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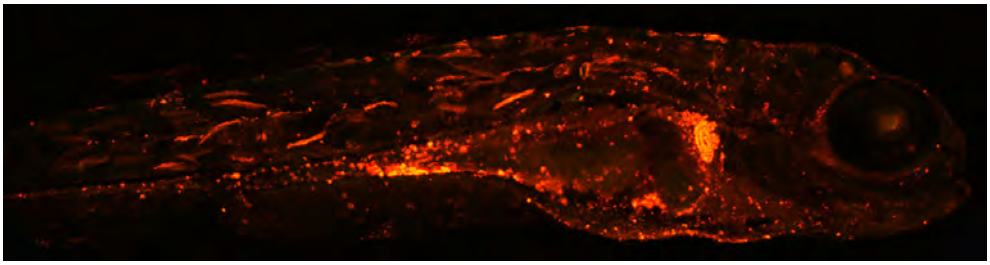
A bifunctional tool for Cre/loxP based genetic lineage labelling in zebrafish



Lovisa Wretman

Degree project in biology, Master of science (2 years), 2011
Examensarbete i biologi 45 hp till masterexamen, 2011
Biology Education Centre and Department of Evolution and Development, Uppsala University
Supervisor: Bettina Ryll

Table of contents

Summary

Cre-mediated site specific recombination has become an invaluable tool for performing genetic lineage labelling in the mouse model system. In the past decades the zebrafish has become a powerful model organism for studying vertebrate development. The main advantage of the zebrafish system is the easily manipulated and transparent embryo, which makes it suitable for analysing visually. Recently researchers have successfully explored the possibility to use the Cre/loxP system on zebrafish. Using the Tol2kit we generated a tool for Cre-mediated genetic lineage labelling in zebrafish. The tool is a bifunctional middle entry clone Cre-2A-mOrange, which is connected to a promoter to make a Cre-driver. The Cre-driver drives the equimolar expression of Cre-recombinase and the fluorophore mOrange. To test the functionality of the middle entry clone, one ubiquitous and one specific Cre-driver were produced and tested in zebrafish. The results of the transient transgenesis indicate that the tool works but stable transgenesis needs to be performed to have unambiguous results. Stable lines are now raised for further breeding. We want to make use of this tool to study craniofacial development of zebrafish. Especially the patterning of the second pharyngeal arch and the regulation of the second arch specific genes are matters we are interested in.

1 Introduction

1.1 General background

One of the main issues in developmental biology is to understand how the very simple embryonic structures develop into complicated organs and structures in the adult organism. One way to establish the relationships between adult structures and their embryological origins is by lineage labelling, also called fate mapping, which allows you to trace a cell population through development. As lineage labelling helps us to understand development of the adult morphology in one species, comparing species will provide information to understand how developmental differences correspond to morphological differences. From a developmental and evolutionary point of view vertebrates are a very fascinating group as the morphological similarity of the embryos of different taxa contrasts with very diverse adult morphologies. Especially, the vertebrate head is intriguing because of its complexity and vast morphological differences between taxa.

In particular the second pharyngeal arch or the hyoid arch is of interest to our group, not only for developmental reasons but also for evolutionary reasons. The second pharyngeal arch has undergone major evolutionary change during the course of evolution and especially during the fish-tetrapod transition. We have chosen to study the development of the second pharyngeal arch in zebrafish for many reasons. Firstly, much is known of the contributions of the second pharyngeal arch to the facial skeleton in mouse and chick, while less is known about the same in zebrafish. Therefore, a detailed study of the development of the second pharyngeal arch of the zebrafish would provide a non-tetrapod dataset, and is therefore of utter most importance. Secondly zebrafish is an excellent model organism because of its short generation time and easily manipulated embryo that is transparent which allows for live imaging and tracing cell populations *in vivo*. The aim of our group is to study the regulation and development of the second pharyngeal arch and acquire information that is still unknown about the development of the hyoid arch in zebrafish.

In order to perform lineage labelling we need genetic tools and so far a suitable tool for studying craniofacial development in zebrafish is missing. In this thesis I describe the construction and testing of a genetic tool for cell lineage labelling in zebrafish that allow for studying aspects of craniofacial development.

1.2 Technical and theoretical background

1.2.1 Genetic lineage labelling allows for ultra specific tracing of cell populations

Fate mapping or lineage labelling is a technique by which cells and their progeny are traced through developing embryo in order to understand the origin and development of specific tissues and organs. Normally fate maps are produced by labelling one cell or a small group of cells in the early embryo with dyes and by following their stained progeny ultimately determine what tissues and organs the cell line gives rise to.

Methods for vertebrate fate mapping were originally developed in avian and amphibian systems because of the ease to manipulate these embryos *in ovo*. Injection of retroviral, fluorescent or vital dyes or by grafting of quail cells into chick cells were widely used techniques (Branda & Dymecki, 2004). However, labelling of cell lineages using these techniques was often crude and not very reliable as they relied on anatomical landmarks and

surgical skills. A genetic technology was developed that could overcome these difficulties. Genetic lineage labelling allows you to label a cell population genetically, which not at all relies on anatomical markers. Genetic lineage labelling is superior to other techniques as it is precise; it actually allows you to isolate a cell population in a homogenous group, which might just differ in that they express one gene that the adjacent cells do not express. There are many methods to accomplish this kind detailed lineage labelling and one of them that offer many advantages is site-specific recombination.

1.2.2 Applying site-specific recombination to genetic lineage labelling

Site-specific recombination (SSR) is a method used for genetic lineage labelling. The method is an ideal tool for genetic lineage labelling as it can generate stable inheritable changes on DNA level, which allows you to trace one specific cell and all of its progeny throughout development.

Site-specific recombination basically involves a protein that cleaves DNA at specific short sequences; this is what implies the high specificity to the system. There are many SSR systems and one of them is the Cre/loxP system, which was first discovered in bacteriophage P1 in 1982 (Hoess *et al*., 1982). The basic mechanism of the DNA recombination involves strand cleavage, exchange and ligation. Cre recombinase catalyses site specific recombination between specific 34-bp sequences, called loxP sites. The enzyme integrase cleaves the loxP sites. If the loxP sites are oriented in the same direction (Figure 1.1, A) the intervening DNA will be excised plus one of the loxP sites will be ligated back on itself, forming a small circular DNA-molecule that is subsequently degraded in the cell (Hoess *et al*., 1985). The main DNA-strand will be ligated, leaving one loxP site intact. If the loxP sites are oriented in the opposite direction (Figure 1.1, B) the fragment will instead be inverted (Hoess *et al*., 1986).

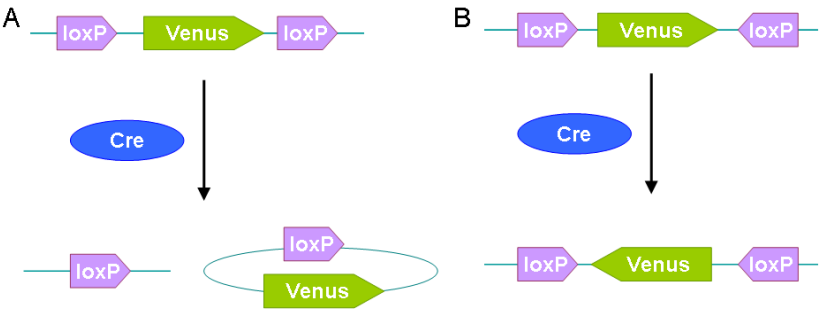


Figure 1.1. The general principle of the Cre/loxP system. Cre is a recombianse that cleaves and recombines DNA at specific sites called loxP sites. The result of recombination depends on the orientation of the loxP sites. Two loxP sites in the same orientation results in the intervening fragment being cut out and recombined with itself as depicted in **A**. The resulting plasmid with Venus is degraded.Two loxP sites in opposite direction results in an inversion of the intervening fragment, as shown in **B**.

Lineage labelling with Cre or any other SSR system makes use of two stable transgenic lines. One line, which expresses Cre recombinase, called the driver line. The Cre-expression is under the control of aspecific promoter, which makes it expressed only in specific cells, those that you want to label. The second so called reporter or indicator line harbours a transgene and loxP sites and the expression is ubiquitous. When the Cre-driver and Cre-reporter lines are crossed, in some cells both the specific Cre-driver and the ubiquitous Cre-reporter will be expressed. In those cells the Cre recombinase will catalyse a recombination of the Cre-reporter, and thereby activating the indicator. In the cell where the excision takes place a switch can be seen from the unrecombined state of the Cre.reporter to the recombined state. One common design of a Cre-reporter is a stop-signal flanked by loxP sites followed by a

transgene that acts as a selective marker. This might be lacZ or a gene coding for a fluorescent protein. When a recombination has occurred the transgene is switched on and can be detected. The activation of the transgene is heritable and all the daughter cells will inherit the recombined state of the reporter, thereby leaving a permanent record of the recombination in that cell lineage. Tracking each cell's progeny and following the cell populations allow very detailed fate mapping. (Dymecki *et al*., 2002). What is neat with this way of labelling is that the endogenous gene expression is never affected. The construct is integrated randomly into the genome but where the endogenous enhancer is active the enhancer of the construct mimics the real expression. Thereby, labelling all the cells where the particular enhancer is active is possible without affecting the natural expression.

Figure 1.2 shows the principle of how a double fluorescent Cre-reporter works. In the unrecombined state Venus is expresses and in the recombined mCherry is expressed. The switch from green to red fluorescence in a specific cell indicates in which cell the heritable recombination, which mediated the tracing of the cell line (Figure 1.3).

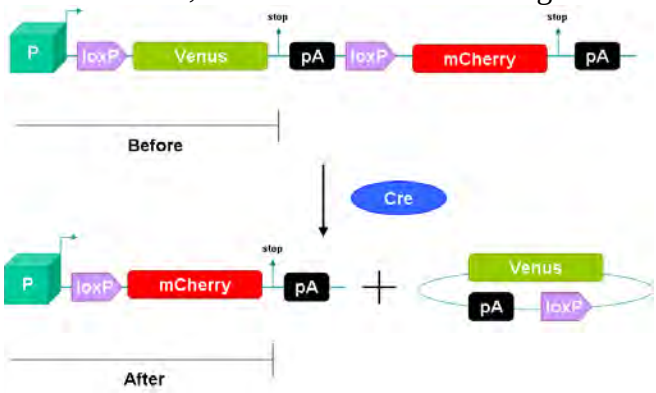


Figure 1.2. The function of a double fluorescent Cre-reporter is to switch from expressing one fluorescent protein to another in the presence of Cre recombinase. In the absence of Cre Venus is expressed. In the presence of Cre Venus with flanking loxP sites is excised and instead mCherry is expressed under the promoter P.

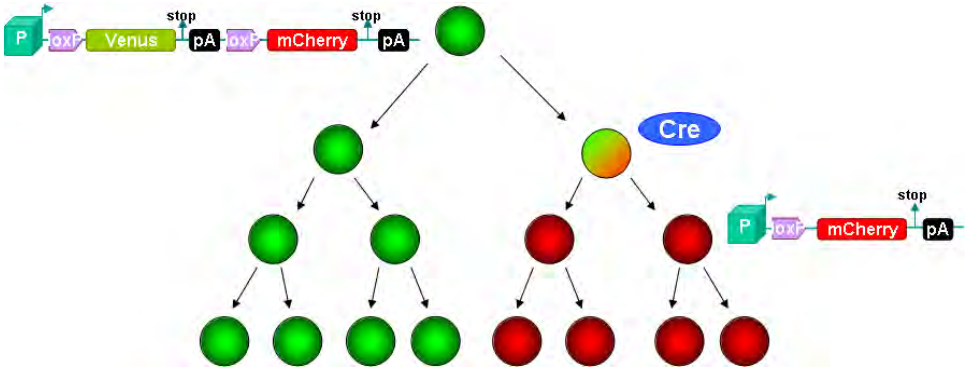


Figure 1.3. Schematic depiction of Cre-mediated genetic lineage labelling. Default expression of the doublefluorescent Cre-reporter is green fluorescence (Venus). All the cells with the Cre-reporter will fluoresce green. When Cre is introduced in one cell the Venus cassette will be excised and mCherry will be expressed instead. As the excision is inherited all progeny of the initial cell will show the red fluorescence.

The first two reports of SSR-based fate mapping in mouse were published in 1998 (Zinyk *et al*., 1998, Dymecki & Tomasiewicz, 1998). The method has proven to be a very powerful tool in fate mapping of mouse, contributing not only to our understanding of development but also gene function, genetic relationships and disease (Branda & Dymecki, 2004). Even though SSR-based lineage labelling works essentially the same in mouse and zebrafish the technique is not widely used in the zebrafish system. The first studies using this technique to

establish transgenic lines in zebrafish were carried out in 2005 (Thummel *et al*., 2005, Pan *et al*., 2005).

In a series of papers the general functionality of the Cre/loxP system and combining it with fluorescent labelling in the zebrafish system has been demonstrated (Pan *et al*., 2005; Thummel *et al*., 2005; Langenau *et al*., 2005; Le *et al*., 2007 and Feng *et al*., 2007). Although it was possible to establish stable Cre-reporter and Cre-driver lines and confirm the Cre recombination the efficiency was still relatively low. The transgenic rate of Cre- mediated genetic lineage labelling was successfully improved by injecting the plasmid constructs with the Tol2 system (Yoshikawa *et al*., 2008; Hans *et al*., 2009).

1.2.3 The Tol2 system has revolutionised zebrafish transgenesis

Transgenic Zebrafish have been generated in three ways. The first transgenic zebrafish was created in 1988 by injecting naked linearised DNA into the cytoplasm of the one cell stage embryo (Stuart *et al*, 1988). By this method fluorescent zebrafish was created in 1997 (Higashijma *et al*., 1997). However, the germline transmission frequency using this method was very low. Another approach that was tried with a near 100 % efficiency was to inject pseudotyped retrovirus (Lin *et al*., 1994; Gaiano *et al*., 1996 from the Kawakami article 2007). Even though the efficiency of this technique is very it has a backside of being very laborious intensive and researchers turned to another technique proved to be very successful in *Drosophila*, namely using transposons. Unfortunately no active transposons had been found in the zebrafish, neither any other vertebrates. First an artificial transposon called *Sleeping Beauty* was constructed (Ivics *et al*., 1997) that proved to work in zebrafish with a trangenesis rate of 30 % (Davidson *et al*., 2003). Another approach that was performed was to test invertebrate transposons on zebrafish. Transposons both from *C. elegans* (Raz *et al*., 1998) and *Drosophila* (Fadool *et al*., 1998) were successfully tested in zebrafish.

In 1996 an active transposable element was discovered in the genome of the medaka fish *Oryzias latipes* (Koga *et al*., 1996), the element was called Tol2. When it was shown to be autonomous and also capable of excision in zebrafish embryos the system's potential use in transgenesis was realised (Kawakami *et al*., 1998), Kawakami & Shima, 1999). A few years later the system was refined to achieve a transgenesis rate of 50 % (Kawakami 2004b), which makes it the most efficient transposon tool developed so far.

Tol2-element is an autonomous transposon that encodes a fully functional transposase, which catalyses transposition of a transposon construct that has 200 to 150 bp of DNA from the left and right of the Tol2-sequence respectively. In between those 200 and 150 bp long sequences inserts of up to 11 kb can be cloned. When performing Tol2 transgenesis mRNA coding for transposase and a transposon-donor plasmid containing the elements intended to be cloned flanked by the 200 and 150 bp long Tol2 sequences are mixed in an injection solution. The solution is injected into the yolk of the 1-2 cell stage embryos (Figure 1.4). The mRNA is translated to transposase, which catalyse the excision of the tranposon construct and integrates it into the genome. The integration does not cause any rearrangements or modification at the target-site. Where in the genome the construct is integrated is completely random. The embryos that are injected with the plasmid always show transient expression. To generate fish with stable expression the F_0 generation must be outcrossed and germline transmission of the construct can be selected for in the F_1 generation. (Kawakami, 2007).

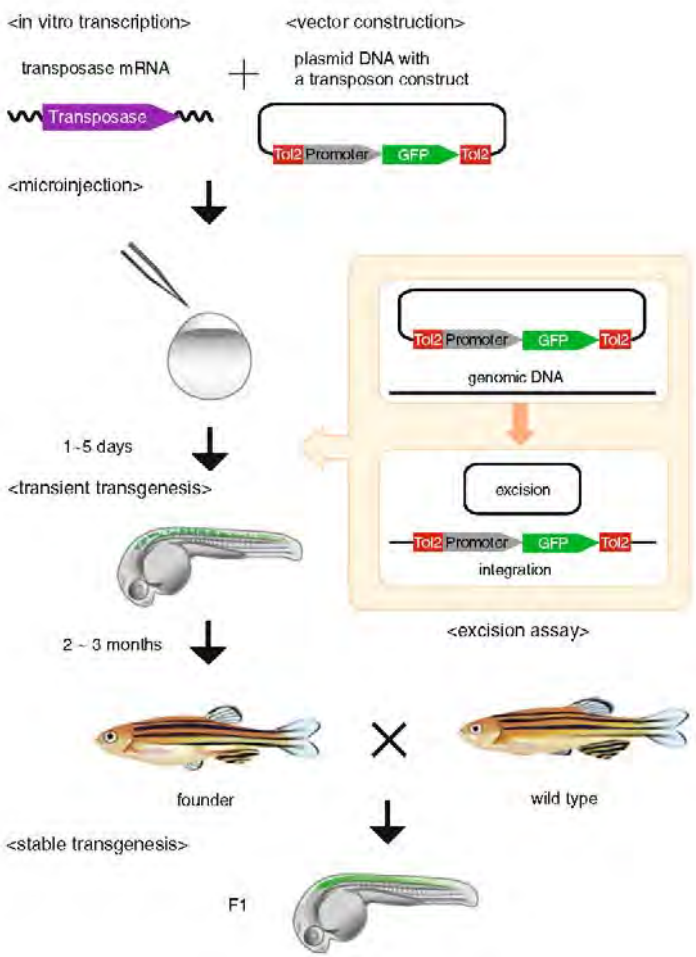


Figure 1.4. Scheme for transient and stable transgenesis in zebrafish. Transposase mRNA and plasmid DNA are coinjected into a fertilised egg. The transposase protein synthesised from the mRNA excises the Tol2 construct and integrates it into the genome. When the GFP gene is connected to the appropriate promoter cells will start to fluoresce in green. The expression is mosaic or transient, meaning that not all cells expressing the promoter synthesise the fluorescent protein. To create stable transgenesis the injected embryo has to be raised and crossed with a wildtype fish. The construct will be transmitted to the offspring, which will show nonmosaic expression. (Figure adopted from Suster *et al*., 2009)

Another reason why transgenesis in zebrafish has advanced so slowly is the labour of creating transgenic constructs. Previously traditional cloning with restriction enzymes and ligases has been used, which is very time-consuming. Another technique for cloning more efficiently and qucker would speed up the generation of constructs, which in turn would optimise zebrafish transgenesis. This technique was called Gateway cloning.

1.2.4 Gateway cloning – a quick and reliable way of generating constructs

The Gateway® technology is a cloning method that has several advantages to conventional cloning methods. Traditionally, restriction enzymes and ligases have been used to extract a DNA-fragment of interest and join it with a vector to make a new DNA construct. This method is laborious and time-consuming, especially when making clones and subsequent subclones. The Gateway® technology, on the other hand, makes use of recombining sequences that allow for cloning of multiple DNA fragments simultaneously. That way the tedious process of cloning and subcloning is avoided. The recombining sites are designed to maintain orientation and reading frame of the gene. Moreover the Gateway® system is more efficient and has a higher incorporation frequency than former cloning systems (Hartley *et al*., 2000).

The Gateway® technology is based on a method developed by Hartley *et al*. in 2000 and they first called it recombinational cloning. Hartley *et al*. took use of site-specific cloning, mediated by enzymes derived from the bacteriophage lambda (Landy *et al*., 1989) to perform directional cloning of PCR-products an subsequent subcloning of DNA-segments into new

vector backbone. Two reactions can be carried out by the system: (1) attB × attP → attL + attR catalysed by integrase (Int) and integration factor host factors (IHF) proteins and (2) attL × attR → attB + attP catalysed by Int, IHF and excisionase (Xis) (Hartley *et al*., 2000). The technique was later refined and made even more powerful by increasing the number of unique recombination sites which enabled cloning of multiple DNA-segments into a backbone in a predefined order (Cheo *et al*., 2004).

Invitrogen made use of the recombinational cloning technique and developed a cloning kit called MultiSite Gateway® Three Fragment Vector Construction Kit. The basic idea of the system is to assemble three entry clones into an expression clone by carrying out two recombination reactions.

Each reaction involves a gene or genes flanked by recombination sites mixed with (1) a vector containing recombination sites and (2) recombinase, will result in the incorporation of the gene or sequence into the vector. Altered sequences of the att sites have been engineered to enable site-specific recombination to ensure that the fragment maintains its direction.

In the first BP-reaction (Figure 1.5) a recombination between the attB and attP sites is catalysed to make attL-sites. The fragment of interest is flanked by attB sites and the donor vector has att P sites. The sites recombine to make attL sites and the fragment is cloned into the donor vector, which is now called an entry clone. The fragment you clone is generally a gene or reporter. In the second LR-reaction (Figure 1.6) the attL sites of the entry clone and attR sites of the destination vector recombines to make attB sites of the expression vector. In this way you can assemble three fragments to make one expression clone in two simple reactions (Figure 1.7).

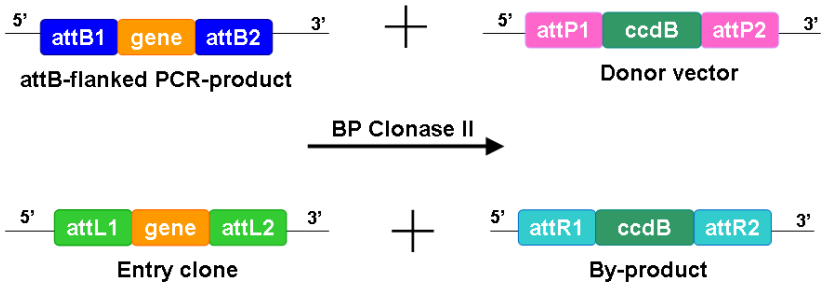


Figure 1.5. Schematic overview of the BP-reaction. An attB flanked PCR-product (e.g. gene) and a donor vector with attP sites recombine to form an entry clone flanked by attL sites and an attR site flanked by-product.

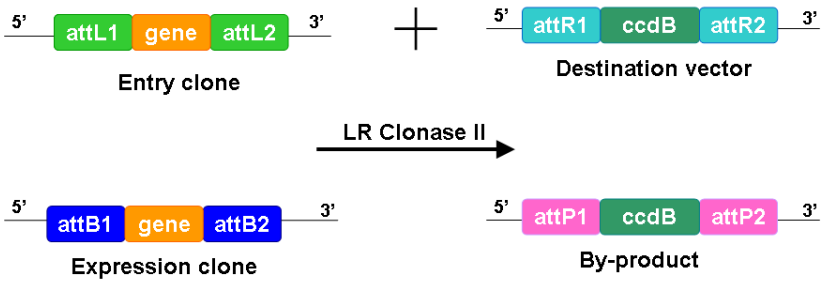


Figure 1.6. Schematic overview of the LR-reaction. An attL site flanked entry clone (produced from the BP-reaction) and a destination vector with attR sites recombine to form an expression clone flanked by attB sites and a by-product with attP sites.

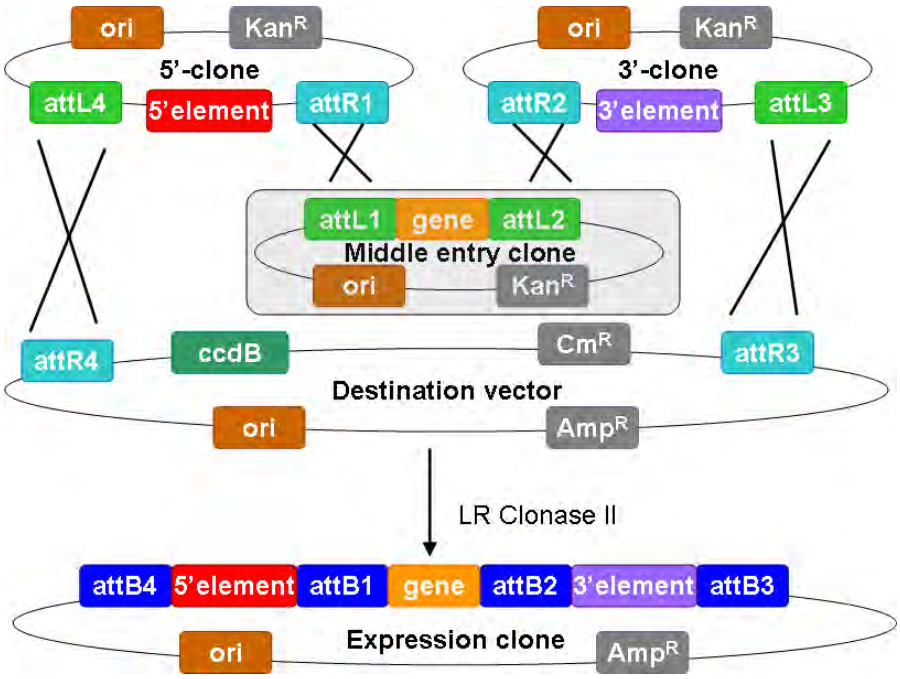


Figure 1.7. The generation of an expression clone requires four parts and one enzymatic reaction. The clones are the 5'-clone, usually an enhancer element, the 3'-clone which is usually a polyA tail and the middle entry clone, (highlighted in the picture) which is generated through the BP-reaction (see Figure 1.5). The forth part is the Destination vector, which contains the attR sites required for the final LR-reaction. In the presence of LR Clonase II the attL and attR sites recombine in a specific manner to generate the expression clone.

1.2.5 The Tol2kit combines the efficiency of the Tol2 system with the quick Gateway cloning technique

Scientists working with zebrafish transgenics have been struggling with three problems. The first being hard and tedious labour of building complex expression constructs using conventional subcloning, secondly, low transgenic rate, meaning mosaic expression and infrequent germline incorporation when injecting with a linearised plasmid and thirdly difficulty to identify germline incorporations. New discoveries and techniques developed to solve these problems separately were later brought together to create a very powerful tool, with which zebrafish transgenics could reach its full potential. The problem of tedious cloning was solved by using recombination-based cloning method called multisite Gateway cloning. The problem of inefficient transgenics was solved with the Tol2 transposon element and detection of successful incorporation with fluorescent proteins. (Kwan el al., 2007)

The Tol2kit is a system that makes use of Multisite Gateway cloning to allow rapid assembly of multiple segments constructs in a Tol2 backbone vector. The kit provides entry clones with different tissue-specific promoters as entry clones, destination vectors with Tol2 transposon sequences and methods by which one can link a gene without a fluorescent gene product to a gene coding for a fluorescent protein in order to visualise gene expression. The final construct is made of three parts, the 5' entry clone (enhancer-promoter), middle entry clone (coding sequence) and 3' entry clone (poly-A). (Kwan *et al*., 2007)

1.3 The aim and purpose of the project

One of the main issues in evolutionary biology today is to understand how differences in developmental patterns correspond to the adult morphologies we see in the different animal groups. Questions that evolutionary developmental biologists ask themselves are how these

patterns changed during the course of evolution and what developmental changes underlie the origin of morphological structures. To address these questions one must start by comparing the developmental patterns of different groups.

One question of evolutionary interest is the development and evolution of the vertebrate head and especially the pharyngeal region, which is constituted by a series of branchial arches that take different form and function in different vertebrate groups. The pharynx has undergone major changes during the course of evolution but almost nothing is known about what changes in the developmental systems that underlie these evolutionary changes. The development of the mouse and chick head and pharynx have been studied in detail by fate mapping, which demonstrates the relation between the embryonic tissues and the adult structures. Of special interest is the second pharyngeal arch, which lies just posterior of the first branchial arch, which constitutes the jaws. The contribution of the second pharyngeal arch to the facial skeleton is known in detail for mouse and bird, but a comparative dataset for zebrafish is still missing.

To study the development and genetics of the hyoid arch in zebrafish we will label lineages of hyoid arch specific enhancers. But to do this we need genetic tools, which are not available. In this thesis I describe the generation and testing of a tool that can be used for genetic lineage labelling of all cells and tissues but will be used primarily to study aspects of craniofacial development.

The tool is a middle entry clone that can be combined with any promoter to make a Cre-driver. The middle entry clone will be comprised of three components. The design of the construct can be viewed in Figure 1.8. The advantage of this construct which makes it unique is that it has two functions. As integration of a construct in transgenesis is random, we cannot know for sure that it is actually integrated in the cells where the endogenous gene is active, therefore we need a readout to control that the construct is actually integrated into the cells that it is supposed to be. The gene mCherry codes for a red fluorescent protein, which entails this readout. The Cre gene codes for the Cre recombinase, which is needed for the Cre-mediated switch and lineage labelling.

To ensure that mCherry and Cre is expressed in equimolar amounts the two are linked via a 2A sequence (Figure 1.9). 2A is a sequence that codes for a peptide, which is naturally used by viruses. Linking two genes by this sequence is a clever way of generating two proteins from one transcript. The peptide is quite deceptively called a 'self-cleaving' peptide. In fact, one peptide-bond is impaired in the 2A-sequence resulting in two separate protein fragments from one translation event (Donnelly *et al*., 2001). In an article from Hsiao *et al*. from 2008, they describe how constructs of different combinations of fluorophores and bacterial/eukaryotic selection markers linked to the same promoter region by a 2A peptide are created. These plasmids are then used to establish stable reporter lines.

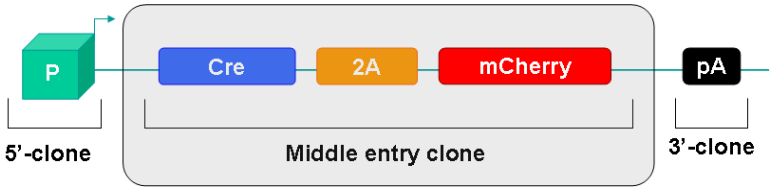


Figure 1.8. The design of the middle entry clone Cre-2A-mCherry.

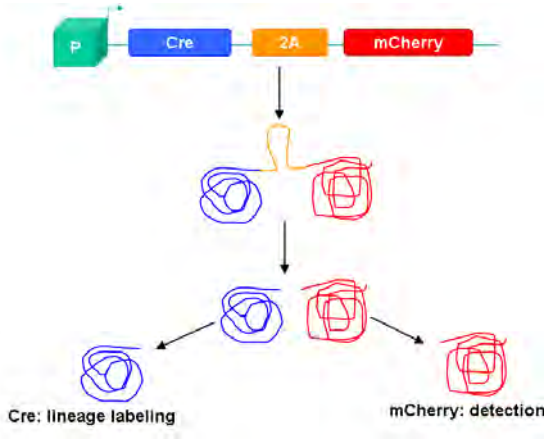


Figure 1.9. A schematic overview of the middle entry clone and the functions of the different parts Cre, 2A and mCherry 2A sequence in the Cre driver construct. The 2A peptide allows for the equimolar expression of two genes under the control of one promoter (P). The purpose of the fluorescent protein is to detect if the construct is successfully integrated with the genome. Cre recombinase acts in Cre-mediated lineage labelling.

The generated middle entry clone will be tested by linking it to one ubiquitous and one specific promoter, using the Tol2kit. Thus the final constructs will be β-actin-Cre-2A-mCherry and clmc2-Cre-2A-mCherry (Figure 1.10). The Cre-drivers' ability to drive a Cre-mediated switch will be tested and thereby their potential to be used in lineage labelling can be evaluated.

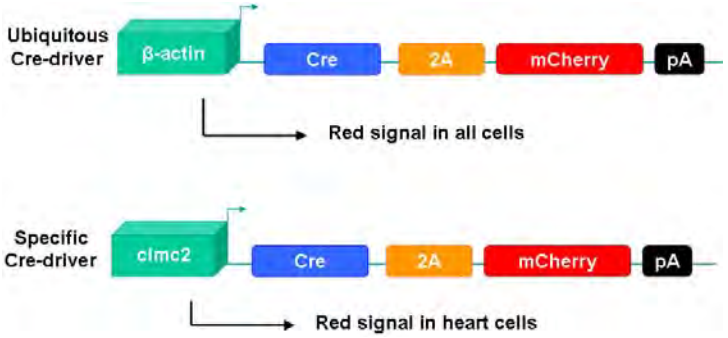


Figure 1.10. The design of the Cre-drivers. The ubiquitous Cre-driver β-actin-Cre-2a-mCherry and the specific Cre-driver clmc2-Cre-2A-mCherry.

In this thesis I describe the generation and testing of a bifunctional tool for Cre/loxP based genetic lineage labelling in zebrafish. The tool is generic but our aim is to use it for studying craniofacial development. Especially, we will use this tool to screen for genetic elements with hyoid arch specific expression. Although knock-out studies have been made on the hyoid arch in zebrafish (Hunter & Prince, 2002), the details of its development are poorly known. With transgenic techniques we will be able to acquire new information at a higher resolution than made before. This tool will contribute to the knowledge and understanding of the development of the second pharyngeal arch in zebrafish. Moreover, it will provide a new dataset that will be interesting to put in a greater phylogenetic context. The tool will be generated with and compatible with the Tol2kit, which allow other researchers to make use of it. The tool can be combined with any promoter of interest to study any aspect of zebrafish development.

2 Material and methods

2.1 General molecular biology

2.1.1 Primers and PCR amplification

Four oligonucleotides as primers were used for PCR reactions in this project. They were designed by Bettina Ryll, using the SeqBuilder. They were all designed to have a GC content as close to 50 % as possible (Table 1). All the primers were 'recombinant', meaning that they consist of both an overlapping part with the sequence that will be amplified plus a 'recombinant' part that will be added to the sequence, as a restriction site or a recombination site (e.g. att sites). All the primers were purchased from Roche and diluted in sterilised water (WFI for cell culture from Invitrogen) to 100 mM.

Table 1. Primers used for PCR amplification, their sequence, melting temperatures and GC contents.

Primer name	Nucleotide sequence (5'→ 3')	T_m (°C)	GC (%)
oBR9	GGGGACAAGTTTGTACAAAAAAGCAGGC TCTCGAGAACGTCGAGGGCAGCGGCGCGACCA	94.6	58.3
oBR10	GGGGACCACTTTGTACAAGAAAGCTGGGT GCTACTTGTACAGCTCGTCCATGCC	87.8	53.7
oBR11	AGACTCGAGGCCACCATGTCCAATTTACTGACCG	80.4	52.9
oBR12	AGACTCGAGATCGCCATCTTCCAGCAGGCGCACC	85.9	61.7

The PCR amplifications were carried out on a Mastercycler Epgradient (Eppendorf). The standardised gradient PCR program (Table 2) started with an initial denaturation step at 94 °C for 20 seconds, then followed 30 cycles of denaturation (94 °C for 30 seconds), annealing (60 °C +/- 10 °C for 30 seconds) and elongation (72 °C for 1 minute. The program ended with a final elongation step at 72 °C running for 10 minutes.

Table 2. The thermocycling conditions of the standard gradient PCR program. The program denaturation-annealing-elongation steps run 30 cycles.

Step	Temperature and time
Initial denaturation	94 °C 20 sec
Denaturation	94 °C 30 sec
Annealing	60 °C +/- 10 °C 30 sec
Elongation	72 °C 1 min
Final elongation	72 °C 10 min

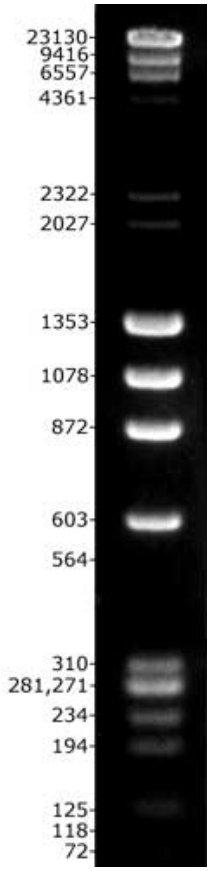
AmpliTaq®DNA Polymerase and buffers were purchased from Applied Biosciences (AmpliTaq® 360 DNA Polymerase Kit) and dNTPs from Roche. One master mix was prepared on ice and divided into 12 reactions of 20 µl volume with final concentrations of 0.2 ng/µl plasmid DNA, 500 µM dNTPs, 0.5 µM primer each, 1 X Magnesium chloride, 1X PCR buffer and 0.125 U/µl AmpliTaq®.

2.1.2 Gel electrophoresis and visualisation of DNA fragments

Restriction digests and PCR results were checked on 1 % Agarose gels (Agarose for routine use, Sigma) in 1X TAE (TAE buffer, Promega) and run at 50 mVolt. Fragments for purification were separated on 1 % High Quality Agarose gels (SeaKem® ME Agarose, Lonza) and run at 25 mV. The gels were stained in a 1X TAE solution containing Ethidium bromide and photographed with an UV-light camera..

2.1.3 Ladder marker

As gel ladder marker for sizing of fragments a mixture of λ*Hind*III DNA and φ174 *Bsu*RI DNA with a final concentration of 0.2 mg/ml was used. Both products were ordered from Fermentas. Ladder marker fragments were sized as indicated below.



2.1.4 Restriction digests and Diagnostic digests

Restriction enzymes were purchased from Fermentas and New England Biolabs. Standard diagnostic digests were performed as 20 µl reactions in a 37°C incubator for one to three hours and then checked on 1 % Standard Agarose gels. Fragments for purification were restricted in 30 µl reactions over night and then run on 1 % High Quality gels and then excised and purified with MinElute® Gel Extraction Kit (Qiagen) according to the manual. Backbone vectors linearised for cloning were also restricted in 30 µl reactions over night and then run on 1 % Standard Agarose gels for verification of complete digest. If so, the restriction enzyme was heat inactivated for 20 minutes at 65°C followed by de-phosphorylation with rAPid Alkaline Phosphatase (Roche). The reaction was carried out in 37°C waterbath over night. The phosphatase was then heat inactivated for 2 minutes in 75°C water incubator.

2.1.5 Plasmid propagation and purification

For propagation, plasmids were grown in chemically competent One Shot TOP10 *E.coli* (Invitrogen). Transformation was performed according to the standard procedure, stated in all protocols used during the project. The cells were thawed on ice, the ligation mixture added to the vial of cells, then incubated on ice for 30 minutes, heat-shocked for 30 seconds at 42°C in a waterbath and then placed on ice again for 2 minutes. After addition of 250 µl room-temperature S.O.C. medium, cells were incubated in a 37°C shaking incubator for one hour and then plated out on pre-warmed selective LB agar plates. The next day colonies were picked and separately put in test tubes with 6 ml LB selective medium. The cells were incubated in a 37°C shaking incubator over night and purified the following day with GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's manual.

2.2 Cloning procedures

2.2.1 Generation of pLW1

Cre, the gene for Cre recombinase was PCR-amplified from pPax2CreERT with the recombinant primers oBR11 and oBR12 to add *Xho*I sites for further cloning. *Xho*I-Cre-*Xho*I was sub-cloned into pCR®II TOPO®, resulting in the plasmid pLW1. The TOPO cloning was performed according to the TOPO TA Cloning® Manual, Version U, Invitrogen without any modifications. The vector pCR®II TOPO® was included with the kit. The insert was sequenced with the primers M13 reverse and T7.

2.2.2 Generation of pLW2

2A-mCherry was amplified from pEnt-mCherry with the recombinant primers oBR9 and oBR10 to add an *Xho*I site and attB sites for further Gateway cloning. The plasmid was ordered through BACPAC CHORI. The primers were designed according to the instructions of the MultiSite Gateway® Three-Fragment Vector Construction Kit, Version D (Invitrogen). The fragment attB1-*Xho*I-2A-mCherry-attB2 was sub-cloned into pDONR221™ according to the section 'Performing the BP recombination reaction' in the manual stated above. However, one deviation from the protocol was made. Instead of adding 1 µl of the BP recombination reaction to a vial of *E.coli* cells 10 µl was added. The insert was sequenced with M13 and M13 reverse. The correctly sequenced plasmid was called pLW2, possessing the components attL1-*Xho*I-2A-mCherry-attL2.

2.2.3 Generation of the middle entry clone: pLW3

In order to sub-clone *Xho*I-Cre-*Xho*I into pLW2 and to create the final middle entry clone, the backbone and insert were prepared. The plasmid pLW2, acting as the backbone was linearised with *Xho*I and dephosphorylated with rAPid Alkaline Phosphatase. The complete linearisation was verified by electrophoresis gel analysis. pLW1 was digested with *Xho*I and the fragment *Xho*I-Cre-*Xho*I was extracted from a high quality gel and purified with MinElute® Gel Extraction Kit (Qiagen) according to the manual. Ligations for sub-cloning *Xho*I-Cre-*Xho*I into the backbones pLW2 and pBRU3 were set up according to the protocols in Table 3 and 4. The backbone to insert ratios were 1:5. The ligation kit used was the Rapid DNA Dephos & ligation kit from Roche. The final plasmid was sequenced along its entire length with primers M13, M13 reverse TOPO and oLW5. This completed middle entry clone was called pLW3.

Table 3. Ligation protocol of *Xho*I-Cre-*Xho*I/pLW2 cloning

Component	Ligation (volume, µl)	Control (volume, µl)
Backbone (pLW2)	2	2
Insert (*Xho*I-Cre-*Xho*I)	2	0
DNA Ligase Buffer	10	10
dH_2O	6	8
T4 DNA Ligase	1	1
Total	21	21

Table 4. Ligation protocol of *Xho*I-Cre-*Xho*I/pBRU3 cloning

Component	Ligation (volume, µl)	Control (volume, µl)
Backbone (pBRU3)	1	1
Insert (*Xho*I-Cre-*Xho*I)	2	0
DNA Ligase Buffer	10	10
dH_2O	7	9
T4 DNA Ligase	1	1
Total	21	21

2.2.4 Generation of the expression clones pLW4 and pLW5

LR-reactions to produce expression clones were performed according to the section 'Performing the MultiSite Gateway LR Recombination' from the manual MultiSite Gateway® Three-Fragment Vector Construction Kit, Version D (Invitrogen). The ubiquitous promoter β-actin (Higashijima *et al*., 1997) from the Tol2kit construct p5E-bactin2 (#299) was used for the construct pLW4 and the specific promoter clmc2a (Joplin *et al*., 2010) for pLW5. The plasmid p302 (polyA) as 3'-clone and p394 as vector were used for both constructs. Six samples of each reaction were digested . The correct clones were called pLW4 (β-actin-Cre-2A-mOrange-pA) and pLW5 (clmc2-Cre-2A-mOrange-pA).

2.3 Zebrafish husbandry, injections and screenings

The zebrafish are obtained on a regular light-dark cycle, with 10 h of light and a temperature of 28°C. The day prior to injections fish were set up for matings in small plastic tanks, consisting of a base tank, a slotted insert, a separator to isolate the females from the males and a plastic lid. The fish were set up with 2 females-2 males or 2 females-3 males or 3 females-3 males per tank. The following morning the separator was removed. After 15-30 minutes the eggs were collected in petri dishes. The needles (Glass Capillaries with filament 1x90mm, Narishige) were prepared and loaded with the injection solution (Table 5), composed according to published procedures (Fisher *et al*., 2006). In the cases where two plasmids were injected the each of them had a volume of 0.5 µl, to keep the total volume of DNA to 1 µl. The injections were performed under a Zeiss dissecting microscope (Stemi 2000 microscope) with a IM3000 Microinjector from Narishige. They were injected at the 1-2 cell stage. The injected eggs were then kept in a 28°C incubator. They were screened under the Zeiss dissecting microscope the first day and then under the Leica MZ FLIII with the UV-lamp to check for positives. Embryos that were underdeveloped, deformed or suffering with oedemas were aborted. The embryos were screened, observed and documented with the Leica DFC490 camera with a magnification of 0.63x for 5 days.

Table 5. The components of the injection solution.

Component	Volume (µl)
Plasmid (125 ng/µl)	1
Transposase RNA (175 ng/µl)	1
Phenol Red	0.5
RNase-free water	2.5

2.4 Confocal microscopy and image processing

The confocal images were acquired on a ZEISS CLSM 710 with objectives 5x, 10x and 20x magnification. Signals in green, red and orange channels were set using the standard scanning settings provided by the manufacturer. The channels and the waavelengts for the different flurophores asre stated in Table 6.

During the scanning the embryos were placed in small petri dishes, positioned laterally in drops of low melting agarose containg Tricaine (1%) to immobilize the fish.

Images of collapsed z-stacks were processed with the Adobe Photoshop Software. In some images brightness, contrast and colour were adjusted. The adjustments were always linear and applied to the entire plane of the image.

Table 6. Channels used for different flurophores and their wavelengths (Shaner, 2005).

Fluorophore	Channel	Excitation Wavelength (nm)
Venus	EYFP	514
mCherry	mCherry	587
mOrange	mOrange	548

2.5 Softwares

For construct design, primer design and map creation SeqBuilder of the Lasergene® software packet from DNAstar was used. For sequence alignments BioEdit was used. All stereomicroscope, confocal images and gel pictures were treated in Adobe Photoshop CS4. Diagrams and schematic figures were produced with Microsoft Office Power Point 2003.

3 Results

3.1 Generation of constructs

3.1.1 Generation of pLW1

The gene Cre was PCR-amplified from pPax2CreERT with the recombinant primers oBR11 and oBR12, the procedure is schematically described in Figure 3.1. The PCR-product was checked on a gel and the band for the 1046 bp long *Xho*I-Cre-*Xho*I-fragment could clearly be seen (Figure 3.2). *Xho*I-Cre-*Xho*I was sub-cloned into pCR®II TOPO®, resulting in the plasmid pLW1 (Figure 3.3).

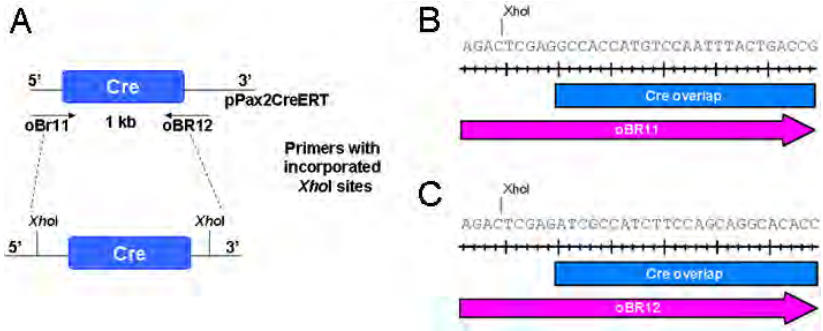


Figure 3.1. **A** Cre was amplified by standard PCR from the plasmid pPax2CreERT with the recombinant primer pair oBR11 and 12 to add restriction sites for *XhoI*. **B** and **C** show the sequences of the primers oBR11 and 12 respectively.

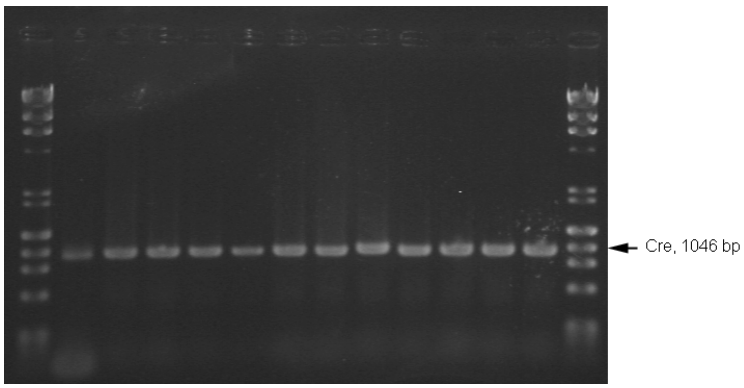


Figure 3.2. Gel picture showing result of PCR-amplification of *Xho*I-Cre-*Xho*I with primers oBR11 and oBR12 from the plasmid pPax2CreERT. The expected band of 1046 bp is indicated by arrow.

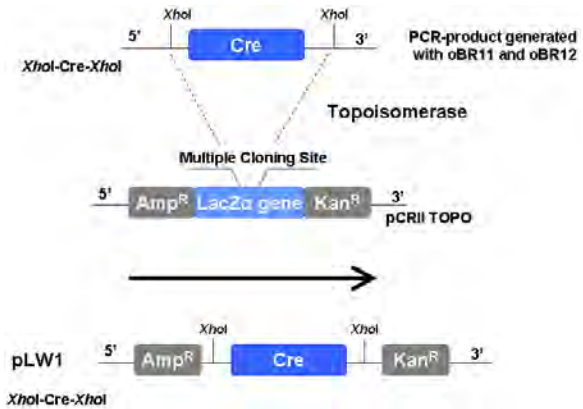


Figure 3.3. The *XhoI*-Cre-*XhoI* fragment generated with oBR11 and oBR12 was cloned into the pCRII-TOPO vector using the TOPO TA Cloning kit. The resulting plasmid was called pLW1.

3.1.2 Generation of pLW2

The fragment 2A-mCherry was PCR-amplified pEntmCherry with the recombinant primers oBR9 and oBR10, the procedure is schematically described in Figure 3.4. The PCR-product was checked on a gel and the band for the ~850 bp long attB1-*Xho*I-2A-mCherry-fragment could clearly be seen (Figure 3.5). The fragment was sub-cloned into pDONR221 resulting in the plasmid pLW2 (Figure 3.6). The plasmid was restricted with *Pvu*II and *Bsr*GI separately to verify successful BP-cloning. Expected bands of successful *Pvu*II restriction were 1942 bp, 1159 bp and 242 bp. Samples 2-6 in Figure 3.7 display the correct bands. Expected bands of successful *Bsr*GI restriction were 2514 bp, 803 bp and 25 bp. Samples 8-12 in Figure 3.7 display the correct bands. The shortest band of 25 bp cannot be seen as it has travelled too far on the gel. Therefore the first sample is discarded and the 5 last ones are kept for further cloning.

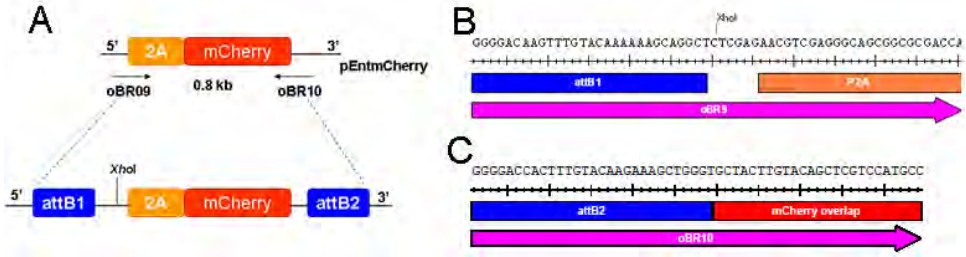


Figure 3.4. **A** 2A-mCherry was amplified by standard PCR from the plasmid pEntmCherry using recombinant primer pairs oBR9 and 10 to add relevant cloning sites. **B** and **C** show the sequences of the primers oBR9 and 10 respectively.

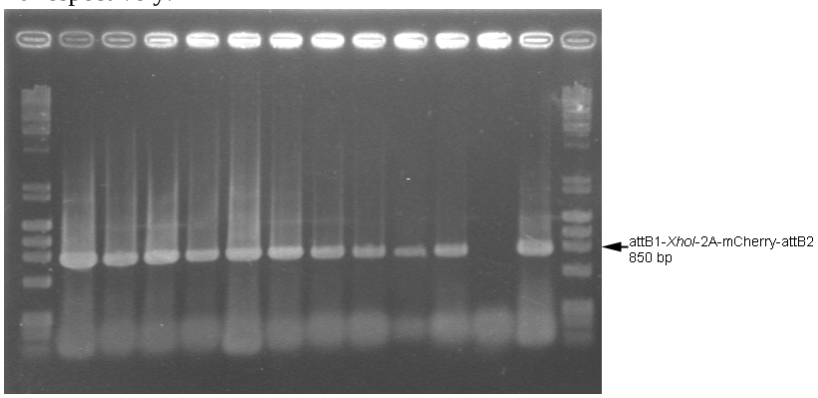


Figure 3.5. 2A-mCherry was amplified using the primers oBR9 and 0BR10 from the plasmid pEntmCherry. The expected band of 850 bp is indicated by the arrow.

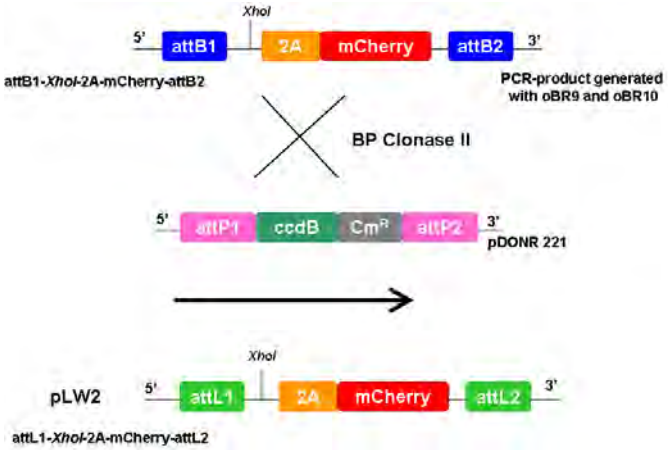


Figure 3.6. The attB1-XhoI-2A-mCherry-attB2 fragment generated with the recombinant primers oBR9 and 10 was cloned into the donor vector pDONR221. The resulting plasmid was called pLW2.

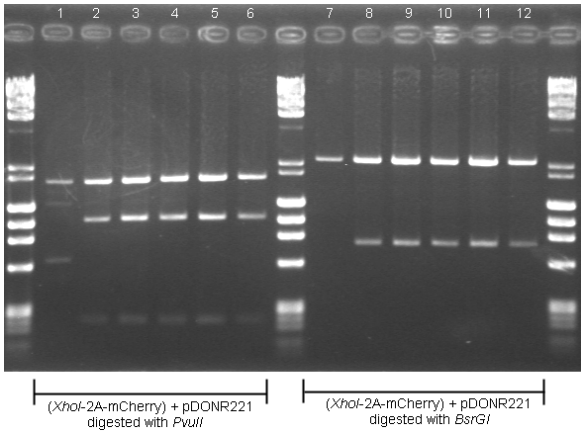


Figure 3.7. The gel picture shows the result of two diagnostic digestions preformed on six samples of the product from the recombination between the fragment attB1-*XhoI*-2A-mCherryattB2 and the vector pDONR221. The six samples were restricted by *PvuII* (1-6) and *BsrG*I (7-12) separately. The bands in rows 2-6 are ~1.9 kbs, ~1.2 kbs and ~200 bp and the bands in rows 8-12 are ~2.5 kps and ~800 bp.

3.1.3 Generation of pLW3

Two samples of pLW2 were linearised with *Xho*I (Figure 3.8, left gel picture). pLW1 was restricted with *Xho*I and the product was run on a high quality gel for further purification (Figure 3.8). A ligation with the purified *Xho*I-Cre-*Xho*I and pLW2 was set up. Figure 3.9 is a gel picture of a diagnostic digest of the first attempt to clone *Xho*I-Cre-*Xho*I into pLW2. The expected result for a successful ligation would be two bands, one of 1046 bp, being the Cre insert and one of 3400 bp, being the backbone. As we can only see bands the length of the backbone it was concluded that the ligation was unsuccessful. Two more ligations were performed and both failed. It was decided that one last ligation with pLW2 as backbone would be made in parallel with pBRU3 as backbone. The plasmid pBRU3, produced by Bettina Ryll has the components attL1-*Sal*I-2A-mOrange-attL2. mOrange is a fluorophore differing only in a few basepairs to mCherry, which results in an orange signal instead of a red. The restriction site for *Sal*I is compatible with *Xho*I, which enable the two ends to ligate and therefore sub-cloning Cre with the flanking *Xho*I sites into attL1-*Sal*I-2A-mOrange-attL2 would be possible. A diagnostic digest with *Eco*RV of the two ligation reactions is shown in Figure 3.10. Samples 1 to 6 are *Xho*I-Cre-*Xho*I /pBRU3 ligations and samples 7 to 12 are *Xho*I-Cre-*Xho*I/pLW2 ligations. Expected bands of successful *Eco*RV-restriction of *Xho*I-Cre-*Xho*I /pBRU3 ligations are 1381 bp and 2993 bp and those can be seen in samples 1, 2, 3 and 6. Expected bands of successful *Eco*RV-restriction of *Xho*I-Cre-*Xho*I /pLW2 ligations are 1387 bp and 2996 bp, which cannot be seen. However, only the backbone of 3400 bp can be seen and it was concluded that this forth attempted ligation of *Xho*I-Cre-*Xho*I and pLW2 had failed (Figure 3.11).

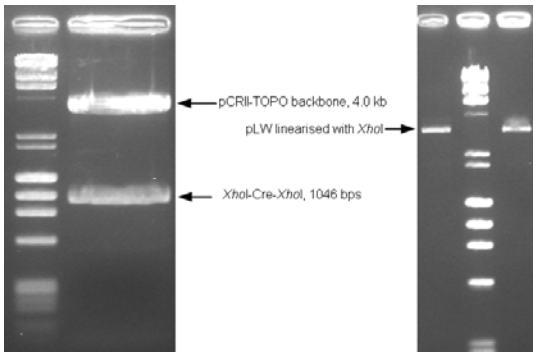


Figure 3.8. The gel picture on the left shows the restriction of pLW1 with XhoI. The expected bands were of 1046 bp XhoI-Cre-XhoI) and 4.0 kbs (backbone). Both are indicated by the arrows.
The gel picture to the right shows the result of the linearization of pLW2 (*XhoI*-Cre-2A-mCherry). Two samples were linearised and run on a gel. The arrow indicates the fully linearised plasmids.

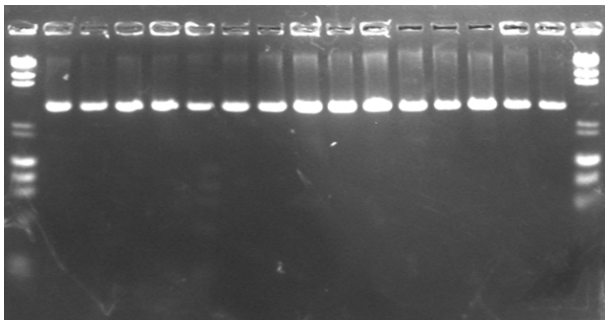


Figure 3.9. Gel picture of diagnostic digest product from ligation reaction of *Xho*I-Cre-*Xho*I cloned into pLW2. The bands are 3400 bp long.

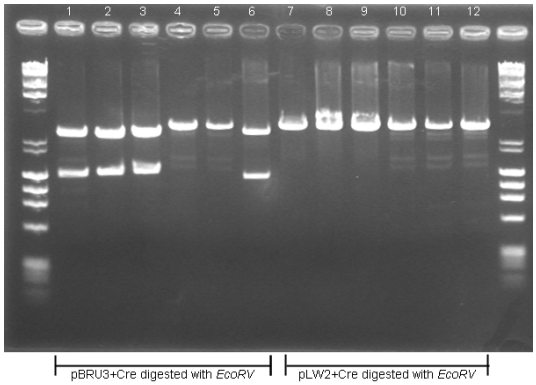


Figure 3.10. The *Xho*I-Cre-*Xho*I fragment of 1046 bp was cloned into the vector pBRU3 and pLW2. The gel picture shows the result of *EcoRV*-restrictions performed on the products from the cloning reactions. Samples 1-6 are restrictions of pBRU3/*Xho*I-Cre-*Xho*I ligation and samples 7-12 are restrictions of pLW2/*Xho*I-Cre-*Xho*I.

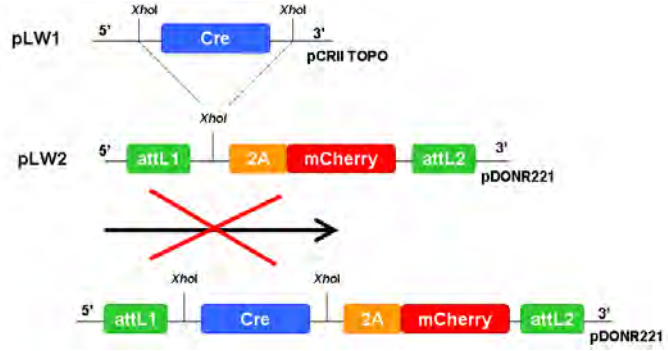


Figure 3.11. The attempt to clone *Xho*I-Cre-*Xho*I into pLW2 failed. pLW2 was linearised with *Xho*I and the attempt to ligate the *Xho*I-Cre-*Xho*I fragment into the pLW2 was not successful.

To determine if the orientation of the insert was correct and not reverse yet another diagnostic digest was performed on the 4 plasmids that had the insert. The result can be seen in Figure 3.12. The expected bands of a correctly oriented *Xho*I-Cre-*Xho*I insert are196 bp, 1577 bp and 2610 bp. Only sample number four had the appropriate bands. The band of 196 bp cannot be seen as the bands were let to travel too far on the gel. The generated plasmid was called pLW3 (Figure 3.13).

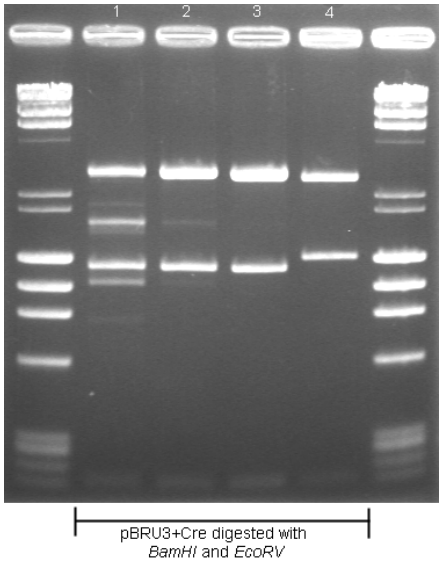


Figure 3.12. The gel picture shows are double restriction with *Bam*HI and *Eco*RV of the products from the cloning of the Cre flagment into the vector pBRU3.

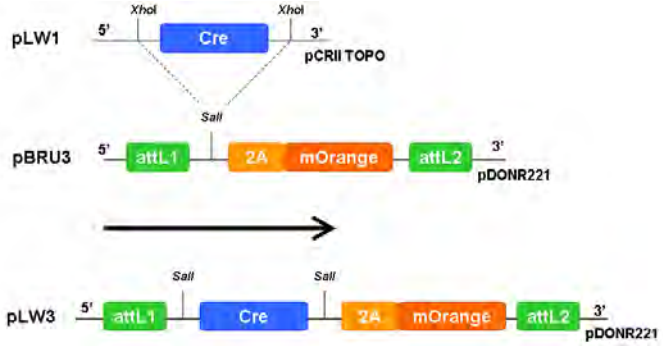


Figure 3.13. The *XhoI*-Cre-*XhoI* fragment was cut out of the pLW1 with *XhoI* and ligated into pBRU3, after it had been opened with *SalI*. The resulting plasmid was called pLW3.

3.1.4 Generation of pLW4 and pLW5

The final expression clones were created with the Gateway LR-reaction. The middle entry clone was linked to 2 promoters, β-actin which is expressed in all cells and clmc2 which is only expressed in heart cells. To determine if the recombination reactions had worked they were digested (Figure 3.14). The expected band of the *Pci*I restriction of the clmc2-construct were 3357 bp, 2500 bp, 789 bp and 692 bp, making samples 1, 2, 4, 5, 6 correct. The expected bands of the *Pvu*II restriction of the β-actin-construct were 5916 bp, 4249 bp, 767 bp and 746 bp, making samples 8, 9, 10 and 12 correct. The complete β-actin-construct was called pLW4 and clmc2-construct pLW5 (Figure 3.15).

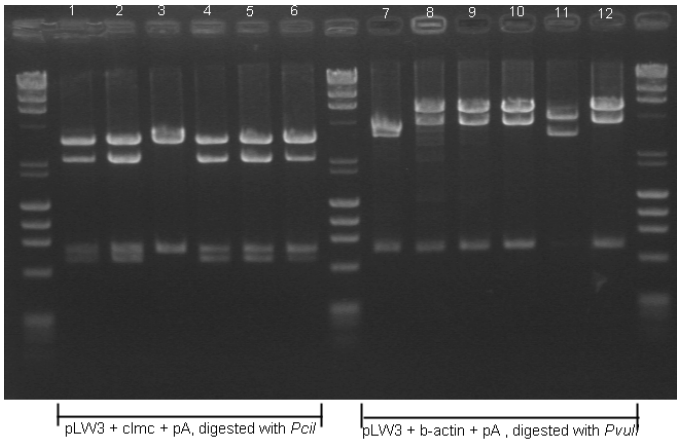


Figure 3.14. The results of the restrictions of the two LR-reactions are shown on the gel picture. The two LR-reactions were performed with pLW3 as middle entry clone, polyA as 3'-clone and clmc2 and β-actin as 5'-clones respectively. The products of the two LR-reactions were digested with *PciI* and *PvuII* respectively.

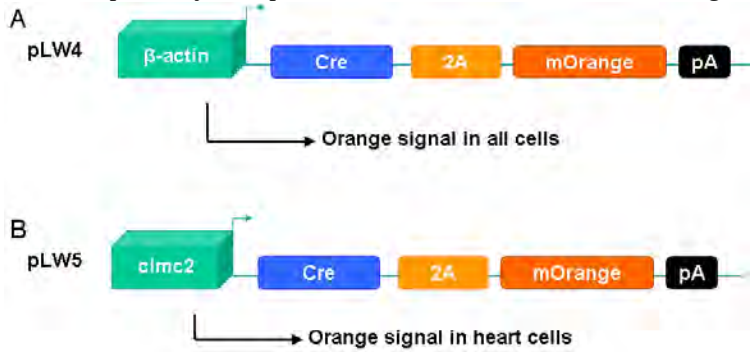


Figure 3.15. The two Cre-drivers generated. **A** shows the β-actin-driver and **B** shows the clmc2-driver.

3.2 Testing of constructs

3.2.1 Overview of injections and some comments about the images

Six rounds of injections were carried out in three parts. In the first part the Cre-reporter was tested, first its fluorescence and then if it was receptive to Cre-mediated recombination by coinjecting it with a Cre-plasmid. The drivers were injected alone to determine if they generate any fluorescence and if they express it in the tissue they were designed to. Both the drivers were coinjected with a double-fluorescent Cre-reporter β-actin-loxP-Venus-loxP-mCherry, in order to test the drivers' ability to perform Cre-mediated recombination.

A few comments should be made about the fluorescence of the fish. Some structures of the fish are autofluorescent, namely the yolk, the retina and also some small cells that can be seen along the edges of the caudal fin. Also, injected plasmid into the yolk fluoresces on its own even though it might not be incorporated into the genome of the cells in the yolk. This fluorescence decreases with time as the plasmid is gradually degraded.

In Figures 3.22 A, B and C, 3.25 A, B and C and 3.26 A, B and C there is a very bright area dorsal and posterior to the heart.

3.2.2 The Cre-reporter is expressed in various cell types and recombines when coinjected with Cre

The double fluorescent Cre-reporter β-actin-loxP-Venus-loxP-mCherry was injected to see the green signal produced by the expression of Venus. Venus and mCherry of the reporter

have membrane-tags, which localises the proteins expressions to the membrane. The signal was visible from 1-2 days. The expression is clearly mosaic, showing in muscle and notochord cells (Figure 3.16). In some embryos signal in heart cells was also visible. The mosaic expression was expected as it is only transient opposed to stable, in which you can expect signal in all cells expressing the promoter you have used. As Venus is membrane-bound you would also expect to see the signal only in the membranes of the cells. This is difficult to detect in Figure 3.16, most probably because the magnification is not high enough.

In order to demonstrate the Cre-mediated shift from green to red the reporter was co-injected with a Cre-plasmid. Confocal images were taken in both the red and green channel. By comparing images B and C in Figure 3.17 we can see that considerably more red than green cells. Therefore we could deduce that the Cre-mediated excision of Venus had worked and moreover that it was efficient. The arrows in the tail of 3.17 indicate a cell with overlapping expression of mCherry and Venus, visible as yellow in 3.17 A. In our opinion the presence of both mCherry and Venus in one cell might be caused by Venus being expressed before the onset of the translation of Cre recombinase. When the recombination started Venus was excised from the genome and mCherry was produced instead.

These fish injected with p104 and the Cre-plasmid showed the same expression pattern as in the embryos injected with only the reporter p104. Muscle, notochord and heart and some epithelial cells showed signal. Additionally nerve cells in the ventral part of the tail were visible in the mCherry channel, indicated by the small arrows in 3.17 A and B. In Figure 3.18 A and B the expression of the notochord is very well displayed. In the close up we could see distinct edges of the cells, indicating that the expression of mCherry is really membranal. Therefore we could conclude that the Cre-reporter p104, Cre-reporter β-actin-loxP-Venus-loxP-mCherry, is expressed in muscle, notochord, epithelial and heart cells and that mCherry is membranal.

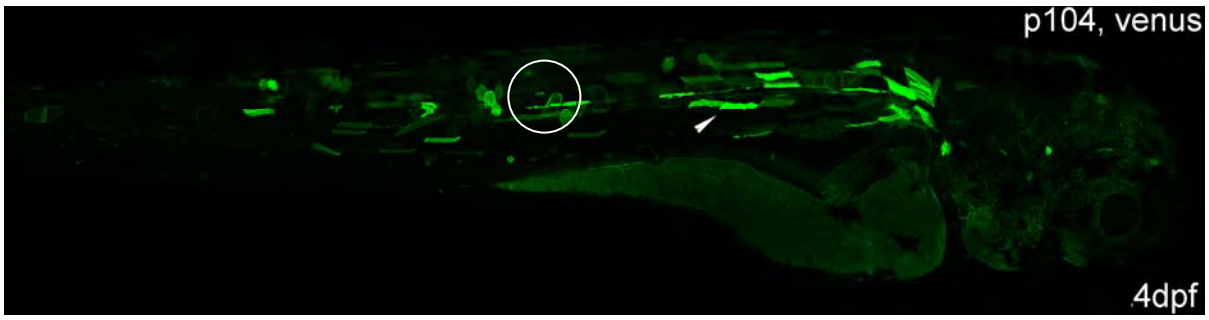


Figure 3.16. Lateral view of a 4 dpf zebrafish, showing mosaic expression of the fluorescent venus protein driven by the β–actin promoter. The image is taken in a confocal microscope (Zeiss LSM 710) in EYFP (514 nm) channel with a 10x magnification. The protein is expressed in muscle cells, of which one is indicated by the arrow and also in cells in the notochord, one is seen in the circle.

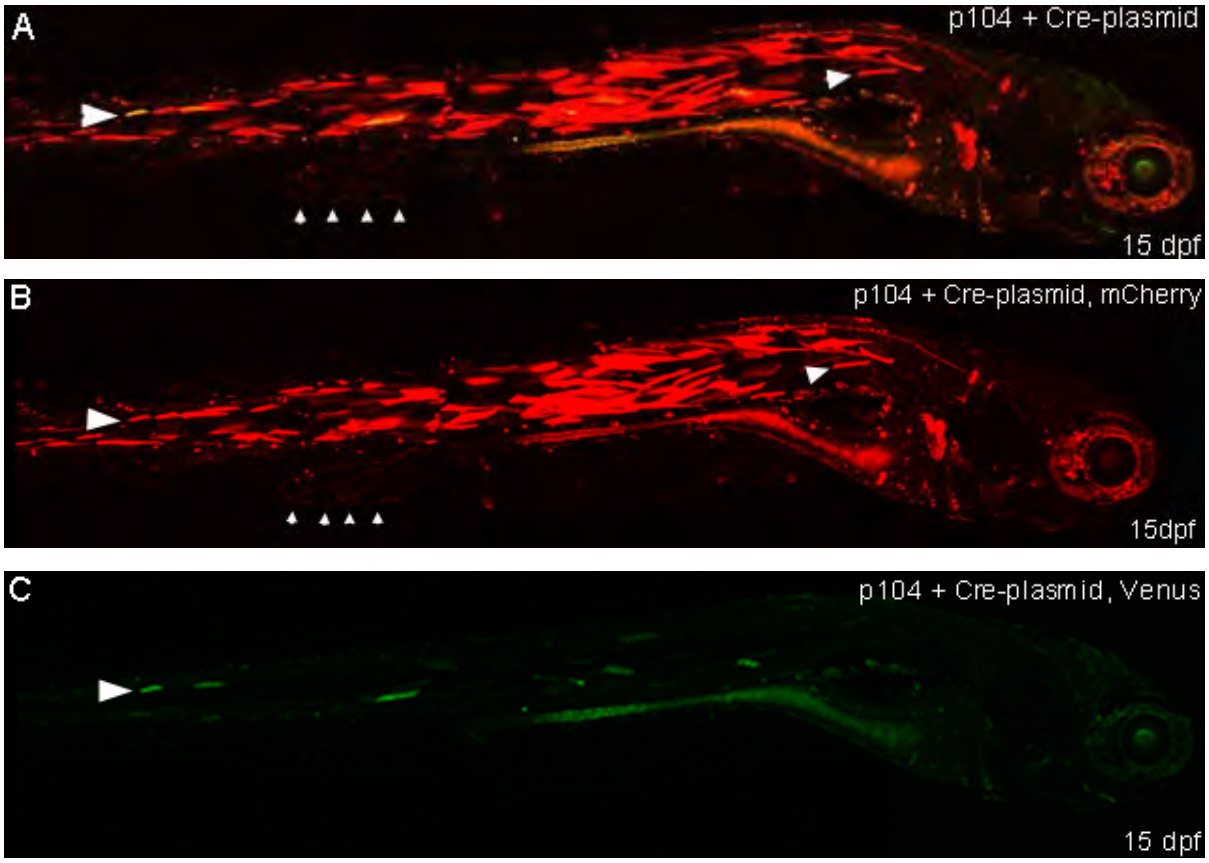


Figure 3.17. Lateral view of a 15 dpf zebrafish, showing mosaic expression of fluorescent proteins driven by the β–actin promoter. The images are taken in a confocal microscope (Zeiss LSM 710) with 10x magnification in the mCherry (587 nm) (**B**) and EYFP (514 nm) (**C**) channels. **A** was created by overlying **B** and **C**. **B** show the expression of mCherry, **C** the expression of Venus and **A** the overlapping expression of mCherry and Venus. The long cells, indicated by the bigger arrows are muscle cells. The four smaller arrows in the ventral part of the caudal fin indicate nerves. The arrows in the tail in **A**, **B** and **C** all indicate the same cell, whose expression is visible in both the mCherry and Venus channel. The cell is orange in the **A**, which is a result of overlying the colours green and red. The arrows in the front part of the fish in **A** and **B** indicate a signal that is only seen in the mCherry channel.

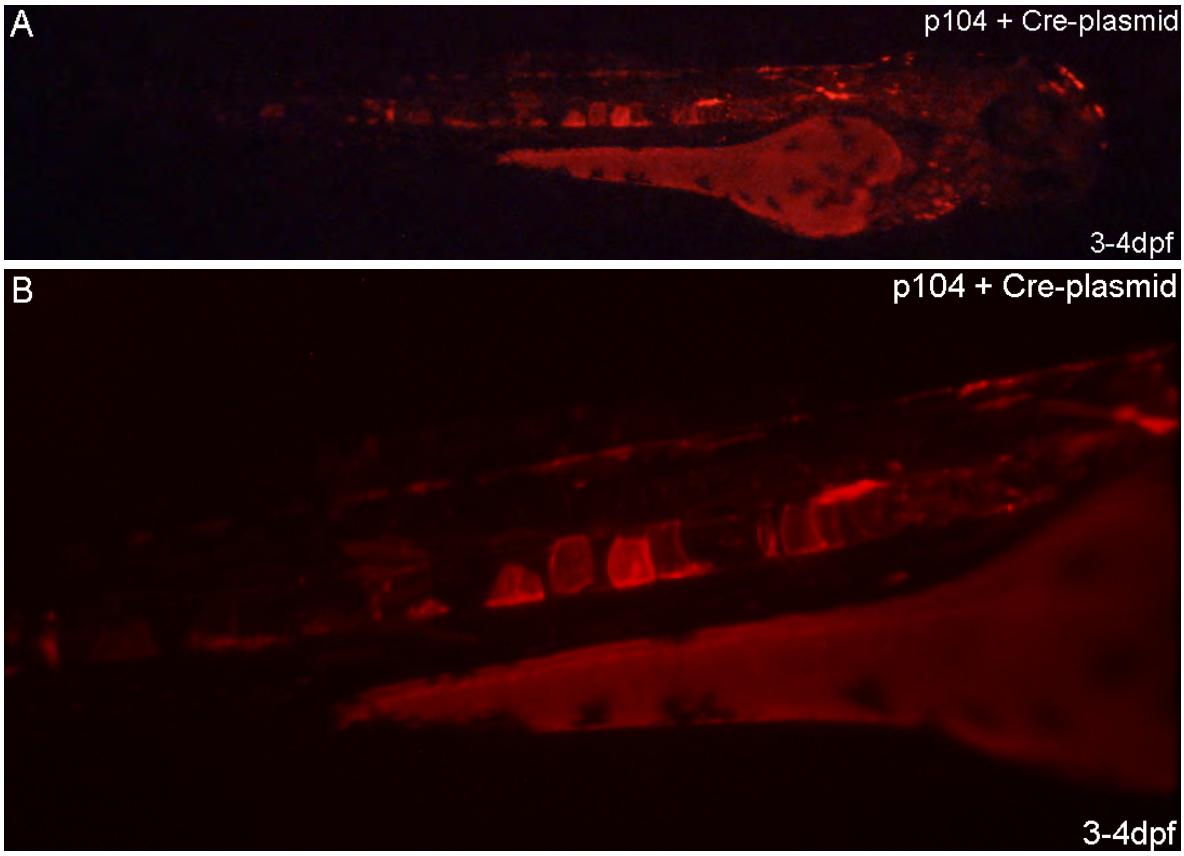


Figure 3.18. Lateral view of 3-4 dpf zebrafish, showing mosaic expression of the fluorescent protein mCherry driven by the β–actin promoter. The images are taken with a stereo microscope (Leica DFC 490). **A** shows the entire fish and **B** a close up of the cells in the notochord.

3.2.3 The ubiquitous Cre-driver is able to perform recombination on Cre-reporter p104 in muscle, notochord and epithelial cells

The construct β-actin-Cre-2A-mOrange, called pLW4, the Cre-driver under the control of the ubiquitous promoter β-actin was injected alone to test the signal of mOrange. The mOrange has no tag and is therefore expressed in cytoplasm and /or nucleus. I could detect an orange signal in muscle cells of the entire body of the embryo (3.19 and 3.20). In 3.20 B we see a close-up of the trunk muscle cells. The diffuse edges of the cells are indicated by arrows.

In order to determine if the Cre recombinase expressed by Cre-driver (β-actin-Cre-2A-mOrange) was fully functional it was coinjected with the Cre-reporter β-actin-loxP-Venus-loxP-mCherry. In the stereo microscope pictues in 3.21 we could clearly see a bright fluorescence in muscle, notochord and epithelial cells. However, it can be difficult to tell mOrange and mCherry fluorescence apart. One indication that it really is mCherry and not mOrange in 3.21 is that the signal is very bright, not at all like the mOrange signal in 3.19. To be able to distinguish between mCherry and mOrange we photographed the embryo in mCherry (3.22 B) and mOrange (3.22 C) channels in the confocal microscope. The expression of mCherry is brighter the mOrange but they seem to overlap more or less. This is in line with expectations because without the Cre-driver there cannot be any switch. In the figure, overlap of mCherry and mOrange expression in one muscle cell and four notochord cells are indicated by arrows. In the EYFP channel to detect expression of Venus (3.22 D) only one cell shows a signal. The explanation for this might be that Venus was expressed before the onset of Cre and the switch to mCherry, exactly as the overlap of Venus and mCherry in 3.17.

As mCherry is membrane-bound and mOrange is not and therefore expressed in the cytoplasm/nucleus we hoped to be able to distinguish between membranal and nuclear/cytoplasmic expression in the same cell. Images of notochord cells were taken in the mCherry and mOrange channels (Figure 3.23). The expression of mCherry in B is bright and clearly membranal. The expression of mOrange in C is very faint but has the exact same pattern as the expression of mCherry.

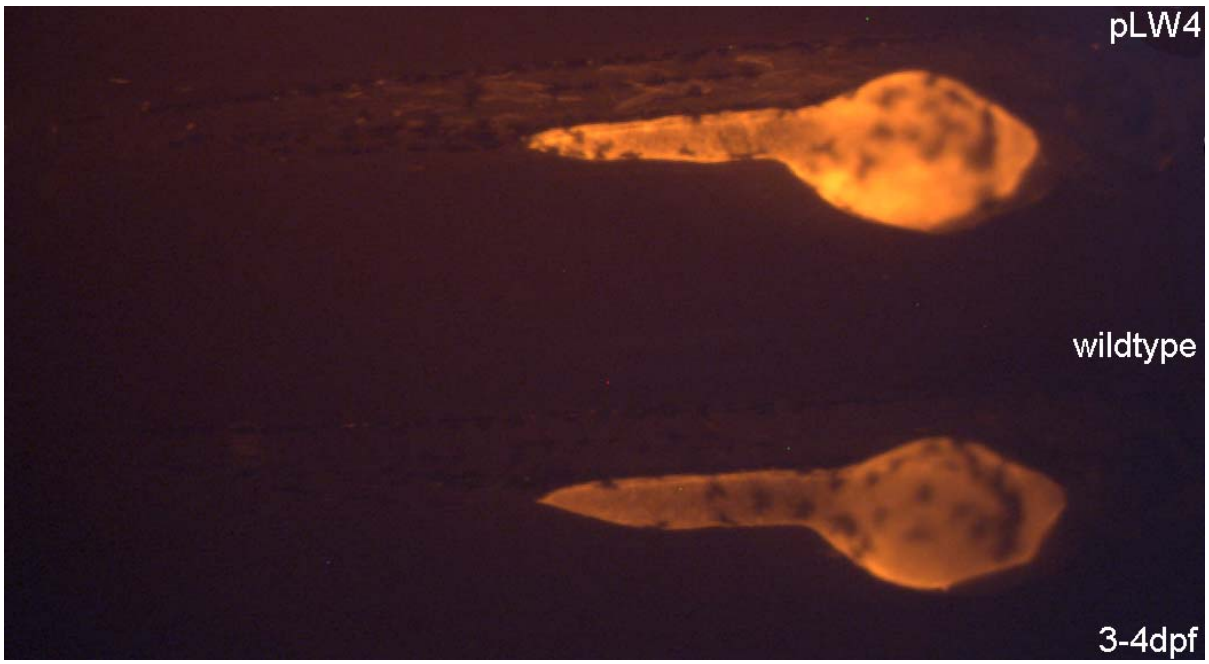
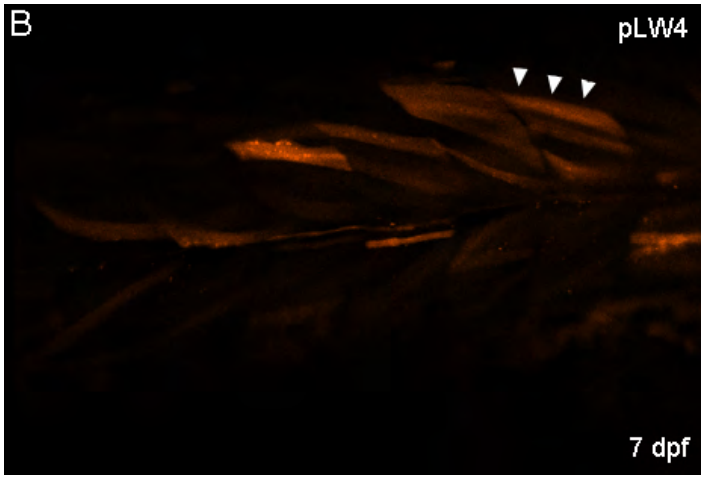


Figure 3.19. Lateral view of two 3-4 dpf zebrafish, both injected with pLW4, the β-actin driven Cre-driver. The upper embryo in the picture is positive and displays the fluorescent protein mOrange, while the lower one is negative and thus displaying the wildtype phenotype. The image was taken in a stereo microscope (Leica DFC 490).

Figure 3.20. Lateral view of a 7 dpf zebrafish, showing mosaic expression of the fluorescent protein mOrange driven by the β–actin promoter. The images were taken in a confocal microscope (Zeiss LSM 710) with 5x (**A**) and 20x (**B**) magnification in the mOrange (548 nm) channel. The large arrow in **A** indicate a muscle cell and the small arrows indicate autoflurescent cells in the caudal fin. **B** is a closeup of muscle cells in the back region. The arrows in **B** indicate the diffuse boundary of the muscle cell.



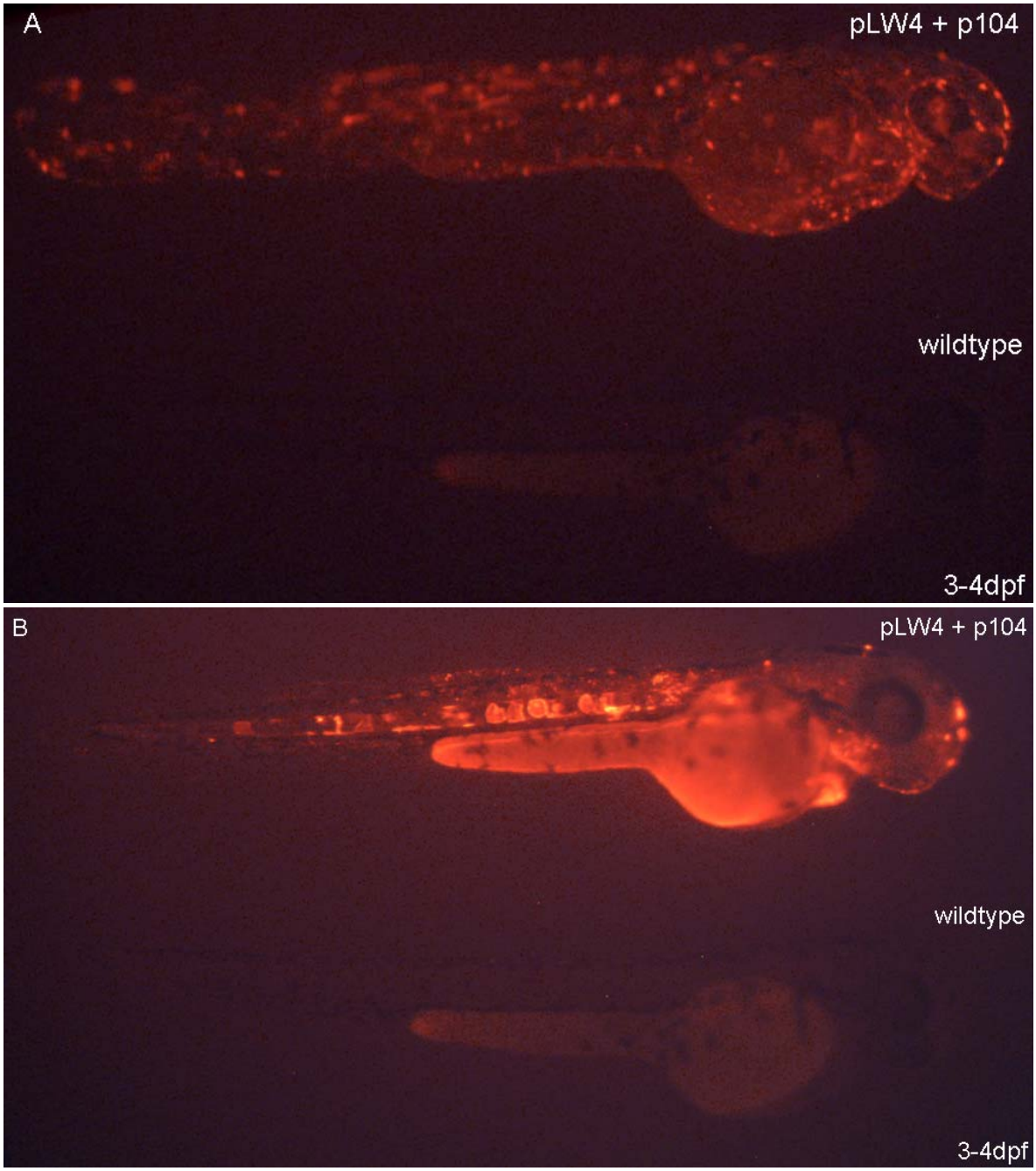


Figure 3.21. Lateral view of a 6 dpf zebrafish coinjected with the β–actin driven double fluorescent Cre-reporter and the β–actin-driven Cre-driver. The images display the difference between a positive and a negative (wildtype) phenotype. In **A** the positive fish show expression in muscle cells and other small cells, while the positive fish in **B** have almost the entire notochord labelled. The images were taken with a stereo microscope (Leica DFC 490).

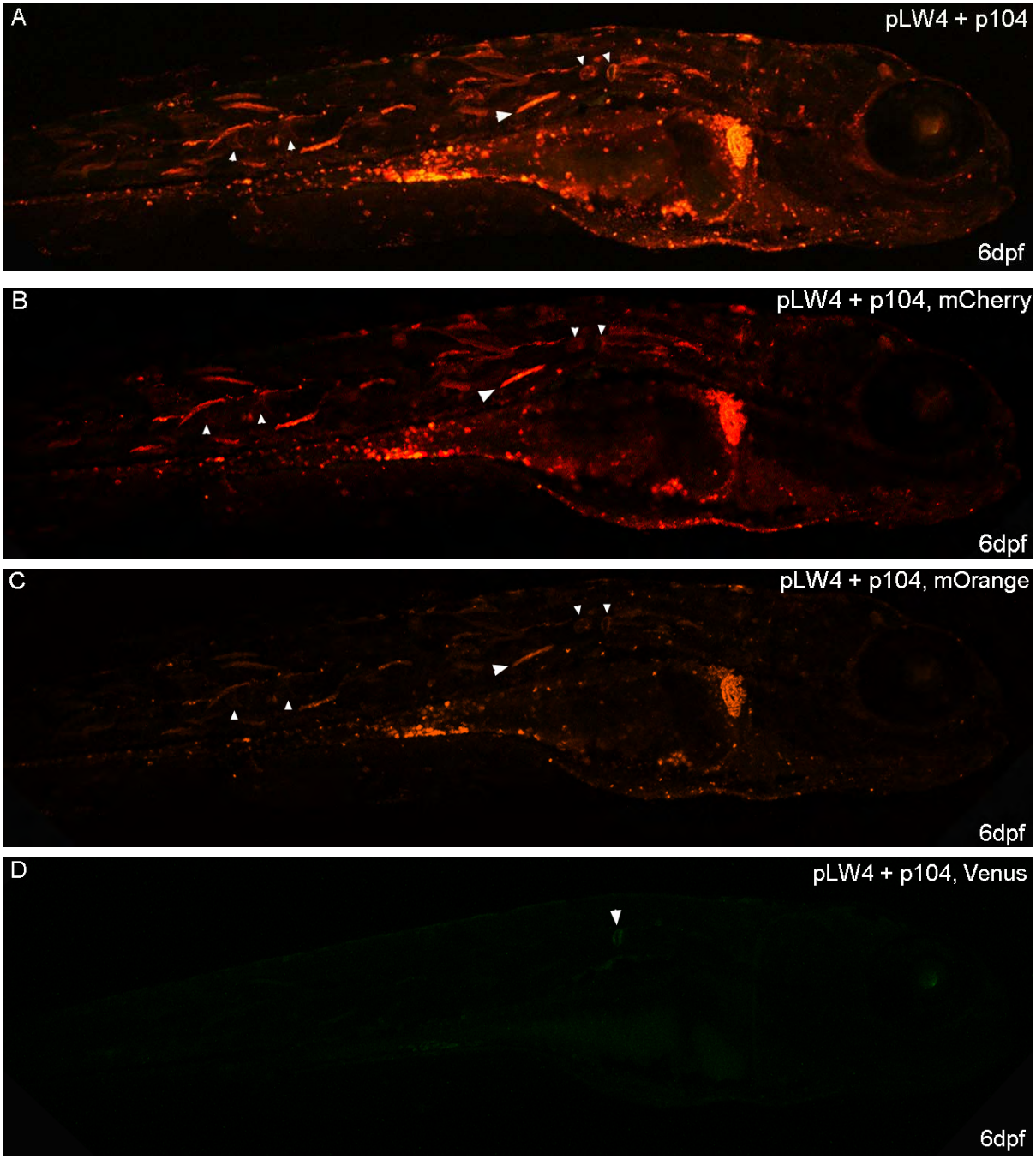


Figure 3.22. Lateral view of a 6 dpf zebrafish coinjected with the β–actin driven double fluorescent Cre-reporter and the β–actin-driven Cre-driver. The images were taken in a confocal microscope (Zeiss LSM 710) with 5x magnification in the mCherry (587 nm) (**B**), mOrange (548 nm) (**C**) and EYFP (514 nm) (**D**) channels. **A** was created by overlying **B**, **C** and **D**. **B, C** and **D** show the expression of mCherry, mOrange and venus respectively. Image **A** show the overlapping expression of mCherry, mOrange and Venus. The large arrows in **A**, **B** and **C** indicate muscle cells while the smaller arrows indicate cells in the notochord.

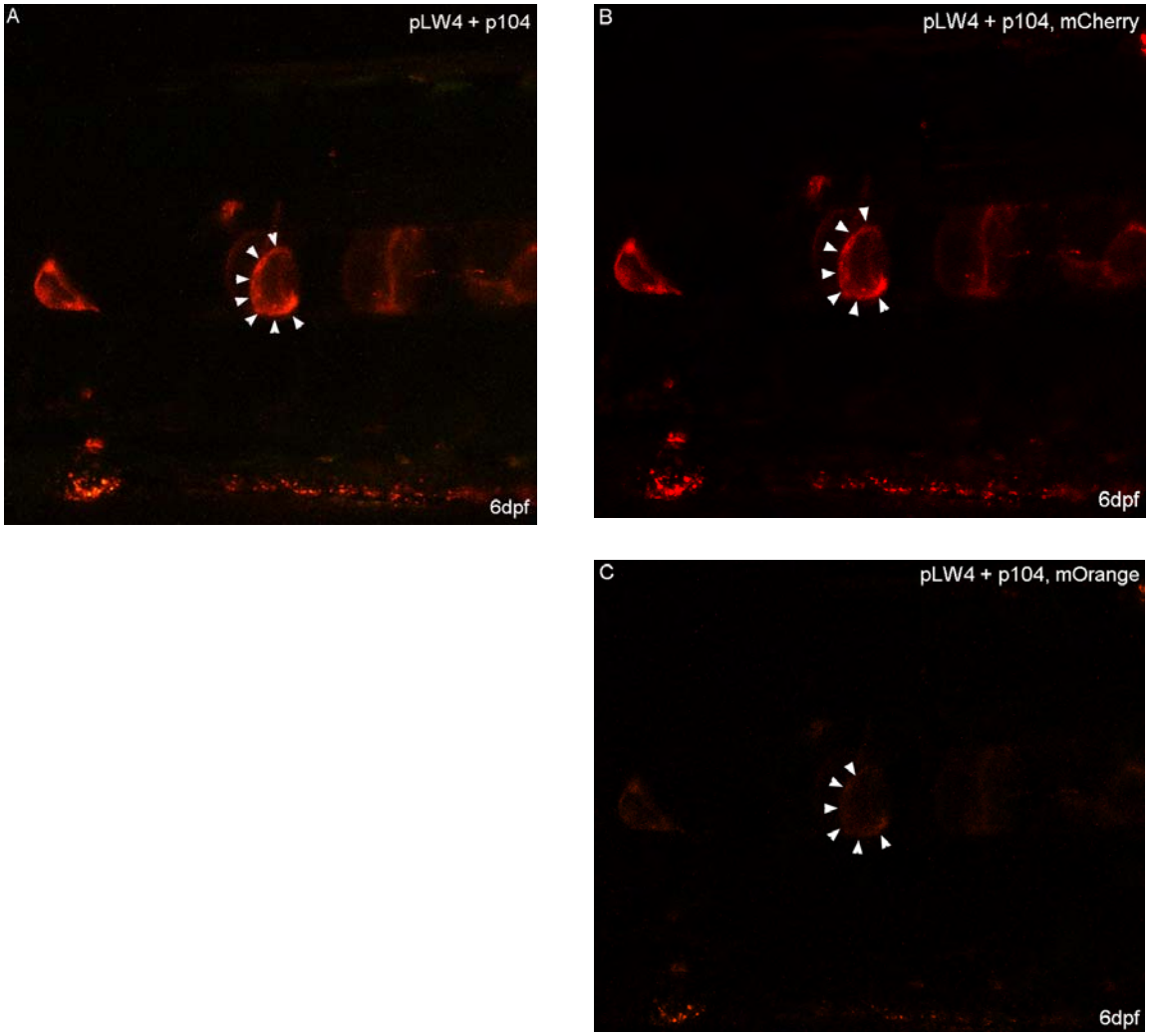


Figure 3.23. Lateral view of a 6 dpf zebrafish coinjected with the β–actin driven double fluorescent Cre-reporter and the β–actin drivenCre-driver. The images show expression of mCherry (**B**), mOrange (**C**) in notochord cells. **A** was created by overlying **B** and **C**. The images were taken in a confocal microscope (Zeiss LSM 710) with 20x magnification in the mCherry (587 nm) (**B**), mOrange (548 nm) channels. The arrows indicate the distinct membrane of a notochord cell.

3.2.4 Injection of the clcm2-specific Cre-driver gives ambiguous results

The Cre-driver under the control of the specific promoter clmc2 was injected in the yolk of 1-2 cell stage embryos. Clmc2 is only expressed in the heart cells and in Figure 3.24 A and B we can see clear mosaic expression of mOrange in the heart, indicated by the rings. The streak of fluorescence located in the tail of the fish in 3.24 A is most probably autofluorescence.

In Figure 3.25 an embryo injected with pLW5 and p104 is shown. There are signals in the mCherry, mOrange and the EYFP channels in the muscle cells of the trunk but the heart can only be detected in the mCherry and mOrange channels. Figure 3.26 shows a close-up of the heart in the mCherry, mOrange and EYFP channels. It is clear that the expression of mCherry is more extensive than the expression of mOrange in the heart cells. There is no expression of Venus in the heart cells. The only fluorescence seen in the EYFP channel seems to be the autofluorescence of the small epithelial cells lining the body. These results are discussed further in the next section Discussion.

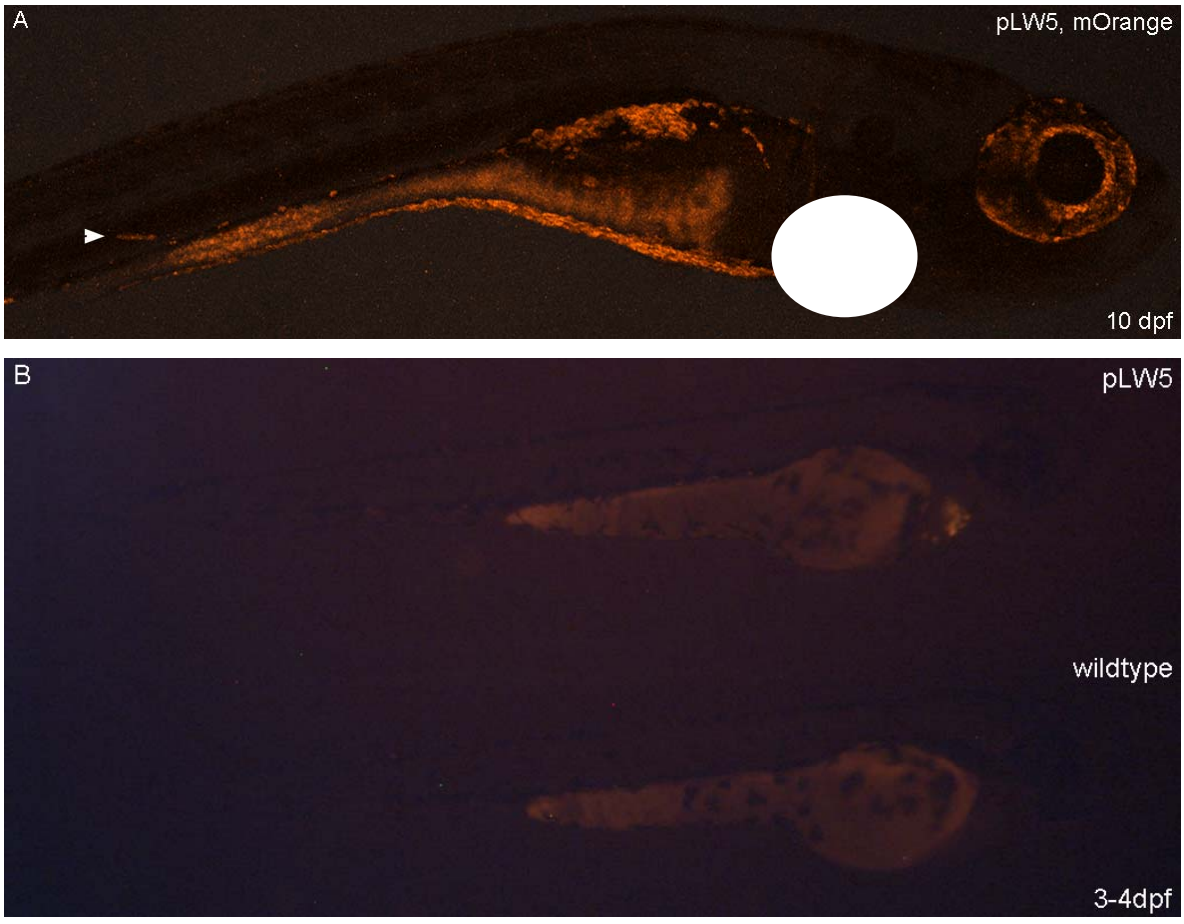


Figure 3.24. Lateral view of zebrafish showing transient expression of the fluorescent protein mOrange driven by the clmc2 promoter. **A** shows a 10 dpf embryo, displaying exclusive expression in heart cells (ellipse). The image was taken in a confocal microscope (Zeiss LSM 710) with 5x magnification in the mOrange (548 nm) channel. **B** is taken in a stereo microscope (Leica DFC 490) and shows two fish (3-4 dpf) displaying the positive and negative (wildtype) phenotype. The ellipse indicates the exclusively fluorescent heart cells in the positive embryo.

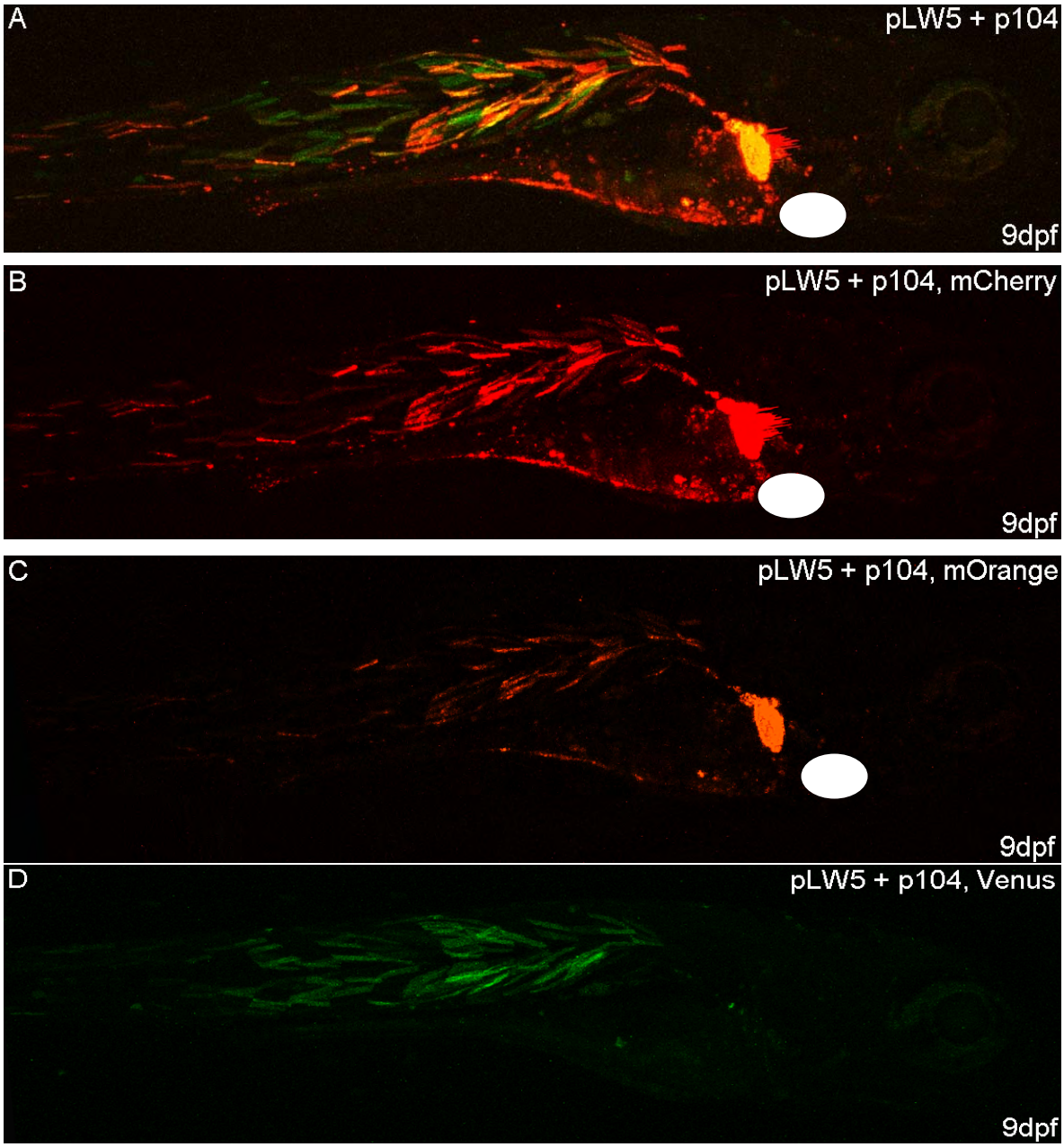


Figure 3.25. Lateral view of a 9 dpf zebrafish coinjected with the β–actin driven double fluorescent Cre-reporter and the clmc2-driven Cre-driver. **B** shows the expression of mCherry, **C** the expression of mOrange and **D** the expression of venus. **A** shows the overlapping expression of mCherry, mOrange and venus. The images were taken in a confocal microscope (Zeiss LSM 710) with 5x magnification in the mCherry (587 nm) (**B**), mOrange

(548 nm) (**C**) and EYFP (514 nm) (**D**) channels. **A** was created by overlying **B**, **C** and **D**. The fluorescent heart cells are indicated by the ellipses in **A, B** and **C**. Fluorescent muscle cells are visible in all channels.

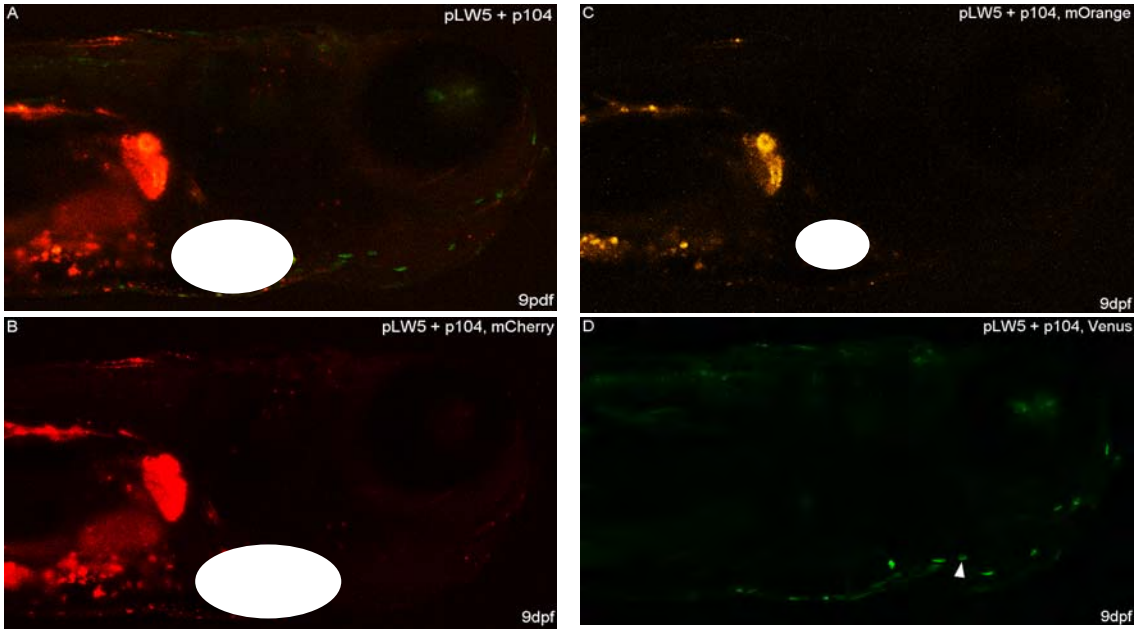


Figure 3.26. Lateral view of a 9 dpf zebrafish coinjected with the β–actin driven double fluorescent Cre-reporter and the clmc2-driven Cre-driver. **B, C** and **D** show the expression of mCherry, mOrange and venus respectively. **A** shows the overlapping expression of mCherry, mOrange and venus. The fluorescent heart cells are indicated by ellipses in **A, B** and **C**. The images were taken in a confocal microscope (Zeiss LSM 710) with 10x magnification in the mCherry (587 nm) (**B**), mOrange (548 nm) (**C**) and EYFP (147 nm) (**D**) channels. **A** was created by overlying **B**, **C** and **D**. The heart cells are indicated by the ellipses.

4 Discussion

4.1 Expression and functionality of the constructs pLW4 and pLW5

4.1.1 Clear membranal expression of Cre-reporter's Venus and mCherry

As expected we could se an overall body expression of Venus when injecting only p104. The expression pattern in the whole body resemble that described in Higashijma's report from 1997 when stable lines zebrafish expressing GFP under control of the β-actin promoter were injected. We expected a switch from green to red when coinjecting the reporter with the Cre-plasmid and this was confirmed (3.17). Some cells did however not display a switch, this probably due to the transient expression. In previous reports, where stable Cre-reporter lines were injected with heat-shock Cre-plasmids (Thummel et al., 2005 and Yoshikawa et al., 2008) the switch was complete. Had we used a stable reporter line we would have expected a complete switch in all the cells expressing the reporter.

Both Venus and mCherry have membrane tags, which make their expression confined to the cell membrane. That mCherry is membrane bound can be seen very clearly in Figure 3.18, where the notochord cells of the trunk are displayed. These cells looks almost exactly like the notochord cells of the CMV-EGFPlyn-injected embryos in Köster & Fraser (2001), where the EGFP-expression is clearly confined to the membranes of the cells. Lyn is a membrane-tag. In that article muscle cells with the same expression are also demonstrated. In the confocal images of 3.16 and 3.17 the expression of the muscle cells is homogenous over the entire cell and not confined the cell membrane as the Köster & Fraser article. Muscle cells with distinct boundaries indicating membranal expression cannot be detected in any of the confocal images displayed in this thesis. Most probably the Venus and mCherry expressions are membranal but the muscle cells are stacked on top of each other making the fluorescence too bright to distinguish any details.

4.1.2 Difficulties to distinguish between cytoplasmic/nuclear mOrange expression and membranal mCherry expression in the same cell

Since there was no membrane-tag on mOrange we expected the expression to be confined to the cytoplasm and/or nucleus. The first thing we could say when viewing the embryos injected with pLW4 under the fluorescent microscope (Figure 3.19) and the confocal (3.20 A) was that the signal was much fainter than mCherry. This seems to be despite the fact that mOrange and mCherry differ only in a few basepairs. Perhaps the expression in the cytoplasm and/or nucleus gives a weaker signal.

Cytoplasmic expression should be evenly distributed in the cell and the signal entirely homogenous. In the higher magnification image 3.20 B the individual muscle cells are clear. The pattern of the expression of these cells is quite different compared to that in the muscle cells expressing membrane-tagged EGFP in Köster & Fraser, 2001. The fluorescent protein

mOrange in Figure 3.20 B seems to be evenly distributed in the cell and there are no distinct edges of the cell, as in the Köster & Fraser article. The pattern of the mOrange expression is in the muscle cells is clearly not membranal. We can conclude that it is cytoplasmic or cytoplasmic and nuclear. Had the expression been only nuclear we would have seen a number of ellipse shaped dots in the syncytic muscle cells (Köster & Fraser, 2001).

When coinjecting the Cre-driver pLW4 with the Cre-reporter p104 we expected a Cre-mediated switch from green to red in the entire body and this was confirmed (Figure 3.21 and 3.22). We also expected mCherry and mOrange to overlap, this could also be confirmed. Thus, we can conclude that the ubiquitous Cre-driver pLW4 is able to perform Cre-mediated recombination of the Cre-reporter p104.

To be able to distinguish between membranal mCherry and cytoplasmic mOrange expression in the same cell we took a close-up image of a notochord cell in the confocal microscopy. We expected mCherry in the membrane and mOrange in the cytoplast. mCherry can clearly be seen in the membrane of the cell (3.23 B). The image (3.23 C) of the cell in the mOrange channel shows the same shape of the expression as in the mCherry channel, although much fainter. As mOrange is cytoplasmic/nuclear the detected signal must be a result of the signal from the mCherry channel leaks over to the mOrange channel. We know that there is an overlap in the wavelength ranges of mCherry and mOrange, which is probably the reason why mCherry leaks over to mOrange.

The original plan was to label the Cre-driver with untagged mCherry. To distinguish the Cre-driver mCherry from the switched reporter Cherry the idea was that Cre-driver derived mCherry would be visible in the nucleus/cytoplasm and reporter derived mCherry would be visible in the membrane. Of some reason we could not ligate Cre into the pLW2 plasmid with mCherry, which lead us to exchange mCherry with pBRU3, which contained untagged mOrange. Why the ligation with pLW2 was unsuccessful we never managed to determine the cause but the problem must have been somewhere in pLW2 and not the Cre-insert since the ligation with the other plasmid pBRU3 worked perfectly. It is unclear whether the nuclear/cytoplasmic mCherry signal would have been brighter than the mOrange signal we have detected in the confocal images. Had it been we would probably be able to distinguish the reporter and driver expression in the same cell apart.

4.1.3 Coinjection of Cre-driver pLW5 and Cre-reporter p104 leads to disruption of pLW5's expression pattern

The Cre-driver pLW5 was as expected only expressed in the heart (Figure 3.24).
The Cre-reporter p104 is ubiquitous, meaning it will be expressed in the entire body, while the Cre-driver pLW5 only will be expressed in the heart cells, therefore resulting in a switch from green to red only in the heart cells. So, the body should show a mosaic expression of Venus, while the heart should express only mCherry. There should be no expression of mCherry in the body. Contrary to expectations, there is Venus, mCherry and mOrange expression in the entire body, mostly epithelial and muscle cells (Figure 3.25). One possible explanation for how mCherry could be expressed in the body and not only heart as expected is that the reporter was recombined by the Cre-driver in the yolk before the reporter was incorporated into the genome of the body cells. We could never see any Venus expressed in the heart (Figure 3.25 and 3.26), which indicated that at least the Cre-driver was working in the heart like it was designed to do. There are many muscle cells that express Venus and apparently some Cre-reporters were incorporated into the genome intact, exactly as we hypothesised. However, the expression in the yolk does not explain why mOrange is

expressed in the body under a heart specific promoter. Probably the signal seen in the mOrange channel is mCherry leaking over. mOrange and mCherry lie very close in the wavelength range. Probably we did not manage to fin-tune the settings of the channels of the confocal microscope.

Somehow, the interaction between the Cre-driver pLW5 and the Cre-reporter p104 must have been disrupted. But we can still conclude that the Cre-driver actually did perform recombination in the tissue it was supposed to, namely the heart tissue.

4.2 Conclusions and future prospects

Firstly, we can conclude that the Cre-reporter used in the study worked on its own, the expression of the Venus promoter was clear. The reporter's ability to recombine was also clear when it was coinjected with a Cre-plasmid and the switch from green to red could be demonstrated (Figure 3.17). Expression driven by the β-actin promoter was detected in muscle, notochord, epithelial and nerve cells. mCherry is membrane-bound and this was very neatly shown in Figure 3.18.

Secondly, we can conclude that both the ubiquitous β-actin-driven Cre-driver was incorporated into the genome in cells expressing β-actin as muscle, epithelial and notochord cells (Figure 3.19-3.23). The expression was shown not to be membranal but rather cytoplasmic/nuclear or cytoplasmic as expected since mOrange is not membrane-bound (3.20 B). We could determine that mOrange was expressed in the cytoplasm. The Cre-activity of the driver was proven to be efficient (3.22). The Figure 3.23 showing an overlap of the mCherry and mOrange signal in the membrane, where mOrange should not possibly be expressed implied that the mCherry signal actually leaked into the mOrange range, making it impossible to fully distinguish between an mCherry and mOrange expression. This of course disturbed our interpretations. We need to be sure that the mOrange expression is always followed by a mCherry expression in the same cell.

The last round of injections were the most important as it tells us about the Cre-driver's capability of using in genetic lineage labelling. When performing genetic lineage labelling you want to distinguish just one cell population from all others, therefore only that cell population must give a specific signal or switch. So, we injected the ubiquitous Cre-reporter with the heart-specific Cre-driver, and expected a Cre-recombination and switch to red solely in the heart cells. This was however not the result. mCherry was showing not only in the heart cells but also in muscle and epithelial cells of the entire body. Since the Cre-driver was specific it could not have been incorporated into the genome of the muscle cells, hence the Cre-mediated recombination must have taken place before the Cre-reporter was incorporated into the genome of the muscle cells. Probably the recombination took place in the yolk when the two plasmids were injected. All in all we can conclude that studying the expression and function of the Cre-reporter in transient transgenic zebrafish is not ideal.

The ideal set-up of the experiment would be to cross a stable Cre-reporter line with a stable Cre-driver line, preferably one driven with a specific promoter as clmc2. In order to produce a stable line the transient transgenic zebrafish (F0) must be raised to adulthood and crossed with the wildtype and the offspring (F1) will be screened for positives, which all carry the transgene in the germline. Then these stable transgenic zebrafish can be crossed with other lines to perform lineage labelling in next generation (F2). Stable lines could not be generated during the 6-month project frame as the generation time of Zebrafish is 3 months. The ideal

set-up for this experiment would be to generate stable p104, pLW4 and pLW5 lines and then cross p104 with the two driver lines respectively as done in Hans et al., 2009. This kind of experiment would generate results that would be unambiguous and tell us if the middle entry clone would be suitable for constructing Cre-drivers and perform genetic lineage labelling.

Zebrafish injected with the Cre-reporter p104 are being raised right now for further interbreeding. Zebrafish injected with the Cre-driver pLW4 are also being raised at the moment but unfortunately the ones injected with pLW5 did not survive the confocal imaging.

Many things could have been done differently in order to make the results of this experiment easier to interpret. For example following the fish frequently the first 48 hours would give us information about the onset of expression of the different constructs. The wavelength ranges mOrange and mCherry overlapped leading to a very crude separation between the two. If we had been able to really fine-tune the channels of the confocal microscope maybe we could have been able to tell them apart. Another way of solving that problem would have been choosing another flurophore for the driver. Maybe some green or cyan fluorophore would have been easier to separate from mCherry.

The purpose of this study was to develop a tool, a middle entry clone that can be used for genetic lineage labelling. We tested the constructs to determine if they met our requirements and if they were suitable for further investigation and development for lineage labelling. All in all, we could conclude that they met our requirements. The results were somewhat ambiguous, which can only be expected from a sub-optimal set-up of the experiment. The design of the experiment was suited for the time frame of the project and the test could only give preliminary results. However, the results gave strong indications that the constructs actually worked. Firstly the Cre-drivers could label the tissues according to the promoters used. Secondly the Cre-activity was confirmed, even though the test of the specific Cre-driver were quite puzzling it was probably just a consequence of double transient transgenic expression. Therefore, to achieve an unambiguous result, we need to cross the stable lines. Generation and testing the middle entry clone pLW3 was just the first step to create constructs used for genetic lineage labelling on the second pharyngeal arch.

In the future pLW3 will be recombined with second pharyngeal arch specific enhancer and lines with stable second pharyngeal arch labelling will be raised. Thereby, this tool will hopefully reveal information about the regulation and development of the hyoid arch in zebrafish at a resolution we have not been able to achieve in previous studies.

5 Acknowledgements

I am most grateful to my supervisor Bettina Ryll who decided to take me on for this project. I would like to thank Bettina for providing me with such an interesting and sometimes very challenging project and for guiding me through the hard bits! Also, I would like to thank you, Bettina for introducing me to developmental biology and I must say, your enthusiasm for the subject is really contagious! I would like to show my appreciation to Tatjana Haitina, who has taught me so many things in the lab; Gateway cloning and injecting transgenes among other things. Moreover I would like to thank you for discussing results with me and giving me constructive critique during the writing process. Your help has been completely indispensable, thank you so much! I would like to thank Judith Habicher for helping me with injections and confocal microscopy. I am grateful to all working in the wet lab facility and for helping me out during the course of this project. Thank you Johan Ledin, Katarina Holmborn Garpenstrand, Beata Filipek Gorniok and Ralf Janssen!

References

Branda CS & Dymecki SM (2004). Talking about a Revolution: The Impact of Site-Specific Recombinases on Genetic Analyses in Mice. Developmental Cell 6: 7-28.

Cheo DL, Titus SA, Byrd DRN, Hartley JL, Temple GF, Brasch MA (2004). Concerted Assembly and Cloning of Multiple DNA Segments Using In Vitro Site-Specific Recombination: Functional Analysis of Multi-Segment Expressio Clones. Genome Research 14: 2111-2120.

Davidson AE, Balciunas D, Mohn D, Shaffer J, Hermanson S, Sivasubbu S, Cliff MP, Hackett PB, Ekker SC (2003). Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon. Developmental Biology 236:191-202.

Donnelly MLL, Luke G, Mehrotra A, Li X, Hughes LE, Gani D, Ryan MD (2001). Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. Journal of General Virology 82: 1013-1025.

Dymecki SM, Rodriguez CI, Awatramani RB (2002). Switching on Lineage Tracers Using Site-Specific Recombination. Turksen K (ed.). Methods in Molecular Biology, vol. 185: Embryonic Stem Cells:Methods and Protocols. Human Press, Totowa, NJ.

Dymecki S & Tomasiewicz H (1998). Using Flp-recombinase to characterize expansion of Wnt1 expressing neural progenitors in mouse. Developmental Biology 201: 57-65.

Fadool JM, Hartl DL, Dowling JE (1998). Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish. PNAS 95: 5182-5186.
Feng et al., 2007

Fisher S, Grice EA, Vinton RM, bessling SL, Urasaki A, Kawakami K, McCallion AS (2006). Evaluation the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. Nature Protocols 1(3): 1297-1305.

Hans S, Kaslin J, Freudenreich D, Brand M. (2009). Temporally-Controlled Site-Specific Recombination in Zebrafish. PLoS ONE 4(2): 1-7.

Hartley JL, Temple GF, Brasch MA (2000). DNA Cloning Using In Vitro Site-Specific Recombination. Genome Research 10:1788-1795.

Higasijma S, Okamoto H, Ueno N, Hotta Y, Eguchi G (1997). High-frequency Generation of Transgenic Zebrfish Which Reliably Express GFP in Whole Muscles ir the Whole Body by Using Promoters of Zebrafish Origin. Developmental Biology 182: 289-299.

Hoess RH, Ziese M, Sternberg N (1982). P1 site-specific recombination:nucleotide sequence of the recombination site. PNAS 79: 3398-3402.

Hoess RH, Wierzbiki A, Abremski K (1985). Formation of small circular DNA molecules via an in vitro site-specific recombination system. Gene 40: 325-329.

Hoess RH, Wierzbiki A, Abremski K (1986). The role of the loxP spacer region in P1 site-specific recombination. Nucleic Acids Research 14: 2287-2300.

Hsiao EC, Yoshinaga Y, Nguyen TD, Musone SL, Kim JE, Swinton P, Espineda I, Manalac C, deJong PJ, Conklin BR (2008). Marking Embryonic Stem Cells with a 2A Self-Cleaving Peptide: A NKX2-5 Emerald GFP BAC Reporter. PLoS ONE 3(7): e2532.

Hunter MP & Prince VE (2002). Zebrafish Hox Paralogue Group 2 Genes Function Redundantly as Selecor Genes to Pattern the Second Pharyngeal Arch. Developmental Biology 247: 367-389.

Ivics Z, Hackett PB, Plasterk RH, Izsvák Z (1997). Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell 91: 501-510.

Joplin C, Sleep E, Raya M, Marti M, Raya A, Belmonte JCI (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and profileration. Nature 464: 606-609.

Kawakami K, Koga A, Hori H, Shima A (1998). Excision of the Tol2 transposable element of the medaka fish, *Oryzias latipes*, in zebrafish, *Danio rerio*. Gene 225: 17-22.

Kawakami K & Noda T (2004). Transposition of the Tol2 element, an Ac-like element from Japanese medaka fish *Oryzias latipes*, in mouse embryonic stem cells. Genetics: 166: 895-899.

Kawakami K & Shima A (1999). Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous Tol2 element in zebrafish *Danio rerio*. Gene 240: 239-244.

Koga A, Suzuki M, Inagaki H, Bessho Y, Hori H (1996). Transposable elemnt in fish. Nature 383: 30.

Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS (2007). The Tol2kit: A Multisite Gateway-Based Construction Kit for Tol2 Transposon Transgenesis Constructs. Developmental Dynamics 236: 3088-3099.

Köster RW & Fraser SE (2001). Tracing Transgene Expression in Living Zebrafish Embryos. Developmental Biology 233: 329-346.

Landy A (1989). Dynamic, structural, and regulatory aspects of λ site-specific recombination. Ann. Rev. Biochem. 58: 913-949.

Langenau DM, Feng H, Berghmans S, Kanki JP, Kutok JL, Look AT (2005). Cre/lox-regulated transgenic zebrafish model with conditional myc-induced T cell acute lymphoblastic leukemia. PNAS 102: 6068-6073.

Le X, Langenau DM, Keefe MD, Kutok JL, Neuberg DS, Zon LI (2007). Heat shock-inducible Cre/lox approaches to induce diverse types of tumors and hyperplasia in transgenic zebrafish. PNAS 204: 9410-9415.

Lin S, Gaiano N, Culp P, Burns JC, Friedmann T, Yee JK, Hopkins N (1994). Integration and germ-line transmission of pseudotyped retroviral vector in zebrafish. Science 265: 666-669.

Pan X, Wan H, Chia W, Tong Y, Gong Z (2005). Demonstration of site-directed recombination in transgenic zebrafish using the Cre/loxP system. Transgenic Research 14: 217-223.

Raz E, van Luenen HG, Schaerringer B, Plasterk RH, Driever W (1998). Transposition of the nematode *Caenorhabditis elegans* Tc3 element in the zebrafish *Danio rerio*. Current Biology 8: 82-88.

Shaner NC, Steinbach PA, Tsien RY (2005). A guide to choosing fluorescent proteins. Nature Methods 2(12): 905-909.

Stuart GW, McMurray JV, Westerfield M (1988). Relication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103: 403-412.

Suster ML, Kikuta H, Urasaki A, Asakawa K, Kawakami K (2009). Transgenesis in Zebrafish with the Tol2 Transposon System. Cartwright EJ (ed.). Transgenesis Techniques, Methods in Molecular Biology, vol. 561: 41-63. Human Press.

Thummel R, Burket CT, Brewer JL, Sarras Jr MP, Li L, Perry M, McDermott JP, Sauer B, Hyde DR, Godwin AR (2005). Cre-Mediated Site-Specific Recombination in Zebrafish Embryos. Developmental Dynamics 233: 1366-1377.

Yoshikawa S, Kawakami K, Zhao XC (2008). G2R Cre Reporter Transgenic Zebrafish. Developmental Dynamics 237: 2460-2465.

Zinyk D, Mercer EH, Harris E, Andersson DJ, Joyner AL (1998). Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Current Biology 8: 665-668.