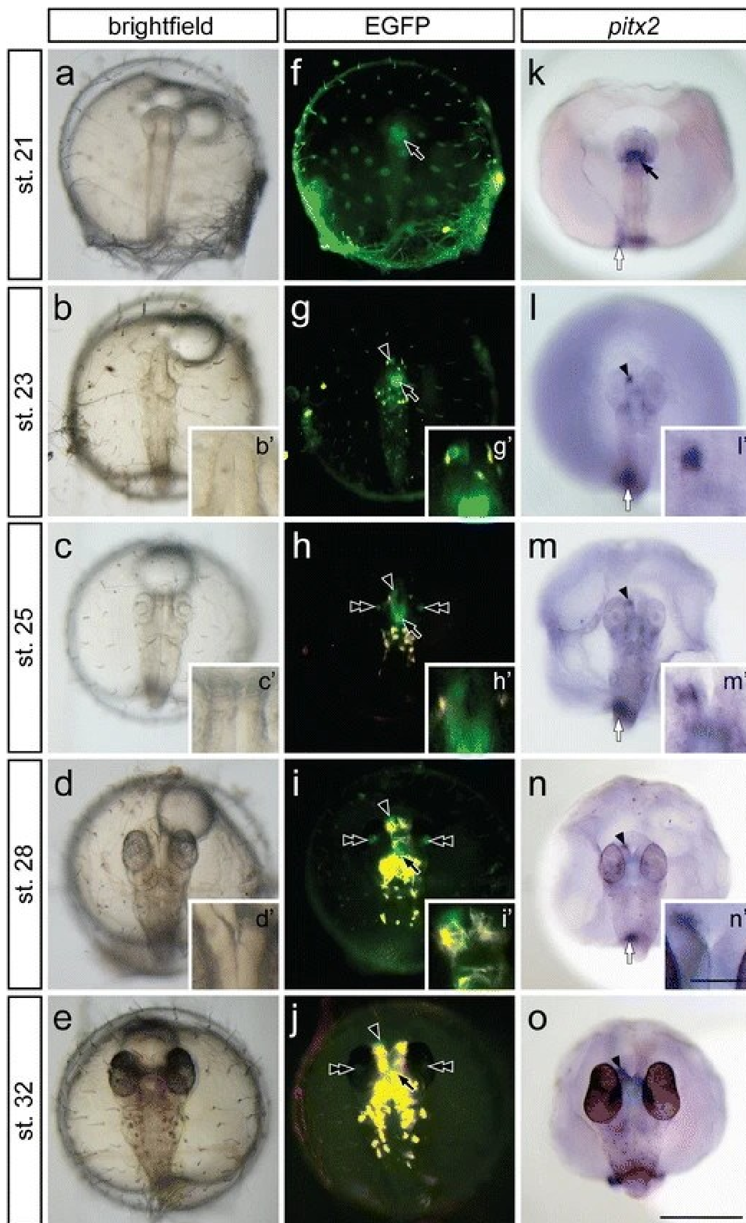


Figure 56: Comparison of endogenous *Pitx2* expression and EGFP expression in the transgenic line across selected developmental stages. A-E: Bright field view of transgenic embryos; B`-D`: Close up views at the diencephalic region; F-J: EGFP expression in the transgenic line; G`-I`: Close up views at the diencephalic region; K-O: In situ hybridization for the endogenous Pitx2; L`-N`: Close up views at the diencephalic region; Black arrow: diencephalic region; White arrow: lateral plate mesoderm; Black arrowhead: epithalamus; Double black arrowhead: additional signal in lens in the transgenic line; Scale is 500 µm and 100 µm in the close ups.

Previous studies demonstrated that asymmetric expression of *Pitx2* is dependent on the nodal signaling pathway (Concha et al., 2000; Long et al., 2003; Roussigné et al., 2009). To investigate, whether our transgenic line, in which EGFP is driven by the *Pitx2* promotor and ASE sequence, responds similarly to nodal signaling, we pharmacologically inhibited the nodal signaling pathway using SB505124 from developmental stage 21 up to stage 24. Following treatments, embryos exhibited a lost the endogenous *Pitx2* expression in the epithalamus and left lateral plate mesoderm, while expression in ventral diencephalon remain unchanged. Similarly, EGFP expression in the epithalamus of the transgenic line was abolished, while signal in the diencephalic and lens signal persisted (Figure 57). These findings indicate that the expression of both endogenous Pitx2 and EGFP in our transgenic line within the left epithalamus is dependent on the nodal signaling pathway. In contrast, the signals observed in the ventral diencephalon and lens in the transgenic line appear to be regulated independently of nodal signaling.

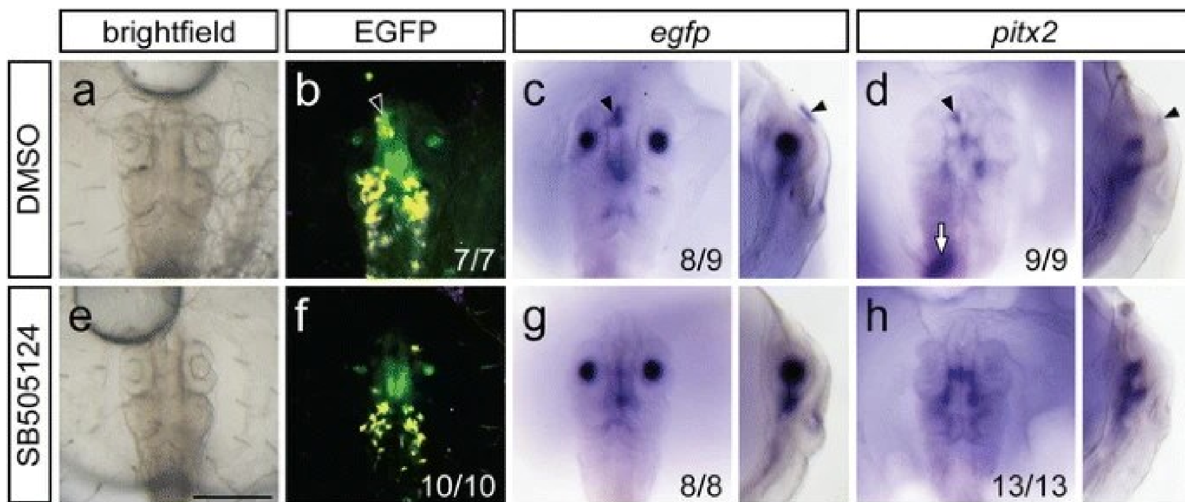


Figure 57: Nodal signaling pathway inhibition treatment. A-D: Control embryos treated with DMSO; E-H: Embryos treated with SB 5050124. Inhibition of the nodal signaling pathway resulted in the loss of both endogenous *Pitx2* and EGFP expression in the epithalamus. The endogenous *Pitx2* signal in the left lateral plate mesoderm also disappeared. However, the expression of *Pitx2* and EGFP in the ventral diencephalon, as well as the EGFP signal in the lens, remained unchanged. Scale bar: 500 µm.

In vertebrates, it is well established that nodal signaling modulates the asymmetric expression of *Pitx2* through foxh1-binding sites located within the asymmetric enhancer (ASE) situated in the last intron of the *Pitx2* gene (Shiratori et al., 2001; Shiratori et al., 2006). Following the confirmation that intron 2 of *Pitx2* is important for the asymmetric expression in the epithalamus of medaka, we aimed to investigate the responsiveness of transgene expression to the loss of individual foxh1 binding sites. We identified three foxh1 binding sites located within the ASE region of the medaka genome. To assess the functional significance of each binding site individually, we have generated a series of constructs, each containing

mutation in one of the binding sites: the first, second or third biding site, as well as combination of mutation affecting the first and second, first and third, second and third, or all three binding sites simultaneously (Figure 58 a). Following the injection of these constructs, embryos were screened for reporter signal expression in left epithalamus. The construct carrying mutation in all three foxh1 binding sites repeatedly failed to drive EGFP expression in left epithalamus compared to control embryos injected with non-mutated construct (Figure 58 b). Embryos injected with constructs carrying mutations that includet the second foxh1 binding site (second, first and second or second and third binding site) showed a significant loss of left epithalamic expression. In contrast, embryos injected with constructs that did not include mutation in the second foxh1 binding site (first, thirdt or first and third binding site) did not exhibit significant loss of left epithalamic expression compared to control embryos (Figure 58 c). These results indicate that the nodal signaling pathway regulates the *Pitx2* expression in left epithalamus through the foxh1 binding sites of ASE, predominantly through the second foxh1 binding site.

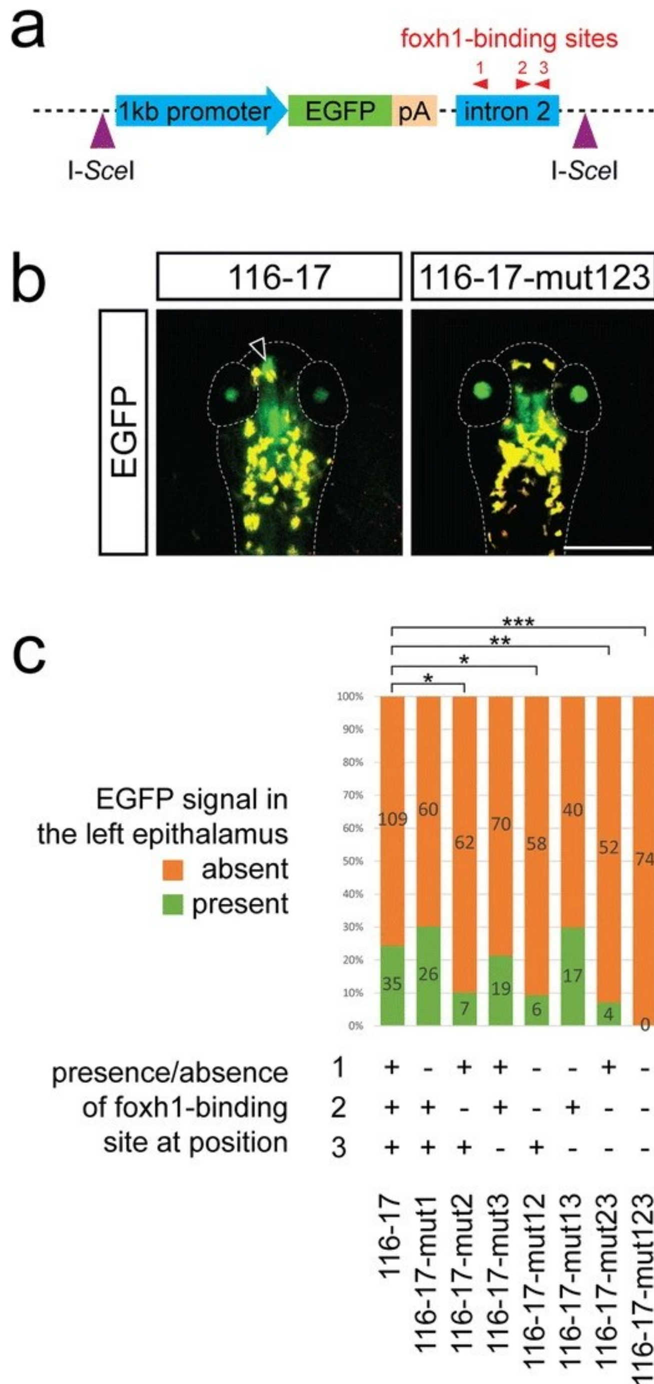


Figure 58: The mutagenesis of foxh1 binding sites present in the ASE region of intron 2 of Pitx2 gene in medaka. A: Schematic representation of the construct with mutated foxh1 binding sites; B: Loss of EGFP expression in left epithalamus following the mutation of all three hoxh1 binding sites; C: Frequency of occurrence of the EGFP signal in the left epithalamus of embryos injected with constructs carrying mutation in different foxh1 binding sites. Embryos that did not eexhibit any EGFP signal following injection were excluded from the analysis; Black aroowhead: left epithalamus; Scale bar: 500 µm.

Discussion

Pitx2 plays a central role in regulating left – right asymmetry in the brain. While its regulation via nodal dependent asymmetric enhancer (ASE) in the lateral plate mesoderm has been described across various vertebrates, it remains unclear whether the same regulatory mechanisms govern *Pitx2* expression in the epithalamus. To address this question, we generated multiple transgenic medaka lines to investigate the regulation of *Pitx2* in the epithalamus. Our findings demonstrate that the promotor alone is not sufficient to drive the expression in the epithalamus, both the promoter and the regulatory region located within the second intron of the gene, which includes the ASE, are required for the asymmetric *Pitx2* expression in the epithalamus. Although the transgenic line does not fully recapitulate the endogenous expression pattern of *Pitx2*, it serves as a valuable tool for identifying some of the regulatory elements of the *Pitx2* gene in medaka. The discrepancies between the EGFP expression in our transgenic line and the endogenous *Pitx2* expression likely arise from the absence of additional regulatory sequences in the transgene.

Interestingly, EGFP expression in the left epithalamus of the transgenic line becomes detectable at stage 22. It has been reported that other nodal related factors, such as *Ndr2* or *Lefty*, as well as endogenous *Pitx2*, are co-expressed in the epithalamus at this stage. Additionally, we confirmed the dependence of ASE in medaka on nodal signaling through pharmacological inhibition. Altogether, data suggests that nodal signaling plays a significant role in establishing epithalamic asymmetry, in a manner similar to what has been observed in zebrafish (Long et al., 2003; Jaszczyszyn et al., 2007; Soroldoni et al., 2007; Roussigné et al., 2009).

The ASE of the *Pitx2* gene in vertebrates contains two or three foxh1 binding sites (Shiratori et al., 2001; Shiratori et al., 2006). In zebrafish, knock-out of the *Foxh1* gene leads to a loss of *Pitx2* expression in the left epithalamus (Concha et al., 2000), indicating that *Pitx2* expression in epithalamus is regulated by nodal dependent transcription factor *Foxh1*. Consistent with this, we have demonstrated that mutagenesis of all three foxh1 binding sites within the ASE results in the loss of EGFP signal in the left epithalamus, while the ventral diencephalon signal remains unaffected. These findings align with data previously published in mouse, where mutagenesis of foxh1 biding sites in the ASE similarly resulted in the loss of *Pitx2* expression in lateral plate mesoderm after (Shiratori et al., 2001). Moreover, we showed that mutagenesis of the second foxh1 binding site, in combination with either of the other sites, is sufficient for the loss of signal in left epithalamus. This indicates that among those three foxh1 binding site, second foxh1 binding site is necessary for the regulation of *Pitx2* expression in the epithalamus.

In summary, our data demonstrate that the asymmetric expression of *Pitx2* in the fish epithalamus is regulated by nodal responsive foxh1 binding sites located within the asymmetric enhancer in the intron of *Pitx2* gene. We provide evidence that the regulatory mechanisms undelaying *Pitx2* expression in left epithalamus of medaka are similar to those previously described in other vertebrates.

Conclusion

This case study focused on investigating the regulation of the *Pitx2* gene in the epithalamus during medaka embryonic brain development. We generated and analyzed several transgenic lines, providing novel insights into the role and regulatory mechanisms of *Pitx2* during medaka brain development. The findings presented in this study extend current knowledge of medaka brain development and contribute to a deeper understanding of the role of the *Pitx2* gene in this process.

Key Conclusions**:**

- *Pitx2* gene is expressed in the epithalamus during medaka brain development.
- *Pitx2* transgenic lines generated in this study partially recapitulate the expression pattern of endogenous *Pitx2* gene.
- The asymmetric enhancer (ASE) carries essential regulatory information for *Pitx2* gene expression in the left epithalamus in medaka.
- The expression of *Pitx2* in epithalamus is regulated by the nodal signaling pathway the ASE.
- The ASE contains three foxh1 binding sides. Our data show that the nodal signaling pathway regulates *Pitx2* expression in the left epithalamus through the foxh1 binding sites of ASE, predominantly via the second foxh1 binding site.

In conclusion, our findings reveal that the asymmetric expression of *Pitx2* in the medaka epithalamus is controlled by nodal-responsive foxh1 binding sites within the asymmetric enhancer located in the intron of the *Pitx2* gene. Our evidence suggests that the regulatory mechanisms governing *Pitx2* expression in the left epithalamus of medaka are conserved and comparable to those observed in other vertebrate species.

<u>Identification of a conserved Pax6 enhancer controlling iris development</u>

In the third study presented in this thesis, we investigate the novel *Pax6* enhancer called IrisE and its role in the iris development in different vertebrates as well as its ability to cooperate with other *Pax6* enhancers (Simo and α enhancer) active in the same area. In this study, we use both medaka and zebrafish, so it's a great opportunity to point out strength of these species individually. It is also a nice example of the use of medaka as a complementary model animal to other model organisms. Data described in this case study are preliminary results, therefore only the data relevant to the topic of this theses will be discussed further.

Overview of the study

The iris is a thin, contractile, colored structure located anterior to the lens in the vertebrate eye. Its primary function is to modulate the amount of light that enters the eye by adjusting the size of the pupil. During embryonic eye development, the iris arises from the retinal pigmented epithelium and peripheral region of the neural retina called the ciliary margin zone (CMZ), as well as from the periocular mesenchyme (Davis-Silberman and Ashery-Padan, 2008; Davis et al., 2009). The mature iris is composed of several layers – pigmented epithelium, iridial muscles and stroma (Davis-Silberman and Ashery-Padan, 2008). Iris morphogenesis is regulated by transcription factors such as homeobox genes *Pax6*, *Meis1*, *Meis2*, *Otx1*, as well as Bmp, FGF and Wnt signaling pathways (Acampora et al., 1996; Zhao et al., 2002; Davis-Silberman et al., 2005; Liu et al., 2007; Zhou et al., 2013; Dupacova et al., 2021). It appears that the *Pax6* gene plays a particularly crucial role in proper iris development. In mouse, studies on complete gene inactivation, conditional deletion in the peripheral retina, or overexpression of *Pax6*, have demonstrated irregular growth and maturation, or structural abnormalities of the iris (Davis-Silberman et al., 2005; Davis et al., 2009). These phenotypes may result either from the direct loss of *Pax6* expression or indirectly as a consequence of disruptions in lens and retina development.

In this study, we have identified a novel cis-regulatory element, named IrisE, which is essential for proper iris development. We elucidate the role of this new iris enhancer in the development of the iris and ciliary body in mouse. Furthermore, the potential cooperative function of IrisE, the α enhancer and the SIMO enhancer has also been tested. Additionally, our findings demonstrate that IrisE is highly conserved across vertebrate species including human, mouse, zebrafish or chick.

It has been established that CMZ and retinal pigmented epithelium (RPE) are the main tissues contributing to the formation of the iris and ciliary body. To investigate the chromatin accessibility at the Pax6 locus, we employed bulk ATAC-seq from CMZ in mouse. Furthermore, we compared our findings with previously published bulk ATAC-seq data from chick RPE and zebrafish (Buono et al., 2021; Tangeman et al., 2022). In addition to the previously characterized *Pax6* enhancers (α and SIMO) (Antosova et al., 2016), we identified a novel cis-regulatory element (IrisE), located within the *Pax6* HS234 enhancer.

Both α and SIMO enhancers are highly conserved across species. Therefore, we investigated whether IrisE exhibits similar conservation among vertebrates (Bhatia et al., 2013; Antosova et al., 2016). Comparative analysis revealed a high level of conservation (75-93%) in IrisE region among human, mouse, chick and fish. Notably, IrisE is present in elephant shark, Mexican tetra, spotted gar, bichir and zebrafish, but is absent in medaka.

In mouse, IrisE is located within the intronic region of the ELP4 gene, specifically in the *Pax6* enhancer HS234 area. To determine whether IrisE is sufficient to drive enhancer activity in the retina, we have generated several reporter gene constructs. A construct with two mouse IrisE elements fused with minimal promoter and a reporter gene under lacZ control was electroporated into developing chick embryos. Whole-mount lacZ staining revealed reporter activity in the peripheral retina and RPE. Given the high conservation of IrisE across species, we also evaluated the activity of elephant shark IrisE in chick embryo. Results demonstrated a lacZ staining pattern in both the retina and RPE, suggesting that the elephant shark IrisE functions similarly to the mouse IrisE element. In addition, we tested the activity of IrisE elements from mouse, zebrafish and spotted gar in zebrafish. All tested elements activated reporter gene expression in

embryonic retina (24-26 hpf) (Figure 59). Although the medaka genome does not contain IrisE element, we examined the activity of elephant shark and spotted gar IrisE in medaka embryos. Both elements were able to promote the expression of the reported gene in the retina (Figure 60). Collectively, these findings confirm that the highly conserved *Pax6* IrisE element can activate *Pax6* expression in the developing retina across various vertebrate species.

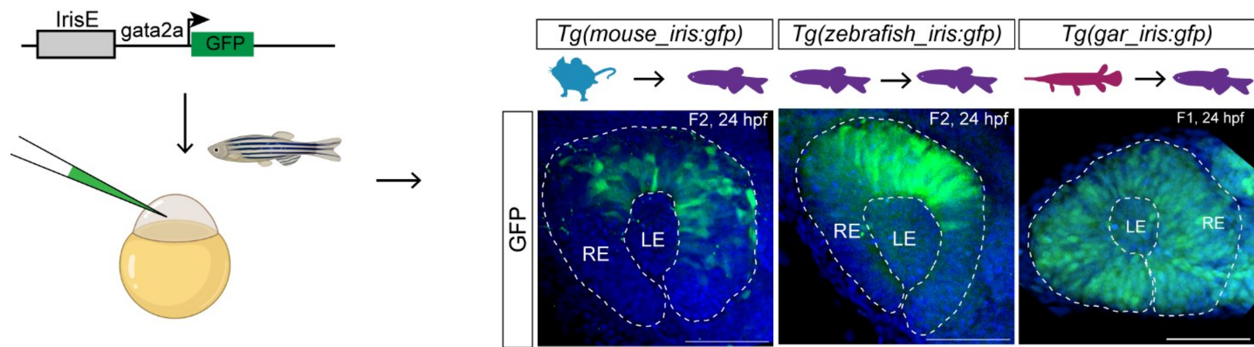


Figure 59: IrisE elements from mouse, zebrafish or gar activate reporter gene expression in embryonic retina of the zebrafish at 24-26 hpf. Scale bar: 50 µm.

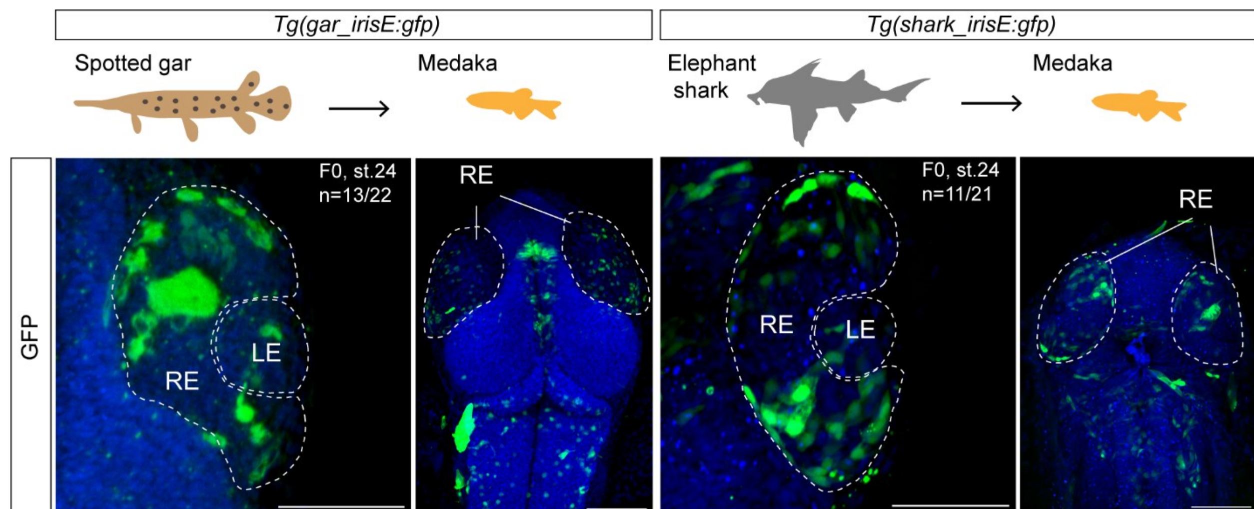


Figure 60: IrisE elements from spotted gar and elephant shark both activate reporter gene expression in embryonic retina of medaka at stage 24. Scale bar: 50 µm.

To further examine the role of IrisE, Simo and α enhancer in iris development, we analyzed mouse models with various knock – out alleles combinations (α, IrisE, α/Simo, α/IrisE). Iris development was assessed by measuring pupil diameter and conducting histological analysis. Mouse with deletion of α or α/Simo

displayed only very mild phenotype, whereas mouse with IrisE or α/IrisE deletion exhibited aniridia, characterized by a shortened iris and hypoplastic ciliary body. These findings demonstrate the importance of the IrisE enhancer in iris and ciliary body development, likely functioning cooperatively with the α enhancer.

To investigate the role of the IrisE and α enhancers, we performed a detailed analysis of retinal morphology in mouse models lacking either the IrisE enhancer, the α enhancer, or both. In the absence of these enhancers, the peripheral region of the RPE showed thickening and hypopigmentation. Immunohistochemical analysis of *Pax6* expression revealed downregulation in both the peripheral retina and RPE in IrisE/ α enhancer deficient mouse. In contrast, mouse lacking only the α enhancer showed reduced *Pax6* expression specifically in the peripheral retina. These results suggest that IrisE enhancer is important for ensuring proper *Pax6* expression in the RPE, while also contributing to *Pax6* regulation in the peripheral retina, likely in cooperation with the α enhancer.

Experiments revealed downregulation of *Pax6* in the RPE and CMZ. Consequently, we wanted to evaluate the development of these structures in IrisE and IrisE/α knock-out mouse using specific markers for RPE (*Mitf*, *Otx2*, *Tyrp1*) (Hodgkinson et al., 1993; Hemesath et al., 1994; Martinez-Morales et al., 2001; Bharti et al., 2012) and CMZ (*Cdo*, *Foxp2*, *Msx1*, *Aqp1*, *Mitf*). Our results demonstrate either a complete absence or significant downregulation of all these markers in IrisE and IrisE/α mutants. Notably, all markers, except *Msx1*, were also downregulated in α enhancer mutant mouse. These findings underscore the critical role of the IrisE enhancer in the proper formation of the RPE. Disruption of the IrisE enhancer leads to a loss of RPE identity in peripheral RPE cells, which subsequently adopt a proximal CMZ fate. Moreover, the distal and medial CMZ, which give rise to the iris and ciliary body, are absent in our mutants (Davis-Silberman and Ashery-Padan, 2008; Davis et al., 2009; Bélanger et al., 2017). These data highlight the significance of the IrisE enhancer, together with α enhancer, in the developmental process of the CMZ. Additionally, deletion of the IrisE enhancer in a mouse with sensitive genetic background ($Pax6^{sey}$) results in more severe ocular phenotype, characterized by albinism, hypercellular RPE, and the absence of lens.

In order to clarify the gene regulatory mechanisms controlling *Pax6* expression via the IrisE element, we investigated the presence of known binding motifs for various signaling pathways and transcription factors within the enhancer region. Specifically, we identified motifs associated with the Wnt (Tcf/Lef), Notch (RBPJ), and BMP (Tgfβ) signaling pathways, as well as motifs for transcription factors such as *Sox*, *Meis*, *Otx1*, and *Vsx2*. To determine whether these factors modulate *Pax6* expression through the IrisE enhancer, we conducted a series of experiments, including luciferase assays, ChIP-seq analyses, and in vivo assays in a mouse model. The results indicate that these transcription factors collaboratively regulate *Pax6* gene expression during retinal development through IrisE.

Additionally, we investigated the role of the IrisE enhancer in teleost fish. Given that transgenic lines showed constructs containing the IrisE enhancer from various species, coupled with GFP sequences, drive expression in the retina of zebrafish or medaka—similar to observations in mouse—we aimed to determine if the IrisE enhancer's function in teleosts fish aligns with that in mouse. Since no *Pax6* IrisE enhancer is present in the medaka genome, our focus turned to zebrafish, where an IrisE enhancer has been identified. We performed targeted mutagenesis on the region of interest and analyzed the phenotype of *Pax6* IrisE homozygous mutants in the eye, particularly in the CMZ and iris regions.

First, we assessed *Pax6.1* gene expression in the developing eye of the *Pax6* IrisE homozygous mutant, which revealed that expression remained unchanged compared to the wild-type (WT) (Figure 61). Collectively, these findings suggest that the function of the IrisE enhancer in mouse and zebrafish is not completely conserved. This divergence may stem from differences in eye development between zebrafish and mouse or the need for zebrafish eyes to adapt to distinct environmental conditions. Another possibility is that deleting the IrisE enhancer alone does not produce a notable phenotype, as it likely collaborates with additional regulatory sequences during zebrafish eye development. It is plausible that another, as-yet unidentified sequence in the zebrafish genome plays a significant role in the development of the iris and CMZ. Further investigation is required to fully elucidate the precise role of the IrisE enhancer in zebrafish eye development.

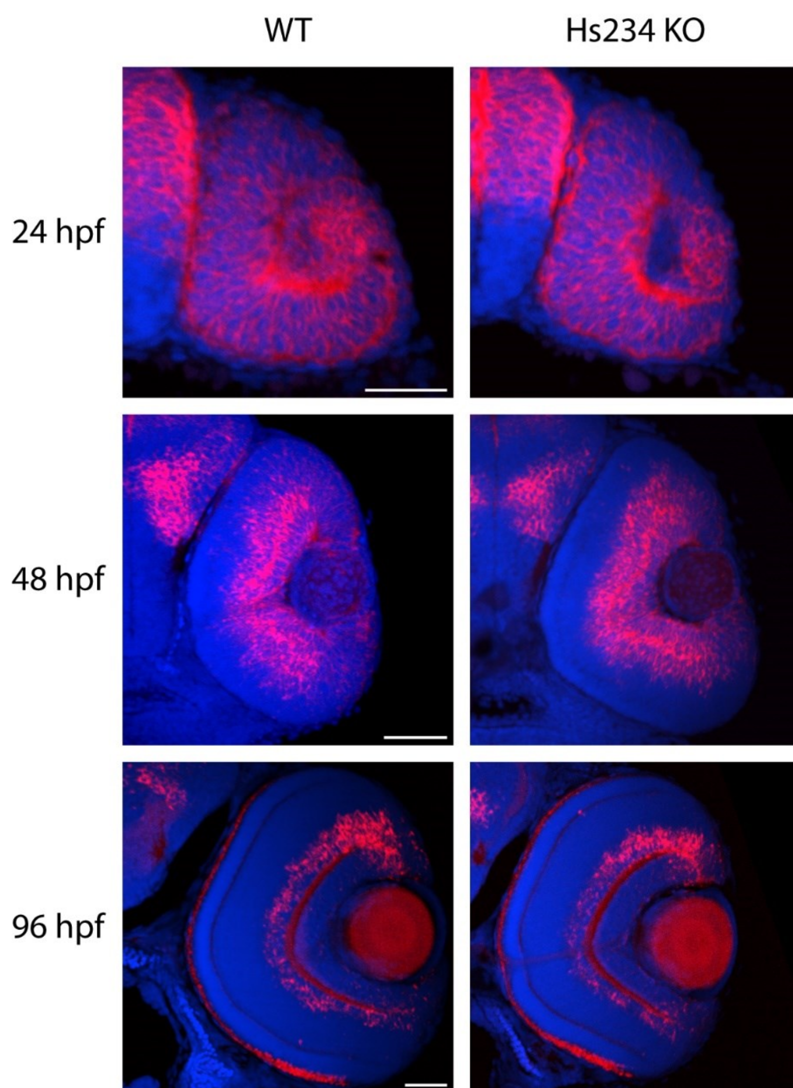


Figure 61: Comparison of *Pax6.1* gene expression in the developing eye of the *Pax6* IrisE homozygous mutant and WT. Scale bar: 50 µm.

Conclusion: Medaka as a model organism for transgenic studies

The advantages of medaka over zebrafish outlined in the previous case study are equally relevant here, highlighting medaka as an exceptional model organism for developmental and genetic studies. Its small size and transparent embryos enable detailed, non-invasive observation throughout various stages of development, making it ideal for high-resolution imaging of biological processes. Medaka's well-characterized genome, coupled with its ability to thrive in laboratory conditions, provides a reliable foundation for reproducible experiments. Additionally, medaka's shorter generation time, which is approximately two months compared to zebrafish's six, allows for faster breeding cycles, significantly accelerating genetic studies. Medaka's well-annotated genome, with fewer repetitive elements, simplifies genomic analyses, while its adaptability to a broader range of temperature and light conditions ensures experimental flexibility across diverse laboratory setups. These advantages collectively make medaka an invaluable model for probing complex genetic and developmental pathways (Wittbrodt et al., 2002; Shima and Mitani, 2004; Kasahara et al., 2007; Kobayashi and Takeda, 2008; Murata et al., 2019).

Medaka's prolific egg production, combined with the slower rate of embryonic development, provides distinct advantages for precise gene expression studies. The extended developmental timeframe allows for more prolonged expression of reporter genes, such as GFP or other markers, ensuring that expression patterns are captured in greater detail. When creating F0 generations via microinjection, the slower pace of medaka's early development grants more time for successful integration of the transgene during the single-cell stage. This reduces mosaicism in later stages and results in more consistent and accurate expression patterns, even within the F0 generation. Consequently, medaka offers a unique capacity to achieve high-resolution, reliable gene expression data that enhances the accuracy of developmental studies.

Both medaka and zebrafish experienced three rounds of whole-genome duplication in their evolutionary history, leading to an initial expansion of their gene repertoires. Over time, each species lost certain duplicated genes, while other genes underwent functional divergence, adapting to fulfill new or modified roles (reviewed in Kobayashi and Takeda, 2008). This process of gene loss and functional specialization has resulted in distinct genomic compositions in each species. Medaka's genome, in particular, is more compact, retaining fewer duplicated genes than zebrafish, which simplifies studies focused on gene regulation and enhancer activity (Freeman et al., 2007; Kasahara et al., 2007; Rouchka, 2010). With a streamlined genome and reduced redundancy, medaka allows researchers to examine regulatory elements and gene function more clearly, as individual gene roles and their associated regulatory sequences are less likely to be influenced by additional paralogues. This unique genomic structure makes medaka an ideal model for detailed studies of gene expression and regulatory mechanisms.

In conclusion, medaka's unique genomic features and experimental advantages make it an exceptional model for studying gene regulation and developmental biology.

Conclusion

In summary, this thesis demonstrates the distinct advantages of medaka as an alternative and complementary model organism for studying transcriptional regulation and developmental processes, particularly in areas where zebrafish may face limitations. The research underscores medaka's suitability for precise gene function analysis, benefiting from its small size, transparent embryos, shorter generation time, and well-annotated, streamlined genome with fewer duplicated genes. This compact genomic structure simplifies studies on regulatory elements, while the retention of certain evolutionary genes, like *Pax6.3*, enables unique investigations into gene conservation and functional divergence, offering an evolutionary perspective that enhances our understanding of vertebrate genetics. Together, these findings reinforce medaka's role as a valuable and powerful model organism, driving forward our insights into complex genetic regulation and developmental biology pathways.

List of publications relevant for this thesis

Genetic analysis of medaka fish illuminates conserved and divergent roles of Pax6 in vertebrate eye development

Mikula Mrstakova S and Kozmik Z (2024) Genetic analysis of medaka fish illuminates conserved and divergent roles of Pax6 in vertebrate eye development. *Front. Cell Dev. Biol.* 12:1448773. doi: 10.3389/fcell.2024.1448773

In this study, I was responsible for mutant generation, which included breeding, microinjections, and DNA mutation screening via sequencing, with the construct preparation conducted by Dr. Zbyněk Kozmik. I also performed mutant analysis through in situ hybridization, immunohistochemistry experiments, and morphological analysis. Additionally, I carried out imaging and processed images to support the presentation and interpretation of the results.

Asymmetric pitx2 expression in medaka epithalamus is regulated by nodal signaling through an intronic enhancer

Soukup, V., Mrstakova, S. & Kozmik, Z. Asymmetric *pitx2* expression in medaka epithalamus is regulated by nodal signaling through an intronic enhancer. *Dev Genes Evol* **228**, 131–139 (2018).

In this study, I contributed to the establishment of transgenic lines, including tasks such as breeding the animals and screening for transgenic signals. Additionally, I supported the visual documentation by assisting with the photography of transgenic animals, ensuring accurate representation of experimental outcomes.

Identification of a conserved *Pax6* enhancer controlling iris development.

Manuscript in preparation

Dupacova N., Mikula Mrstakova S., Bendova M., Antosova B., Sunny, S.S., Kozmik-Jr. Z., Paces J., Bartunek P., Kozmik Z.

In this study, I conducted experiments on zebrafish and medaka, including the generation of transgenic lines, where I handled breeding, microinjections, and screening for transgenic signals, with the construct preparation provided by Dr. Zbyněk Kozmik. I also carried out the analysis of transgenic lines through in situ hybridization experiments and live imaging. For knock-out line generation, I was responsible for breeding, microinjections, and DNA screening for mutations, again with construct preparation by Dr. Kozmik. Additionally, I performed mutant analysis using in situ hybridization, morphological analysis, and handled the processing of images for accurate data representation.

References

Acampora, D., Mazan, S., Avantaggiato, V., Barone, P., Tuorto, F., Lallemand, Y., Brûlet, P. and Simeone, A. (1996). "Epilepsy and brain abnormalities in mice lacking the Otx1 gene." Nat Genet 14(2): 218-222.

Adelmann, H. B. (1936). "The problem of cyclopia." Q. Rev. Biol. 11: 161-182.

Amato, M. A., Arnault, E. and Perron, M. (2004). "Retinal stem cells in vertebrates: parallels and divergences " Int J Dev Biol 48: 993-1001.

Antosova, B., Smolikova, J., Borkovcova, R., Strnad, H., Lachova, J., Machon, O. and Kozmik, Z. (2013). "Ectopic Activation of Wnt/β-Catenin Signaling in Lens Fiber Cells Results in Cataract Formation and Aberrant Fiber Cell Differentiation." Plos one 8(10).

Antosova, B., Smolikova, J., Klimova, L., Lachova, J., Bendova, M., Kozmikova, I., Machon, O. and Kozmik, Z. (2016). "The Gene Regulatory Network of Lens Induction Is Wired through Meis-Dependent Shadow Enhancers of Pax6." PLoS Genet 12(12): e1006441.

Asai, T., Senou, H. and Hosoya, K. (2011). "Oryzias sakaizumii, a new ricefish from northern Japan (Teleostei: Adrianichthyidae)." Ichthyological Exploration of Freshwaters 22(4): 289.

Ashery-Padan, R., Marquard, T., Zhou, X. and Gruss, P. (2000). "Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye." Genes Dev 14(21): 2701-2711.

Audette, D. S., Anand, D., So, T., Rubenstein, T. B., Lachke, S. A., Lovicu, F. J. and Duncan, M. K. (2016). Prox 1 and fibroblast growth factor receptors form a novel regulatory loop controlling lens fiber differentiation and gene expression.

Bassett, E. A. and Wallace, V. A. (2012). "Cell fate determination in the vertebrate retina." Trends in Neurosciences 35(9): 565-573.

Bélanger, M. C., Robert, B. and Cayouette, M. (2017). "Msx1-Positive Progenitors in the Retinal Ciliary Margin Give Rise to Both Neural and Non-neural Progenies in Mammals." Dev Cell 40(2): 137-150.

Belecky-Adams, T., Tomarev, S., Li, H. S., Ploder, L., McInnes, R. R., Sundin, O. and Adler, R. (1997). "Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells." Investigative opthalmology & visual science 38(7): 1293-1303.

Bharti, K., Gasper, M., Ou, J., Brucato, M., Clore-Gronenborn, K., Pickel, J. and Arnheiter, H. (2012). "A regulatory loop involving PAX6, MITF, and WNT signaling controls retinal pigment epithelium development." PLoS Genet 8(7): e1002757.

Bhatia, S., Bengani, H., Fish, M., Brown, A., Divizia, M. T., de Marco, R., Damante, G., Grainger, R., van Heyningen, V. and Kleinjan, D. A. (2013). "Disruption of autoregulatory feedback by a mutation in a remote, ultraconserved PAX6 enhancer causes aniridia." Am J Hum Genet 93(6): 1126-1134.

Bringmann, A., Pannicke, T., Grosche, J., Francke, M., Wiedermann, P., Skatchkov, S. N., Osborne, N. N. and Reichenbach, A. (2006). "Muller cells in the healthy and diseased retina." Prog Retin Eye Res 25: 397-424.

Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L. and Glaser, T. (1998). "Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis." Development 125(23): 4821-4833.

Buono, L., Corbacho, J., Naranjo, S., Almuedo-Castillo, M., Moreno-Marmol, T., de la Cerda, B., Sanabria-Reinoso, E., Polvillo, R., Díaz-Corrales, F.-J., Bogdanovic, O., Bovolenta, P. and Martínez-Morales, J.-R. (2021). "Analysis of gene network bifurcation during optic cup morphogenesis in zebrafish." Nature Communications 12(1): 3866.

Burgy-Roukala, E., Miellet, S., Mishra, A. K. and Sprecher, A. G. (2013). Early Eye Development: Specification and Determination. Molecular Genetics of Axial Patterning, Growth and Disease in the Drosophila Eye. New York, Springer: 1-36.

Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploder, L., Hawes, N. L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V. I., Roderick, T. H., Taylor, B. A., Hankin, M. H. and McInnes, R. R. (1996). "Occular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation." Nat. Genet. 12: 376-384.

Callaert, P., Hlader, G. and Gehring, W. J. (1997). "Pax6 in development and evolution." Annu Rev Neurosci 20: 483-532.

Cardiff, T. i. o. m. g. i. (2020). The human gene mutation database.

Centanin, L., Hoeckendorf, B. and Wittbrodt, J. (2011). "Fate Restriction and Multipotency in Retinal Stem Cells." Cell Stem Cell 9(6): 553-562.

Centanin, L. and Wittbrodt, J. (2014). "Retinal neurogenesis." Development 141(2): 241-244.

Chamberlain, C. G. and McAvoy, J. W. (1987). "Evidence that fibroblastgrowth factor promotes lens fibre differentiation." Curr Eye Res 6: 1165-1169.

Chao, L.-Y., Huff, V., Strong, L. C. and Saunders, G. F. (2000). "Mutation in the PAX6 gene in twenty patients with aniridia." Human Mutation 15(4): 332-339.

Chen, H., Khanna, H., Oh, E. C. T., Hicks, D., Mitton, K. P. and Swaroop, A. (2004). "Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors." Human Molecular Genetics 13(15): 1563-1575.

Chen, J., Ratter, A. and Nathans, J. (2005). "The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes." J. Neurosci 25: 118-129.

Chou, C. Y., Horng, L. S. and Tsai, H. J. (2001). "Uniform GFP-expression in transgenic medaka (Oryzias latipes) at the F0 generation." Transgenic Res 10(4): 303-315.

Chow, R. L., Altmann, C. R., Lang, R. A. and Hemmati-Brivanlou, A. (1999). "Pax6 induces ectopic eyes in a vertebrate." Development 126: 4213-4222.

Chow, R. L. and Lang, R. A. (2001). "Early eye development in vertabrates." Annu. REv. Cell Dev. Bio. 17: 255-296.

Chuang, J. C. and Raymond, P. A. (2001). "Zebrafish Genes rx1 and rx2 Help Define the Region of Forebrain That Gives Rise to Retina." Develomental biology 231(1): 13-30.

Cid, E., Santos-Ledo, A., Parrilla-Monge, M., Lillo, C., Arevalo, R., Lara, J. M., Aijon, J. and Velasco, A. (2010). "Prox1 expression in rod precursors and Müller cells." Experimental eye research 90(2): 267-276.

Collinson, J. M., Hill, R. E. and West, J. D. (2000). "Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye." Development 127(5): 945-956.

Concha, M. L., Burdine, R. D., Russell, C., Schier, A. F. and Wilson, S. W. (2000). "A nodal signaling pathway regulates the laterality of neuroanatomical asymmetries in the zebrafish forebrain." Neuron 28(2): 399-409.

Coolen, M., Sii-Felice, K., Bronchain, O., Mazabraud, A., Bourrat, F., Retaux, S., Felder-Schmittbuhl, M. P., Mazan, S. and Plouhunec, J. L. (2005). "Phylogenomic analysis and expression patterns of large Maf genes in Xenopus tropicalis provide new insights into the functional evolution of the gene family in osteichthyans." Dev Genes Evol 215: 327-339.

Coulombre, J. L. and Coulombre, A. J. (1963). "Lens development: fibre elongation and lens orientation." Science 142: 1489-1490.

Cunha, D. L., Arno, G., Corton, M. and Moosajee, M. (2019). "The Spectrum of PAX6 Mutations and Genotype-Phenotype Correlations in the Eye." Genes 10(12).

Cvekl, A. and Dunkan, M. K. (2007). "Genetic and epigenetic mechanisms of gene regulation during lens development." Prog Retin Eye Res 26: 555-597.

Cvekl, A. and Piatigorsky, J. (1996). "Lens development and crystallin gene expression: many roles for Pax-6." Bioessays 18: 621-630.

Czerny, T. and Busslinger, M. (1995). "DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5)." Mol Cell Biol 15: 2858-2871.

Czerny, T., Halder, G., Kloter, U., Souabni, A., Gehring, W. J. and Busslinger, M. (1999). "twin of eyeless, a Second Pax-6 Gene of Drosophila, Acts Upstream of eyeless in the Control of Eye Development." Molecular CEll 3(3): 297-307.

Czerny, T., Schaffner, G. and Busslinger, M. (1993). "DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site." Genes & Development 7: 2048-2061.

Daniels, J. T., Secker, G. A. and Shortt, A. J. (2010). "Ocular surface restoration." Ocular Disease: 83-90.

Davis-Silberman, N. and Ashery-Padan, R. (2008). "Iris development in vertebrates; genetic and molecular considerations." Brain Research 1192: 17-28.

Davis-Silberman, N., Kalich, T., Oron-Karni, V., Marquardt, T., Kroeber, M., Tamm, E. R. and Ashery-Padan, R. (2005). "Genetic dissection of Pax6 dosage requirements in the developing mouse eye." Hum Mol Genet 14(15): 2265-2276.

Davis, L. K., Meyer, K. J., Rudd, D. S., Librant, A. L., Epping, E. A., Sheffield, V. C. and Wassink, T. H. (2008). "Pax6 3′ deletion results in aniridia, autism and mental retardation." Humman Genetic 123: 371-378.

Davis, N., Yeffe, C., Ravis, S., Antes, R., Berger, J., Holzman, S., Stoykova, A., Overbeek, P. A., Tamm, E. R. and Ashery-Padan, R. (2009). "Pax6 dosage requirements in iris and ciliary body differentiation." Dev Biol 333: 132-142.

Deguchi, T., Fujimori, K. E., Kawasaki, T., Ohgushi, H. and Yuba, S. (2009). "Molecular cloning and gene expression of the prox1a and prox1b genes in the medaka Oryzias latipes." Gene expression patterns 9: 341-347.

Dimanlig, P. V., Faber, S. C., Auerbach, W., Makarenkova, H. P. and Lang, R. A. (2001). "The upstream ectoderm enhancer in Pax6 has an important role in lens induction." Development 128(22): 4415-4424.

Dorsky, R. I., Chang, W. S., Rapaport, D. H. and Harris, W. A. (1997). "Regulation of neuronal diversity in the Xenopus retina by Delta signalling." Nature 385: 67-70.

Dupacova, N., Antosova, B., Paces, J. and Kozmik, Z. (2021). "Meis homeobox genes control progenitor competence in the retina." Proc Natl Acad Sci U S A 118(12).

Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). "Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina." Nat Genet 34(1): 53-58.

Ekstrom, P. and Johansson, K. (2003). "Differentiation of ganglion cells and amacrine cells in the rat retina: correlation with expression of HuC / D and GAP-43 proteins." Developmental Brain Research 145: 1-8.

Epstein, J. A., Glaser, T., Cai, J., Jepeal, L., Walton, D. S. and Maas, R. L. (1994). "Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing." Genes & Development 8: 2022-2034.

Esteve, P. and Bovolenta, P. (2006). "Secreted inducers in vertebrate eye development: more functions for old morphogens." Current Opinion in Neurobiology 16: 13-19.

Faber, S. C., Dimanling, P., Mamarenkov, H. P., Shirke, S., Ko, K. and Lang, R. A. (2001). "Fgf receptor signaling plays a role in lens induction." Development 128(22): 4425-4438.

Favor, J., Gloeckner, C. J., Neuhauser-Klaus, A., Pretsh, W., Sandulache, R., Saule, S. and Zaus, I. (2008). "Relationship of Pax6 activity levels to the extent of eye development in the mouse, Mus musculus." Genetics 179: 1345-1355.

Fisher, A. J. and Reh, T. (2000). "Identification of a proliferating marginal zone of retinal progenitors in postnatal chickens." Dev Biol 220: 197-210.

Freeman, J. L., Adeniyi, A., Banerjee, R., Dallaire, S., Maguire, S. F., Chi, J., Ng, B. L., Zepeda, C., Scott, C. E., Humphray, S., Rogers, J., Zhou, Y., Zon, L. I., Carter, N. P., Yang, F. and Lee, C. (2007). "Definition of the zebrafish genome using flow cytometry and cytogenetic mapping." BMC Genomics 8(1): 195.

Fujimura, N. (2016). "WNT/β-Catenin Signaling in Vertebrate Eye Development." Front Cell Dev Biol 4: 138.

Fujitani, Y., Fujitani, S., Luo, H., Qiu, F., Burlison, J., Long, Q., Kawaguchi, Y., Edlund, H., MacDonald, R. J., Furukawa, T., Fujikado, T., Magnuson, M. A., Xiang, M. and Wright, C. V. E. (2006). "Ptf1a determines horizontal and amacrine cell fates during mouse retinal development." Development 133(22): 4439-4450.

Furukawa, T., Morrow, E. M. and Cepko, C. L. (1997). "Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation." Cell 91(4): 531-541.

Furukawa, T., Morrow, E. M., Li, T., Davas, F. C. and Cepko, C. L. (1999). "Retinopathy and attenuated circadian entrainment in Crx-deficient mice." Nat Genet 23: 466-470.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. and Cepko, C. L. (2000). "rax, Hes1, and notch1 promote the formation of Müller glia by postnatal retinal progenitor cells." Neuron 26(2): 383-394.

Furuta, Y. and Hogan, B. L. M. (1998). "BMP4 is essential for lens induction in the mouse embryo." Genes Dev 12: 3764-3775.

Furutani-Seiki, M. and Wittbrodt, J. (2004). "Medaka and zebrafish, an evolutionary twin study." Mechanisms of development 121(7): 629-637.

Garcia, C. M., Yu, K., Zhao, H., Ashery-Padan, R., Ornitz, D. M., Robinson, M. L. and Beebe, D. C. (2005). "Signaling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fiber cell differentiation." Dev Dyn 233(2): 516-527.

Gehring, W. J. and Ikeo, K. (1999). " Pax 6: mastering eye morphogenesis and eye evolution." Trends in Genetics 15(9): 371-377.

Glasauer, S. M. K. and Neuhauss, S. C. F. (2014). "Whole-genome duplication in teleost fishes and its evolutionary consequences." Molecular genetics and genomics 289(6): 1045-1060.

Glaser, T., Walton, D. S. and Maas, R. L. (1992). "Genomic structure, evolutionary conservation and aniridia mutations in the human PAX6 gene." Nature Genetics 2(3): 232-239.

Glasow, E. and Tomarev, S. I. (1998). "Restricted expression of the homeobox gene prox1 in developing zebrafish." Mechanisms of development 76: 175-178.

Gotoh, N., Ito, M., Yamamoto, S., Yoshino, I., Song, N., Wang, Y., Lax, I., Schlessinger, J., Shibuya, M. and Lang, R. A. (2004). "Tyrosine phosphorylation sites on FRS2α responsible for Shp2 recruitment are critical for induction of lens and retina." PNAS 101(49): 17144-17149.

Grabher, C. and Wittbrodt, J. (2007). "Meganuclease and transposon mediated transgenesis in medaka." Genome Biology 8(1): S10.

Graw, J. (2009). "Crystallins. Cataract and beyond." Exp. Eye Res 88: 173-189.

Graw, J. (2009). "Mouse models of cataract." J. Genet 88: 469-486.

Graw, J. (2010). "Eye development." Curremt topics in developmental biology: 343-386.

Greiling, T. M. and Clark, J. I. (2008). "The transparent lens and cornea in the mouse and zebra fish eye." Semin Cell Dev Biol 19: 94-99.

Greiling, T. M. S., Aose, M. and Clark, J. I. (2009). "Cell Fate and Differentiation of the Developing Ocular Lens." Investigative Ophtalmology & Visual Science 51(3): 1540-1546.

Griffin, C., Kleinjan, D. A., Doe, B. and van Heyningen, V. (2002). "New 3′ elements control Pax6 expression in the developing pretectum, neural retina and olfactory region." Mech. Dev. 112: 89-100.

Grindley, J. C., Davidson, D. R. and Hill, R. E. (1995). " The role of Pax-6 in eye and nasal development." Development: 1433-1442.

Halder, G., Callaerts, P. and Gehring, W. (1995). "Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila." Science 267: 1788-1892.

Hanson, I. M. (2003). "PAX6 and Congenital Eye Malformations." Pediatric Research 54(6): 791-796.

Harding, P. and Moosajee, M. (2019). "The Molecular Basis of Human Anophthalmia and Microphthalmia." Journal of developmental biology 7(3).

Hatakeyama, J., Tomita, K., Inoue, T. and Kageyama, R. (2001). "Roles of homeobox and bHLH genes in specification of a retinal cell type." Development 128: 1313-1322.

Heavner, W. and Pevny, L. (2012). "Eye development and retinogenesis." Cold Spring Harb Perspect Biol. 4.

Heine, P., Dohle, E., Bumsted-O`Brien, K., Engelkamp, D. and Schulte, D. (2008). "Evidence for an evolutionary conserved role of homothorax/Meis1/2 during vertebrate retina development." Development 135(5): 805-811.

Hemesath, T. J., Steingrímsson, E., McGill, G., Hansen, M. J., Vaught, J., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., Jenkins, N. A. and Fisher, D. E. (1994). "microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family." Genes Dev 8(22): 2770-2780.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D. and Lewis, J. (1997). "Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina." Curr. Biol. 7: 661-670.

Herder, F. and Behrens-Chapuis, S. (2010). "Oryzias hadiatyae, a new species of ricefish (Atherinomorpha: Beloniformes: Adrianichthyidae) endemic to Lake Masapi, Central Sulawesi, Indonesia." The Raffles bulletin of zoology 58: 269-280.

Hilgers, L. and Schwarzer, J. (2019). "The untapped potential of medaka and its wild relatives." Elife 8.

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, M., Prosser, J., Jordan, T. and Hastie, N. D. (1991). "Mouse small eye results from mutations in a paired-like homeobox-containing gene." Nature 354: 522-555.

Hodgkinson, C. A., Moore, K. J., Nakayama, A., Steingrímsson, E., Copeland, N. G., Jenkins, N. A. and Arnheiter, H. (1993). "Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein." Cell 74(2): 395-404.

Hogan, B. L., Horsburg, G., Cohen, J., Hetherington, C. M., Fisher, G. and Lyon, M. F. (1986). "Small eyes (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse." J Embryol Exp Morphol 97: 95-110.

Hoge, M. A. (1915). "Another gene in the fourth chromosome of Drosophila." Am Nat 49: 47-49.

Hong, S., Kim, C., Yoo, K., Kim, H., Kudoch, T., Dawid, I. B. and Huh, T. (2002). "Isolation and expression of a novel neuron-specific onecut homeobox gene in zebrafish." Mechanisms of development 112(1-2): 199-202.

Huang, J., Liu, Y., Oltean, A. and Beebe, D. C. (2015). "Bmp4 from the Optic Vesicle Specifies Murine Retina Formation." Dev Biol 402: 119-126.

Hyer, J., Mima, T. and Mikawa, T. (1998). "FGF1 patterns the optic vesicle by directing the placement of the neural retina domain." Development 125: 869-877.

Inoue, T., Hojo, M., Bessho, Y., Tano, T., Lee, J. E. and Kageyama, R. (2002). "Math3 and NeuroD regulate amacrine cell fate specification in the retina." Development 129(4): 831-842.

Ishikawa, Y., Inohaya, K., Yamamoto, N., Maruyama, K., Yoshimoto, M., Iigo, M., Oishi, T., Kudo, A. and Ito, H. (2015). "The Parapineal Is Incorporated into the Habenula during Ontogenesis in the Medaka Fish." Brain Behav Evol 85(4): 257-270.

Iwamatsu, T. (2004). "Stages of normal development in the medaka Oryzias latipes." Mechanisms of development 121(7-8): 605-618.

Jadhav, A. P., Roesch, K. and Cepko, C. L. (2009). "Development and neurogenic potential of Muller glial cells in the vertebrate retina." Prog Retin Eye Res 28: 249-262.

Jain, V., Ravindran, E. and Dhingra, N. K. (2012). "Differential expression of Brn3 transcription factors in intrinsically photosensitive retinal ganglion cells in mouse." J Comp Neurol 520(4): 742-755.

Jaszczyszyn, Y., Haeussler, M., Heuzé, A., Debiais-Thibaud, M., Casane, D., Bourrat, F. and Joly, J.-S. (2007). "Comparison of the expression of medaka (Oryzias latipes) pitx genes with other vertebrates shows high conservation and a case of functional shuffling in the pituitary." Gene 406(1): 42-50.

Jordan, D. S. and Snyder, J. O. (1906). "A review of the poeciliidae or killifishes of Japan." Proc. US Natl Mus. 31: 287-290.

Jordan, T., Hanson, I., Zaletayev, D., Hodgson, S., Prosser, J., Seawright, A., Hastie, N. and van Heyningen, V. (1992). "The human PAX6 gene is mutated in two patients with aniridia." Nature Genetics 1(5): 328-332.

Kagawa, N. (2014). "Comparison of aggressive behaviors between two wild populations of Japanese medaka, Oryzias latipes and O. sakaizumii." Zoolog Sci 31(3): 116-121.

Kammandel, B., Chowdhury, K., Stoykova, A., Aparicio, S., Brenner, S. and Gruss, P. (1999). "Distinct cis-essential modules direct the time–space pattern of the Pax6 gene activity." Dev. Biol. 205: 79-97.

Kasahara, M., Nasure, K., Sasaki, S., Natakani, Y., Qu, W., Ahsan, B., Yamada, T., Nagayasu, Y., Doi, K., Kasai, Y., Jindo, T., Kabayashi, D., Shimada, A., Toyoda, A., Kuroki, Y., Fujiyama, A., Sasaki, T.,

Shimizu, A., Asakawa, S., Shimizu, N., Hashimoto, S., Yang, J., Lee, Y., Matsushima, K., Sugano, S., Sakaizumi, M., Narita, T., Ohishi, K., Haga, S., Ohta, F., Nomoto, H., Nogota, K., Morishita, T., Endo, T., Shin-I, T., Takeda, H., Morishita, S. and Kohara, Y. (2007). "The medaka draft genome and insights into vertebrate genome evolution." Nature 447: 714-719.

Kimura, T., Shimada, A., Sakai, N., Mitani, H., Naruse, K., Takeda, H., Inoko, H., Tamiya, G. and Shinya, M. (2007). "Genetic analysis of craniofacial traits in the medaka." Genetics 177(4): 2379-2388.

Kleinjan, D. A., Bancewicz, R. M., Gautier, P., Dahm, R., Schonthaler, H. B., Damante, G., Seawright, A., Hever, A. M., Yeyati, P. L., van Heyningen, V. and Coutino, P. (2008). "Subfunctionalization of Duplicated Zebrafish pax6 Genes by cis-Regulatory Divergence." PLOS Genetics 4(29).

Klimova, L., Antosova, B., Kuzelova, A., Strnad, H. and Kozmik, Z. (2015). "Onecut1 and Onecut2 transcription factors operate downstream of Pax6 to regulate horizontal cell development." Developmental biology 402(1): 48-60.

Klimova, L. and Kozmik, Z. (2014). "Stage-dependent requirement of neuroretinal Pax6 for lens and retina development." Development 141: 1292-1302.

Klimova, L. and Kozmik, Z. (2014). "Stage-dependent requirement of neuroretinal Pax6 for lens and retina development." Development 141(6): 1292-1302.

Klimova, L., Lachova, J., Machon, O., Sedlacek, R. and Kozmik, Z. (2013). "Generation of mRx-Cre transgenic mouse line for efficient conditional gene deletion in early retinal progenitors." Plos one 8(5): e63029.

Kobayashi, D. and Takeda, H. (2008). "The medaka genome project." Brief Funct Genomic Proteomic 7(6): 415-426.

Kozmik, Z. (2005). "Pax genes in eye development and evolution." Curr Opin Genet Dev 15: 430-438.

Krauss, S., Johansen, T., Korz, V. and Fjose, A. (1991). "Expression pattern of zebrafish pax genes suggests a role in early brain regionalization." Nature 353(6341): 267–270.

Kreslova, J., Machon, O., Ruyickova, J., Lachova, J., Wawrousek, E. F., Kemler, R., Krauss, S., Piatigorsky, J. and Kozmik, Z. (2007). "Abnormal Lens Morphogenesis and Ectopic Lens Formation." Genesis 45(4): 157-168.

Kroeber, M., Davis, N., Holzmann, S., Kritzenberger, M., Shelah-Goraly, M., Ofir, R., Ashery-Padan, R. and Tamm, E. R. (2010). "Reduced expression of Pax6 in lens and cornea of mutant mice leads to failure of chamber angle development and juvenile glaucoma." Human Molecular Genetics 19(17): 3332-3342.

laboratory, T. J. (1996). Mouse genome informatics.

Lang, R. A. (2004). "Pathways regulating lens induction in the mouse." Int J Dev Biol 48: 783-791.

Li, H., Tierney, C., Wen, L., Wu, J. Y. and Rao, Y. (1997). "A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate." Development 124: 603-615.

Li, S., Mo, Z., Yang, X., Price, S. M., Shen, M. M. and Xiang, M. (2004). "Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors." Neuron 43(6): 795-807.

Li, X., Perissi, V., Liu, F., Rose, D. W. and Rosenfeld, M. G. (2002). "Tissue-specific regulation of retinal and pituitary precursor cell proliferation." Science 297: 1180-1183.

Lin, Y., Ouchi, Y., Satoh, S. and Watanabe, S. (2009). "Sox2 Plays a Role in the Induction of Amacrine and Müller Glial Cells in Mouse Retinal Progenitor Cells." Investigative ophthalmogy & visual science 50(1): 68-74.

Liu, H., Xu, S., Wang, Y., Mazerolle, C., Thurig, S., Coles, B. L., Ren, J. C., Taketo, M. M., van der Kooy, D. and Wallace, V. A. (2007). "Ciliary margin transdifferentiation from neural retina is controlled by canonical Wnt signaling." Dev Biol 308(1): 54-67.

Liu, W., Lagustin, O. V., Mende, M., Streit, A. and Oliver, G. (2006). "Six3 activation of pax6 expression is essential for mammalian lens induction and specification." EMBO J 25: 5383-5395.

Liu, W., Mo, Z. and Xiang, M. (2001). "The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development." Proc. Natl. Acad. Sci. U.S.A 98: 1649-1654.

Locker, M., Yakoubi, W. E., Mazurier, N., Dullin, J. P. and Perron, M. (2010). "A decade of mammalian retinal stem cell research." Archives Italiennes de Biologie 148: 59-72.

Logan, M., Pagán-Westphal, S. M., Smith, D. M., Paganessi, L. and Tabin, C. J. (1998). "The Transcription Factor Pitx2 Mediates Situs-Specific Morphogenesis in Response to Left-Right Asymmetric Signals." Cell 94(3): 307-317.

Long, S., Ahmad, N. and Rebagliati, M. (2003). "The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry." Development 130(11): 2303-2316.

Loosli , F., Staub, W., Finger-Baieer, K. C., Ober, E. A., Verkade, H., Wittbrodt, J. and Baier, H. (2003). "Loss of eyes in zebrafish caused by mutation of chokh/rx3." EMBO reports 4: 894-899.

Loosli, F., Winkler, S. and Wittbrodt, J. (1999). "Six3 overexpression initiates the formation of ectopic retina." Genes Dev 13: 649-654.

Lovicu, F. J. and Overbeek, P. A. (1998). "Overlapping effects of different members of the FGF family on lens fibre differentiation in transgenic mice." Development 125: 3365-3377.

Lovicu, F. J., Steven, P., Saika, S. and McAvoy, J. W. (2004). "Aberrant Lens Fiber Differentiation in Anterior Subcapsular Cataract Formation: A Process Dependent on Reduced Levels of Pax6." Investigative Ophthalmology & Visual Science 45: 1946-1953.

Lu, M.-F., Pressman, C., Dyer, R., Johnson, R. L. and Martin, J. F. (1999). "Function of Rieger syndrome gene in left–right asymmetry and craniofacial development." Nature 401(6750): 276-278.

Lyu, J. and Joo, C. K. (2004). "Wnt signaling enhances FGF2-triggered lens fiber cell defferentiation." Development 131: 1813-1824.

Machon, O., Kreslova, J., Ruzickova, J., Vacik, T., Klimova, L., Fujimura, N., Lachova, J. and Kozmik, Z. (2010). "Lens Morphogenesis is Dependent on Pax6-Mediated Inhibition of the Canonical Wnt/Beta-Catenin Signaling in the Lens Surface Ectoderm." Genesis 48: 86-95.

Magtoon, W. (2010). "Oryzias songkhramensis, A New Species of Ricefish (Beloniformes; Adrianichthyidae) from Northeast Thailand and Central Laos." Tropical Natural History 10: 107-129.

Malandrini, A., Mari, F., Palmeri, S., Gambelli, S., Berti, G., Bruttini, M., Bardelli, A., Williamson, K., Van Heyningen, V. and Renieri, A. (2001). "PAX6 mutation in a family with aniridia, congenital ptosis, and mental retardation." Clinical Genetics 60(2): 151-154.

Mandagi, I., Mokodongan, D., Tanaka, R. and Yamahira, K. (2018). "A New Riverine Ricefish of the Genus Oryzias (Beloniformes, Adrianichthyidae) from Malili, Central Sulawesi, Indonesia." Copeia 106: 297-304.

Marcos, S., Gonzales-Lazero, M., Beccari, L., Carramoline, L., Martin-Bermejo, M. J., Amarie, O., Mateos-San Martin, D., Torroja, C., Bogdanovic, O., Doohan, R., Puk, O., Hrabe de Angelis, M., Graw, J., Gomez-Skarmeta, J. L., Casares, F., Torres, M. and Bovolenta, P. (2015). "Meis1 coordinates a network of genes implicated in eye development and microphthalmia." Development 142(17): 3009-3020.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P. (2001). "Pax6 Is Required for the Multipotent State of Retinal Progenitor Cells." Cell 105(1): 43-55.

Martinez-Morales, J., Rodrigo, I. and Bovolenta, P. (2004). "Eye development: a view from the retina pigmented epithelium." BioEssays 26: 766-777.

Martinez-Morales, J. R., Signore, M., Acampora, D., Simeone, A. and Bovolenta, P. (2001). "Otx genes are required for tissue specification in the developing eye." Development 128(11): 2019-2030.

Marusich, M. F., Furneaux, H. M., Henion, P. D. and Weston, J. A. (1994). "Hu neuronal proteins are expressed in proliferating neurogenic cells." J Neurobiol 25(2): 143-155.

Mathers, P. H., Grinberg, A., Mahon, K. A. and Jamrich, M. (1997). "The Rx homeobox gene is essential for vertebrate eye development." Nature 387: 603-607.

Mathers, P. H. and Jamrich, M. (2000). "Regulation of eye formation by the Rx and pax6 homeobox genes." Cell Mol Life Sci 57: 186-194.

Mayer, E. J., Hughes, E. H., Carter, D. A. and Dick, A. D. (2000). "Nestin positive cells in adult human retina and in epiretinal membranes." British Journal of Ophthalmology 87(9): 1154-1158.

McAvoy, J. W. and Chamberlain, C. G. (1989). "Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration." Development 107: 221-228.

McAvoy, J. W., Chamberlain, C. G., de Iongh, R. U., Hales, A. M. and Lovicu, F. J. (1999). "Lens development." Nature 13: 425-437.

Mellough, C., Collin, J., Sernagor, E., Wride, N., Steel, D. H. and Lako, M. (2014). "Lab generated retina: Realizing the dream." Visual Neuroscience 31(4-5): 1-16.

Mercier, P., Simeone, A., Cotelli, F. and Boncinelli, E. (1995). "Expression pattern of two otx genes suggests a role in specifying anterior body structures in zebrafish." The international journal of developmental biology 39: 559-573.

Mic, F. A., Molotov, A., Molotova, N. and Duester, G. (2004). "Raldh2 expression in optic vesicle generates a retinoic acid signal needed for invagination of retina during optic cup formation." Dev Dyn 231: 270-277.

Miles, A. and Tropepe, V. (2021). "Retinal Stem Cell 'Retirement Plans': Growth, Regulation and Species Adaptations in the Retinal Ciliary Marginal Zone." Int J Mol Sci 22(12).

Miller, S. A. and Harley, J. P. (2007). Zoology, McGraw-Hill Higher Education.

Mishra, R., Gorlov, I. P., Chao, L. Y., Singh, S. and Saunders, G. F. (2002). "PAX6, Paired Domain Influences Sequence Recognition by the Homeodomain*." Journal of Biological Chemistry 277(51): 49488-49494.

Mokodongan, D., Tanaka, R. and Yamahira, K. (2014). "A New Ricefish of the Genus Oryzias (Beloniformes, Adrianichthyidae) from Lake Tiu, Central Sulawesi, Indonesia." Copeia 2014.

Moore, F. E., Reyon, D., Sander, J. D., Martinez, S. A., Blackburn, J. S., Khayter, C., Ramirez, C. L., Joung, J. K. and Langenau, D. M. (2012). "Improved Somatic Mutagenesis in Zebrafish Using Transcription Activator-Like Effector Nucleases (TALENs)." Plos one 7(5): e37877.

Moshiri, A., Close, J. and Reh, T. A. (2004). "Retinal stem cells and regeneration " Int J Dev Biol. 48(8-9): 1003-1014.

Moshiri, A., Gonzales, E., Tagawa, K., Maeda, H., Wang, M., Frishman, L. J. and Wang, S. W. (2008). "Near complete loss of retinal ganglion cells in the math5/brn3b double knockout elicits severe reductions of other cell types during retinal development." Developmental biology 316: 214-227.

Murakami, Y., Ogasawara, M., Sugahara, F., Hirano, S., Satoh, N. and Kuratuni, S. (2001). "Identification and expression of the lamprey Pax6 gene: evolutionary origin of the segmented brain of vertebrates." Development 128(18).

Murata, K., Kinoshita, M., Naruse, K., Tanaka, M. and Kamei, Y. (2019). Medaka and Oryzias Species as Model Organisms and the Current Status of Medaka Biological Resources. Medaka: Biology, Management, and Experimental Protocols, John Wiley & Sons Ltd: 31-48.

Murata, Y., Oda, S. and Mitani, H. (2012). "Allelic expression changes in Medaka (Oryzias latipes) hybrids between inbred strains derived from genetically distant populations." Plos one 7(5): e36875.

Nakamura, S., Saito, D. and Tanaka, M. (2008). "Generation of transgenic medaka using modified bacterial artificial chromosome." Development, Growth & Differentiation 50(6): 415-419.

Nathans, J. (1992). "Rhodopsin: Structure, Function, and Genetics." Biochemistry 31(21): 4923-4931.

Newman, E. A. and Reichenbach, A. (1996). " The Mu¨ller cell: a functional element of the retina." Trends Neurosci 19: 307-317.

Nishida, A., Furukawa, A., Koike, C. Y., Aizawa, S., Matsuo, I. and Furukawa, T. (2003). "Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development." Nat Neurosci 6(12): 1255-1263.

Nornes, S., Clarkson, M., Mikkola, I., Pedersen, M., Bardsley, A., Martinez, J. P., Krauss, S. and Johansen, T. (1998). "Zebrafish contains two pax6 genes involved in eye development." Mech Dev 77(2): 185-196.

Norris, D. P., Brennan, J., Bikoff, E. K. and Robertson, E. J. (2002). "The Foxh1-dependent autoregulatory enhancer controls the level of Nodal signals in the mouse embryo." Development 129(14): 3455-3468.

Ochocinska, M. J. and Hitchcock, P. F. (2007). "Dynamic expression of the basic helix-loop-helix transcription factor neuroD in the rod and cone photoreceptor lineages in the retina of the embryonic and larval zebrafish." The journal of comparative neurology 501(1): 1-12.

Ohno, S. (1970). Evolution by gene duplication. New York, Springer.

Ohsawa, R. and Kageyama, R. (2008). "Regulation of retinal cell fate specification by multiple transcription factors." Brain Research 1192: 90-98.

Oliver, G., Loosli, F., Koster, R., Wittbrodt, J. and Gruss, P. (1996). "Ectopic lens induction in fish in response to the murine homeobox gene six3." Mech Dev 60: 233-239.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995). "Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development." Development 121: 4045-4045.

Oron-Karni, V., Farhy, C., Elgart, M., Marquardt, T., Remizova, L., Yaron, O., Xie, Q., Cvekl, A. and Ashery-Padan, R. (2008). "Dual requirement for Pax6 in retinal progenitor cells." Development 135(24): 4037-4047.

Pak, W., Hindges, R., Lim, Y. S., Pfaff, S. L. and O`Leary, D. D. M. (2004). "Magnitude of Binocular Vision Controlled by Islet-2 Repression of a Genetic Program that Specifies Laterality of Retinal Axon Pathfinding." Cell 119(4): 567-578.

Pan, L., Deng, M., Xie, X. and Gan, L. (2008). "ISL1 and BRN3B co-regulate the differentiation of murine retinal ganglion cells." Development 135: 1981-1990.

Pan, Q., Lu, K., Luo, J., Jiang, Y., Xia, B., Chen, L., Wang, M., Dai, R. and Chen, T. (2023). "Japanese medaka Olpax6.1 mutant as a potential model for spondylo-ocular syndrome." Funct Integr Genomics 23(2): 168.

Parenti, L., Hadiaty, R., Lumbantobing, D. and Herder, F. (2013). "Two New Ricefishes of the Genus Oryzias (Atherinomorpha: Beloniformes: Adrianichthyidae) Augment the Endemic Freshwater Fish Fauna of Southeastern Sulawesi, Indonesia." Copeia 2013: 403-414.

Parenti, L. R. (2008). "A phylogenetic analysis and taxonomic revision of ricefishes, Oryzias and relatives (Beloniformes, Adrianichthyidae)." Zoological Journal of the Linnean Society 154(3): 494-610.

Passini, M. A., Kurtzman, A. L., Canger, A. K., Asch, W. S., Wray, G. A., Raymond, P. A. and Schechter, N. (1998). "Cloning of zebrafish vsx1: expression of a paired-like homeobox gene during CNS development." Dev Genet 23(2): 128-141.

Patterson, C. and Rosen, D. E. (1977). Review of ichthyodectiform and other Mesozoic teleost fishes, and the theory and practice of classifying fossils. Bulletin of the AMNH ; v. 158, article 2.

Perron, M. and Harris, W. A. (2000). "Retinal stem cells in vertebrates." BioEssays 22(8): 685–688.

Perron, M., Kanekar, S., Vetter, M. L. and Harris, W. A. (1998). "The genetic sequence of retinal development in the ciliary margin of the Xenopus eye " Dev Biol 199(2): 185-200.

Philips, G. T., Stair, C. N., Young Lee, H., Wroblewski, E., Berberoglu, M. A., Brown, N. L. and Mastick, G. S. (2005). "Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type." Dev Biol 279(2): 308-321.

Piatigorsky, J. and Kozmik, Z. (2004). "Cubozoan jellyfish: an Evo/Devo model or eyes and other sensory system." Int J Dev Biol 48: 719-729.

Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D., Alt, F. and Westphal, H. (1997). "Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development." Development 124: 2935-2944.

Quinn, J. C., West, J. D. and Hill, R. E. (1996). "Multiple functions for Pax6 in mouse eye and nasal development." Genes&DEv. 10: 435-446.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J. (1994). "Homology of the eyeless gene of Drosophila to the Small eye gene in mice and Aniridia in humans." Science 265: 785-789.

Ramaesh, T., Collinson, J. M., Ramaesh, K., Kaufman, M. H., West, J. D. and Dhillon, B. (2003). "Corneal Abnormalities in Pax6 +/− Small Eye Mice Mimic Human Aniridia-Related Keratopathy." Investigative Ophthalmology & Visual Science 44(5): 1871-1878.

Ravi, V., Bhatia, S., Gautier, P., Loosle, F., Tay, B. H., Tay, A., Murdoch, E., Countino, P., van Heyningen, V., Brenner, S., Venkatesh, B. and Kleinjan, D. A. (2013). "Sequencing of Pax6 Loci from the Elephant Shark Reveals a Family of Pax6 Genes in Vertebrate Genomes, Forged by Ancient Duplications and Divergences." PLOS Genetics 9(1).

Raymond, P. A., Barthel, L. K., Bernardos, R. L. and Perkowski, J. J. (2006). "Molecular characterization of retinal stem cells and their niches in adult zebrafish." BMC Developmental Biology 6(36).

Reinhardt, R., Centanin, L., Tavhelidse, T., Inoue, D., Wittbrodt, B., Concordet, J. P., Martinez-Morales, J. R. and Wittbrodt, J. (2015). "Sox2, Tlx, Gli3, and Her9 converge on Rx2 to define retinal stem cells in vivo." EMBO J 34(11): 1572-1588.

Rembold, M., Loosli, F., Adams, R. J. and Wittbrodt, J. (2006). "Individual cell migration serves as the driving force for optic vesicle evegination." Science 313: 1130-1134.

Rich, K. A., Figueroa, S. L., Zhan, Y. and Blanks, J. C. (1995). "Effects of Muller cell disruption on mouse photoreceptor cell development." Exp Eye Res 61: 235-248.

Riesenberg, A. N., Le, T. T., Willardsen, M. I., Blackburn, D. C., Vetter, M. L. and Brown, N. L. (2009). "Pax6 regulation of Math5 during mouse retinal neurogenesis." Genesis 47(3): 175-187.

Robinson, M. L., MacMillan-Crow, L. A., Thompson, J. A. and Overbeek, P. A. (1995). "Expression of a truncated FGF receptor results in defective lens development in transgenic mice." Development 121: 3959-3967.

Rouchka, E. C. (2010). "Database of exact tandem repeats in the Zebrafish genome." BMC Genomics 11(1): 347.

Roussigné, M., Bianco, I. H., Wilson, S. W. and Blader, P. (2009). "Nodal signalling imposes left-right asymmetry upon neurogenesis in the habenular nuclei." Development 136(9): 1549-1557.

Roussigne, M., Blader, P. and Wilson, S. W. (2012). "Breaking symmetry: the zebrafish as a model for understanding left-right asymmetry in the developing brain." Dev Neurobiol 72(3): 269-281.

Rowan, S., Siggers, T., Lachke, S. A., Yue, Y., Bulyk, M. L. and Maas, R. L. (2010). "Precise temporal control of the eye regulatory gene Pax6 via enhancer-binding site affinity." Genes Dev 24(10): 980-985.

Ryan, A. K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Peña, J., Sabbagh, W., Greenwald, J., Choe, S., Norris, D. P., Robertson, E. J., Evans, R. M., Rosenfeld, M. G. and Belmonte, J. C. I. (1998). "Pitx2 determines left–right asymmetry of internal organs in vertebrates." Nature 394(6693): 545-551.

Saijoh, Y., Adachi, H., Sakuma, R., Yeo, C.-Y., Yashiro, K., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M., Miyazono, K., Whitman, M. and Hamada, H. (2000). "Left–Right Asymmetric Expression of lefty2 and nodal Is Induced by a Signaling Pathway that Includes the Transcription Factor FAST2." Molecular CEll 5(1): 35-47.

Sato, Y. and Nishida, M. (2010). "Teleost fish with specific genome duplication as unique models of vertebrate evolution." Environmental Biology of Fishes 88(2): 169–188.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Reuden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012). "Fiji: an open-source platform for biological-image analysis." Nature methods 9: 676-682.

Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M. and Gruss, P. (2000). "Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6." Development 127: 4325-4334.

Sheng, G., Harris, E., Bertuccioli, C. and Desplan, C. (1997). "Modular organization of Pax/homeodomain proteins in transcriptional regulation." Biol Che 378(8): 863-872.

Shi, X., Luo, Y., Howley, S., Dzialo, A., Foley, S., Hyde, D. R. and Vihtelic, T. S. (2006). "Zebrafish foxE3: Roles in ocular lens morphogenesis through interaction with pitx3." Mechanisms of development 123: 761-782.

Shima, A. and Mitani, H. (2004). "Medaka as a research organism: past, present and future." Mechanisms of development 121(7-8): 599-604.

Shiratori, H., Sakuma, R., Watanabe, M., Hashiguchi, H., Mochida, K., Sakai, Y., Nishino, J., Saijoh, Y., Whitman, M. and Hamada, H. (2001). "Two-Step Regulation of Left–Right Asymmetric Expression of Pitx2: Initiation by Nodal Signaling and Maintenance by Nkx2." Molecular CEll 7(1): 137-149.

Shiratori, H., Yashiro, K., Shen, M. M. and Hamada, H. (2006). "Conserved regulation and role of Pitx2 in situs-specific morphogenesis of visceral organs." Development 133(15): 3015-3025.

Simpson, E. M., Lam, S. L., Korecki, A., Tam, B. M., Moritz, O. L. and Hickmott, J. (2017). "Towards Corneal Gene Therapy in the Aniridia Mouse Model Pax6Sey/+." Investigative Ophthalmology & Visual Science 58(8).

Singh, S., Stellrecht, C. M., Tang, H. K. and Saunders, G. F. (2000). "Modulation of PAX6 Homeodomain Function by the Paired Domain*." Journal of Biological Chemistry 275(23): 17306-17313.

Smith, A. N., Miller, L. D., Song, N., Taketo, M. M. and Lang, R. A. (2005). "The duality of β-catenin function: A requirement in lens morphogenesis and signaling suppression of lens fate in periocular ectoderm." Dev Biol 285: 477-489.

Smith, A. N., Radice , G. and Lang, R. A. (2009). "hich FGF ligands are involved in lens induction?" Dev Biol 337(2): 195-198.

Soroldoni, D., Bajoghli, B., Aghaallaei, N. and Czerny, T. (2007). "Dynamic expression pattern of Nodal-related genes during left-right development in medaka." Gene expression patterns 7(1): 93-101.

Srinivasan, Y. and Overbeek, P. A. (1996). "Expression of lens specific fibroblast growth factor (FGF5) causes altered epithelial cell morphology and degeneration of fibre cells." Invest Ophtalmol Vis Sci 37: 924.

Sunny, S. S., Lachova, J., Dupacova, N., Zitova, A. and Kozmik, Z. (2020). "Generation and characterization of Aldh3-Cre transgenic mice as a tool for conditional gene deletion in postnatal cornea." Scientific Reports 10(1): 9083.

Suzuki, J., Sakurai, K., Yamazaki, M., Abe, M., Inada, H., Sakimura, K., Katori, Y. and Osumi, N. (2015). "Horizontal Basal Cell-Specific Deletion of Pax6 Impedes Recovery of the Olfactory Neuroepithelium Following Severe Injury." Stem Cells Dev 24(16): 1923-1933.

Swindell, E. C., Zilinski, C. A., Hashimoto, R., Shah, R., Lane, M. E. and Jamrich, M. (2008). "Regulation and function of foxe3 during early zebrafish development." Genesis 46: 177-183.

Takamiya, M., Wewger, B. D., Schindler, S., Beil, T., Yang, L., Armant, O., Ferg, M., Schlunck, G., Reinhard, T., Dickmeis, T., Rastegar, S. and Strahle, U. (2015). "Molecular Description of Eye Defects in the Zebrafish Pax6b Mutant, sunrise, Reveals a Pax6b-Dependent Genetic Network in the Developing Anterior Chamber." Plos one 10(2).

Tang, H. K., Singh, S. and Sauders, G. F. (1998). "Dissection of the transactivation function of the transcription factor encoded by the eye developmental gene PAX6." J Biol Chem 273: 7210-7221.

Tangeman, J. A., Pérez-Estrada, J. R., Van Zeeland, E., Liu, L., Danciutiu, A., Grajales-Esquivel, E., Smucker, B., Liang, C. and Del Rio-Tsonis, K. (2022). "A Stage-Specific OTX2 Regulatory Network and Maturation-Associated Gene Programs Are Inherent Barriers to RPE Neural Competency." Front Cell Dev Biol 10: 875155.

Tittle, R. K., Sze, R., Ng, A., Nuckels, R. J., Swartz, M. E., Anderson, R. M., Bosch, J., Stainier, D. Y. R., Eberhart, J. K. and Gross, J. M. (2011). "Uhrf1 and Dnmt1 are required for development and maintenance of the zebrafish lens." Developmental biology 350(1): 50-63.

Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemont, F. and Kageyama, R. (2000). "Mammalian achaete–scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system." EMBO J. 19: 5460-5472.

Tomita, K., Nakanishi, S., Guillemont, F. and Kageyara, R. (1996). "Mash1 promotes neuronal differentiation in the retina." Genes Cells 1: 765-774.

Ton, C. C. T., Hirvonen, H., Miwa, H., Weil, M. M., Monaghan, P., van Heyningen, V., Hastie, N. D., Meijers-Heijboer, H., Drechsler, M., Royer-Pokora, B., Collins, F., Swaroop, A., Strong, L. C. and

Saunders, G. F. (1991). "Positional cloning and characterization of a paired box and homeobox-containing gene from the aniridia region." Cell 67: 1059-1074.

Tropepe, V., Coles, B. L., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R. and Van der Kooy, D. (2000). "Retinal stem cells in the adult mammalian eye." Science 287: 2032-2036.

Turner, D. L., Snyder, E. Y. and Cepko, C. L. (1990). "Lineage-independent determination of cell type in the embryonic mouse retina." Neuron 4: 833-845.

van Heyningen, V. and Williamson, K. A. (2002). "PAX6 in sensory development." Human Molecular Genetics 11(10): 1161-1167.

Vandepoele, K., Vos, W. D., Taylor, J. S., Meyer, A. and Peer, Y. V. d. (2004). "Major events in the genome evolution of vertebrates: Paranome age and size differ considerably between ray-finned fishes and land vertebrates." Proceedings of the National Academy of Sciences 101(6): 1638-1643.

Varga, Z. M., Wegner, J. and Westerfield, M. (1999). "Anterior movement of ventral diencephalic precursors separates the primordial eye field in the neural plate and requires cyclops." Development 126: 5533-5546.

Varshney, G. K., Carrington, B., Pei, W., Bishop, K., Chen, Z., Fan, C., Xu, L., Jones, M., LaFave, M. C., Ledin, J., Sood, R. and Burgess, S. M. (2016). "A high-throughput functional genomics workflow based on CRISPR/Cas9-mediated targeted mutagenesis in zebrafish." Nature Protocols 11(12): 2357-2375.

Vetrivel, S., Tiso, N., Kügler, A., Irmler, M., Horsch, M., Beckers, J., Hladik, D., Giesert, F., Gailus-Durner, V., Fuchs, H., Sabrautzki, S., Hrabě de Angelis, M. and Graw, J. (2019). "Mutation in the mouse histone gene Hist2h3c1 leads to degeneration of the lens vesicle and severe microphthalmia." Experimental eye research 188: 107632.

Walther, C. and Gruss, P. (1991). "Pax-6, a murine paired box gene, is expressed in the developing CNS." Development 113: 1435-1449.

Wang, S. W., Kim, B. D., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L. (2001). "Requirement for math5 in the development of retinal ganglion cells." Genes Development 15: 24-29.

Watakabe, I., Hashimoto, H., Kimura, Y., Yokoi, S., Naruse, K. and Higashijima, S.-i. (2018). "Highly efficient generation of knock-in transgenic medaka by CRISPR/Cas9-mediated genome engineering." Zoological Letters 4(1): 3.

Wawersik, S., Purcell, P., Rauchman, M., Dudley, A. T., Robertson, E. J. and Maas, R. (1999). "BMP7 acts in murine lens placode development." DEv Bio 207: 176-188.

Westerfield, M. (2007). The Zebrafish Book; A guide for the laboratory use of zebrafish (Danio rerio).

Wetts, R. and Fraser, S. E. (1988). "Multipotent precursors can give rise to all major cell types of the frog retina." Science 239: 1142-1145.

White, T., Lu, T., Metlapally, R., Katowitz, J., Kherani, F., Wang, T. Y., Tran-Viet, K. N. and Young, T. L. (2008). "Identification of STRA6 and SKI sequence variants in patients with anophthalmia/microphthalmia." Mol Vis 14: 2458-2465.

Williams, S. C., Altmann, C. R., Chow, R. L., Hemmati-Brivanlou, A. and Lang, R. A. (1998). "A highly conserved lens transcriptional control element from the Pax-6 gene." Mech. Dev. 73: 225-229.

Wilson, S. W. and Houart, C. (2004). "Early Steps in the Development of the Forebrain." Dev Cell 6(167-181).

Winker, S., Loosli, F., Henrich, T., Wakamatsu, Y. and Wittbrodt, J. (2000). "The conditional medaka mutation eyeless uncouples patterning and morphologenesis of the eye." Development 127: 1911-1919.

Wittbrodt, J., Shima, A. and Schartl, M. (2002). "Medaka - a model organism from the far east." Nature reviewes 3: 53-64.

Wolf, L. W., Yang, Y., Wang, J., Xie, Q., Braunger, B., Tamm, E. R., Zavadil, J. and Cvelk, A. (2009). "Identification of Pax6-Dependent Gene Regulatory Networks in the Mouse Lens." Plos one 4(3).

Wu, F., Sapkota, D., Li, R. and Mu, X. (2012). "Onecut 1 and Onecut 2 Are Potential Regulators of Mouse Retinal Development." J Comp Neurol. 520(5): 952-969.

Xiang, M., Zhou, L., Macke, J. P., Yoshioka, T., Hendry, S. H. C., Eddy, R. L., Shows, T. B. and Nathans, J. (1995). "The Brn-3 Family of POU-Domain Factors: Primary Structure, Binding Specificity, and Expression in Subsets of Retinal Ganglion Cells and Somatosensory Neurons." The journal of Neuroscience 15(7): 4762-4785.

Xie, Q., Ung, D., Khafizov, K., Fiser, A. and Cvekl, A. (2014). "Gene regulation by PAX6: Structural-functional correlations of missense mutants and transcriptional control of Trpm3/miR-204." Mol Vis 20: 270-282.

Xu, H. E., Rould, M. A., Xu, W., Epstein, J. A., Maas, R. L. and Pabo, C. O. (1999). "Crystal structure of the human Pax6 paired domain–DNA complex reveals specific roles for the linker region and carboxy-terminal subdomain in DNA binding." Genes & Development 13(10): 1263-1275.

Yamamoto, Y. (1976). "Growth of lens and ocular environment: role of neural retina in the growth of mouse lens as revealed by implantation experiment." Dev Growth Differ 18: 273-278.

Yang, R. W. (1985). "Cell differentiation in the retina of the mouse." The anatomical record 212(2): 199-205.

Yang, X. J. (2004). "Roles of cell-extrinsic growth factors in vertebrate eye pattern formation and retinogenesis." cell and developmental biology 15: 91-103.

Yang, Z., Ding, K., Pan, L., Deng, M. and Gan, L. (2003). "Math5 determines the competence state of retinal ganglion cell progenitors " Dev. Biol.: 240-254.

Yun, S., Saijoh, Y., Hirokawa, K. E., Kopinke, D., Murtaugh, L. C., Monuki, E. S. and Levine, E. M. (2009). "Lhx2 links the intrinsic and extrinsic factors that control optic cup formation." Development 136: 3895-3906.

Zaghloul, N. A., Yan, B. and Moody, A. (2012). "Step-wise specification of retinal stem cells during normal embryogenesis." Biology of the cell 37: 321-327.

Zhao, S., Chen, Q., Hung, F. C. and Overbeek, P. A. (2002). "BMP signaling is required for development of the ciliary body." Development 129(19): 4435-4442.

Zhao, S., Hung, F. C., Colvin, J. S., White, A., Dai, W., Lowicu, F. J., Ornitz, D. M. and Overbeek, P. A. (2001). "Patterning the optic neuroepithelium by FGF signaling and Ras activation." Development 128: 5051-5060.

Zhou, Y., Tanzie, C., Yan, Z., Chen, S., Duncan, M., Gaudenz, K., Li, H., Seidel, C., Lewis, B., Moran, A., Libby, R. T., Kiernan, A. E. and Xie, T. (2013). "Notch2 regulates BMP signaling and epithelial morphogenesis in the ciliary body of the mouse eye." Proc Natl Acad Sci U S A 110(22): 8966-8971.

Zinkevich, N. S., Bosenko, D. V., Link, B. A. and Semina, E. V. (2006). "laminin alpha 1gene is essential for normal lens development in zebrafish." BMC Developmental Biology 6(1): 13.

Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G. and Harris, W. A. (2003). "Specification of the vertebrate eye by a network of eye field transcription factors." Dvelopment 130: 5155-5167.