CLASS RESTRICTION IN ODORANT RECEPTOR GENE REGULATION

by

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To my dear sister…

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ABSTRACT

CLASS RESTRICTION IN ODORANT RECEPTOR GENE REGULATION

Odorant receptors which partake in the detection of both water-soluble and airborne chemicals constitute the largest family of seven-transmembrane G-Protein-Coupled Receptors in the majority of vertebrates. Since their identification as GPCR proteins, the mechanism of gene regulation in ORs has been extensively studied. It has been shown that ORs in mammals are divided into two major classes, Class I and Class II, and this phenomenon is believed to play an important restrictive role in various aspects of OR gene expression, one of which is the second choice mechanism by which olfactory sensory neurons switch to an alternate OR in case their first attempt fails at the expression of a functional OR. Zebrafish OR repertoire consists of 136 intact OR genes, including only a single Class II OR. The relatively small size of the OR repertoire and the presence of a single Class II gene, OR101-1, makes the zebrafish species an ideal model organism to study class restriction.

In order to ascertain its nature as a Class II OR, the phylogenetic background of OR101-1 was analyzed by the construction of two different phylogenetic trees which include zebrafish and human OR gene repertoires. OR101-1 was shown to be much more closely related to human ORs. Olfactory epithelium-specific expression of OR101-1 was shown through RT-PCR analysis of cDNA from 8 different zebrafish tissues. Early developmental expression patterns of OR101-1 was assessed by RT-PCR, qRT-PCR of embryonic cDNA, and expression of OR101-1 as early as 24 hours post-fertilization was observed. In situ hybridization studies using OR101-1-specific antisense RNA riboprobes also corroborated our previous findings. Finally, two transgenic BAC constructs that would allow the visualization of OR101-1 expression were designed and partially cloned: an OR101-1-IRES-Venus tag construct and a YFP deletion construct replacing the OR101-1 coding sequence to study the second choice mechanism in OR101-1 expressing OSNs.

ÖZET

KOKU RESEPTÖRÜ GEN REGÜLASYONUNDA SINIF KISITLAMASI

Suda ve havada bulunan kimyasalların algılanmasında rol alan koku reseptörleri çoğu omurgalıda yedi-transmembran G protein-kenetli reseptörlerinin en büyük grubunu oluştururlar. GPKR olarak tanımlanmalarından beri KR'nin gen regülasyonu detaylı olarak çalışılmıştır. KR'nin memelilerde iki ana sınıfa, Sınıf I ve Sınıf II, ayrıldığı gösterilmiştir ve bu olgunun KR gen ekspresyonunun çeşitli aşamalarında kısıtlayıcı bir rol oynadığına inanılmaktadır. Bu aşamalardan biri, koku alıcı nöronların ilk denemelerinde fonksiyonel bir KR ifade edememeleri durumunda alternatif bir KR seçtikleri ikinci seçim mekanizmasıdır. Zebrafish KR repertuarı tek bir adet Sınıf I KR (OR101-1) dahil olmak üzere 136 fonksiyonel KR geninden oluşmaktadır. KR repertuarının nispeten küçük boyutu, ve sadece tek bir Sınıf II KR'nün varlığı, zebrafish türünü sınıf kısıtlamasını çalışmak için ideal kılmaktadır.

Soyunun Sınıf II'ye dayandığını kanıtlamak için OR101-1'in filogenetik geçmişi, insane ve zebrafish gen repertuarlarını içeren iki adet filogenetik ağaç hazırlanarak analiz edildi. OR101-1'in insan KR'lerine çok daha yakın olduğu gözlemlendi. 8 değişik zebrafish dokusuna RT-PCR analizi yapılarak, OR101-1'in koku epiteline spesifik ekspresyonu gösterildi. OR101-1'in erken gelişme dönemindeki ekspresyon motifi embryonik cDNA üzerine yapılan RT-PCR ve qRT-PCR aracığıyla gösterildi. 24 saat post-fertilizasyon kadar erken bir safhada OR101-1 ekspresyonu gözlemlendi. OR101-1'e spesifik antisens RNA riboprobları kullanılarak yapılan in situ hibridizasyon çalışmaları da önceki bulgularımızı destekledi. Son olarak, OR101-1 ekspresyonunu gözlememizi sağlayacak iki transgenik konstrakt tasarlandı ve kısmen klonlandı: OR101-1-IRES-Venus işaretleme konstraktı ve OR101-1 ekspres eden koku nöronlarında ikinci seçim mekanizmasını çalışmamıza olanak sağlayacak, OR101-1 kodlayıcı sekansının yerini YFP (sarı flöresan protein) sekansı ile değiştiren, YFP delesyon konstraktı.

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LIST OF SYMBOLS / ABBREVIATIONS

β2-AR	β2 adrenergic receptor
A1R	Adenosine A1 receptor
BAC	Bacterial artificial chromosome
bp	Base pairs
$CaCl_2$	Calcium chloride
cAMP	Cyclic AMP
cDNA	Complementary DNA
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FISH	Fluorescent in situ hybridization
GPCR	G protein-coupled receptor
HB	Hybridization mix
Ig	Immunoglobulin
ISH	In situ hybridization
Kan	Kanamycin
LiCl	Lithium chloride
NaOAc	Sodium acetate
O-MACS	Olfactory specific medium-chain acyl CoA synthetase
OB	Olfactory bulb
OCAM	Olfactory cell adhesion molecule
OE	Olfactory epithelium
OR	Olfactory receptor
OSN	Olfactory sensory neuron
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Real-time reverse-transcription polymerase chain reaction
RecA	Recombinase A
RNA	Ribonucleic acid

RT-PCR	Reverse-transcription polymerase chain reaction
zOMP	Zebrafish olfactory marker protein

1. INTRODUCTION

1.1. Chemo-sensing

Chemo-sensing is among one of the first sensory characteristics evolutionarily developed by organisms, since it goes hand-in-hand with the most important aspects of life and survival: finding food and avoiding danger. Therefore, nearly every living organism has an intrinsic system dedicated to the detection of chemicals present in their surroundings such as chemotaxis in bacteria (Adler, 1966) and chemotropism in plants (Filippenko, 2001). In higher organisms in possession of an established nervous system, this mechanism has evolved into an intricate olfactory system, able to detect, characterize and differentiate among diverse olfactory cues constituted of an extremely wide range of diverse chemicals, or their combinations (Buck, 2000, 2005).

1.2. Olfaction Anatomy and Physiology

In vertebrates, the detection of odorants is achieved by the presence of olfactory sensory neurons (OSN) that are located in a specialized sensory epithelium. These OSNs express a large family of specific seven transmembrane G-protein coupled receptors (GPCRs), which are proteins that detect the presence of specific odorants (Buck and Axel, 1991). The genome of higher vertebrates can contain up to 1.500 different genes coding for these olfactory receptor (OR) proteins (Niimura and Nei., 2007), underlining the importance of the sense of smell as well as the inherent challenges of designing a sensory system that is capable of detecting the wide structural diversity of the different stimuli that the organism meets in its everyday environment. Interestingly, every OSN seems to express only a single member of the large repertoire of ORs. It has been shown that the structural design of the olfactory bulb (OB), the first relay in the signaling pathway from the nose to the brain, proves to be of high importance for the physiological detection and classification of this multitude of odorants with utmost precision (Mori *et al.*, 1999). Generally, the activation of different sets of ORs that respond to a certain odorant or a combination of odorants is transformed into a neuronal pattern of activity in the brain, which then is interpreted by the brain as a distinctive perception of smell for the respective

stimulus. OSN expressing a given OR are scattered in the olfactory epithelium (OE), yet their axons converge into individual anatomical structures called glomeruli, that are arranged in a topographical manner on the surface of the OB (Figure 1.1; Shepherd and Greer, 1998). In turn, each individual glomerulus only receives innervation from OSNs expressing the same OR, thus the variety of OSNs expressing all of the different ORs is mapped onto an anatomical map of glomeruli in the OB. Accordingly, the physiological activation of specific OSN subpopulations (defined by their OR expression) by a given odorant translates into a distinct activity pattern of glomeruli at the level of the OB. OR expression is not randomly scattered across the entire extent of the OE but is rather defined to distinct expression domains called zones, which are arranged dorsal to ventral in the OE (Ressler *et al*., 1993; Vassar *et al*., 1993; Miyamichi *et al*., 2005). OSNs from the dorsal OE establish axonal connections with dorsal glomeruli and ventral OSNs with ventral glomeruli, respectively (Mori *et al*., 1999).

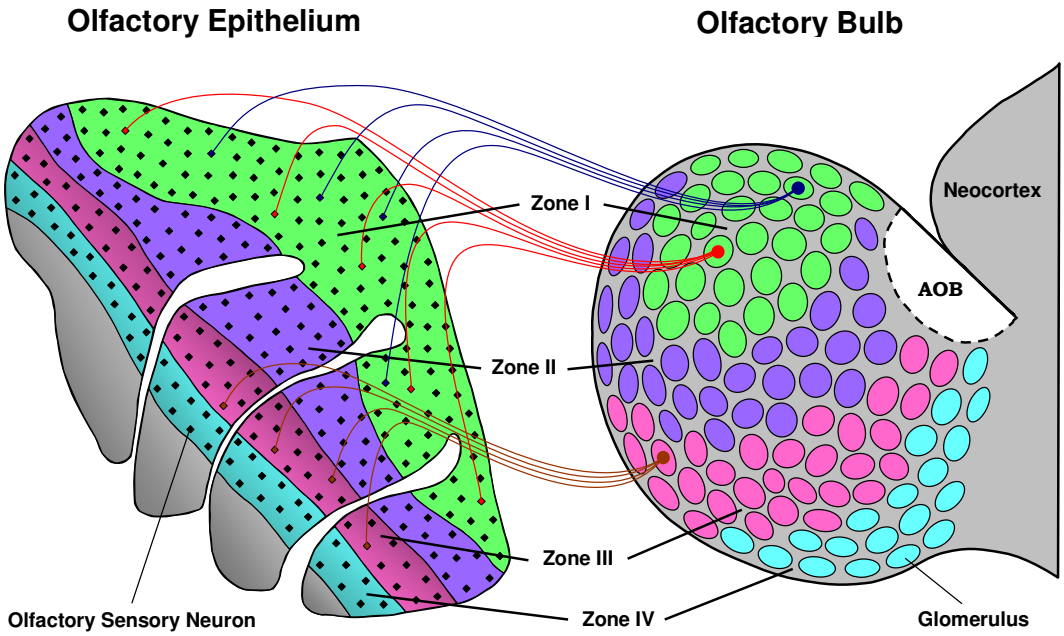


Figure 1.1. Mouse olfactory anatomy. OSNs (black diamonds) situated in the olfactory epithelium of the nose project their axons toward the olfactory bulb. OSNs that express the same OR (shown by different colors) converge onto spherical formations called glomeruli that are topographically arranged on the surface of the OB. Thus, individual glomeruli represent specific ORs at the level of the OB. A zone-to-zone correlation between the OE and the OB can be observed. OSNs expressing a given OR are arranged in four or more narrow strips or expression zones and do not cover the entire extent of the OE. OSNs situated in the dorsal OE project to dorsal glomeruli and ventral OSNs to ventral glomeruli, respectively (derived from Mori *et al.*, 1999; Fuss and Ray, 2009).

OR proteins interact with the odorant molecules that are inhaled into the nose at the molecular level. Upon excitation of an OR by an odorant ligand, the GPCR activates a heterotrimeric $G_{olf\alpha/\beta/\gamma}$ complex that includes the olfactory specific subunit, $G_{olf\alpha}$ (Jones and Reed, 1989). The $G_{olf\alpha}$ subunit, in turn, stimulates the olfactory specific adenyly cyclase type III (ACIII), thereby increasing the level of cytosolic cAMP by converting ATP into cAMP. The elevated amount of cAMP in the neuron then opens a cyclic-nucleotide gated cation channel which allows Na^+ and Ca^{2+} ions to enter the cells, which results in an initial depolarization of the OSN cilia membrane (Dhallan *et al.*, 1990; Nakamura and Gold,

1987). Ca^{2+} entry into the OSN then opens a Ca^{2+}-sensitive Cl^- channel that mediates an outflux of Cl^- ions, which depolarizes the OSN even further and which is strong enough to propagate to the trigger zone of the OSN axon (Nakamura and Gold, 1987). Thus, binding of odorants to ORs is transformed into a membrane action potential of the OSNs (Figure 1.2). The produced signal is propagated to the OB where the activation of specific OSNs that are characterized by the OR that they express is transformed into the activity of glomeruli that are specific for those OSNs. In the glomeruli, OSN axons form synapses with local interneurons and higher order output neurons, so called mitral cells and tufted cells (Mori *et al.*, 1999). Mitral cells convey olfactory signals to cortical areas that are involved in olfactory processing, such as the primary olfactory cortex, the enthorhinal cortex, the amygdala and others, while tufted cells primarily establish intrabulbar and interbulbar connections (Zou *et al.*, 2001).

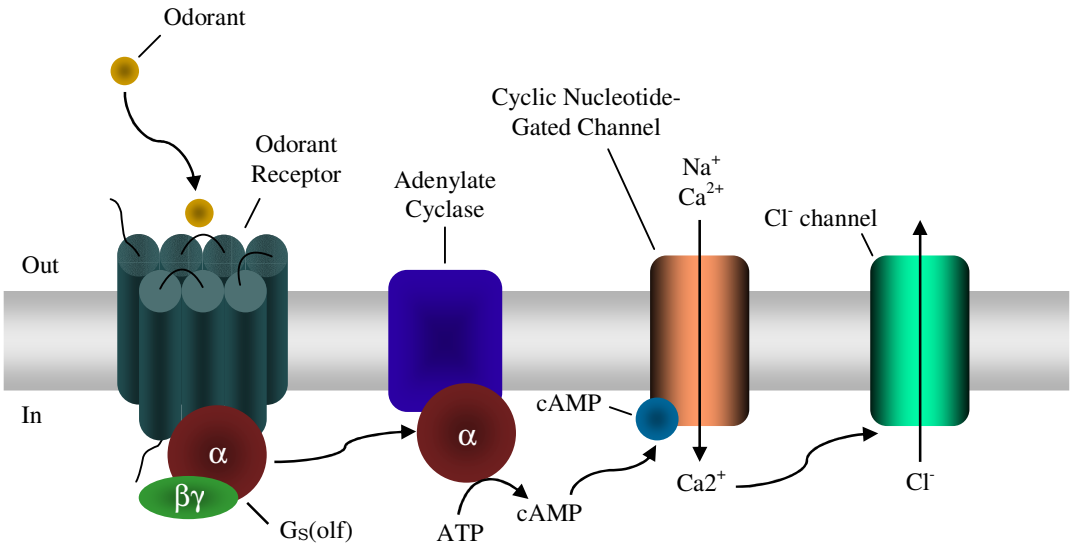


Figure 1.2. Olfactory signal transduction pathway. Stimulation of an OR protein by an odorant results in a G-protein mediated increase of cAMP concentrations which in turn causes Na^+/Ca^{2+} channels to open and to initially depolarize the neuron. Ca^{2+} entry opens a Cl^- channel that mediates a Cl^- outflux, which further depolarizes the cell and generates the receptor potential of OSNs (Derived from Buck and Axel, 1991).

1.3. Olfactory Receptors

The ORs are classified under the rhodopsin-like A family of seven-transmembrane GPCRs, and constitute the largest known multigene superfamily in almost any vertebrate species. One impressive example of the size of the repertoire can be seen in the mouse, which possesses ~1300 genes (of which 3% are pseudogenes; Zhang and Firestein, 2002), which makes up about 3% of all the genes in the mouse genome. The size of the OR repertoire, however, differs largely across species and it can range from some 100 genes in fish to more than 2000 genes in cows (Niimura and Nei, 2007). Generally OR genes are arranged in clusters in the genome, which are distributed over most chromosomes and a single cluster may contain up to 100 OR genes, as seen for the Olfr7 cluster on mouse chromosome 9 (Zhang and Firestein, 2002). There are about 69 OR gene clusters distributed over 19 chromosomes in the mouse genome (Niimura and Nei, 2005a). Zebrafish, on the other hand, only have a repertoire of 136 intact OR genes (Alioto and Ngai, 2005), yet they are still distributed across 13 clusters over 5 chromosomes (Bayramli and Fuss, unpublished).

In humans, the proportion of pseudogenes to functional genes appears to be much higher than in rodents. While the mouse has only around 3% of pseudogenes (Zhang and Firestein, 2002), the human genome contains up to 72% pseudogenes (Zozulya *et al*., 2001). Using microarray analysis including probes for all predicted human ORs, it has been shown that the expressed pool decreases to 295 intact OR genes and 142 expressed pseudogenes (Zhang *et al*. 2007). The expression of pseudogenes, however, poses a big problem for the correct specification of OSNs as pseudogenes fail to produce a functional protein due to transcriptional and/or translational errors. These ORs, therefore, cannot express a functional protein and thus would not be responsive to odorant stimulation.

For zebrafish, Alioto and Ngai (2005) have identified 136 intact genes and 10 pseudogenes through genome data mining studies. In spite of the limited size of the OR gene repertoire in zebrafish, they have found (by comparison with OR genes from two pufferfish species such as *Takifugu rubipes* and *Tetraodon nigroviridis sp.*) that these ~150 genes can be classified into eight distinct subfamilies (A-H), which share 40% average amino acid identity among members of the same clade. A similar analysis by Niimura and

Nei (2005b), in which they phylogenetically analyzed zebrafish, fugu, Xenopus and chicken OR genes, presents comparable families. The aforementioned Family A corresponds to Group β, Family B to Group γ, Family C to Group ε, Families D and G to Group ζ, Families E and F to Group δ, and Family H to Group η. It is suggested that 8 ancestral genes/families expanded to form the OR gene repertoire of zebrafish, while the OR genes from Family A (OR112-1, OR113-1, OR113-2 and OR114-1) and Family B (OR101-1) may have evolved to form the two major branches of ORs in mammals, the Class I and Class II ORs, respectively (Figure 4.2). While Class I ORs, are the predominant type seen in fish, Class II ORs, constitute the majority of ORs in terrestrial vertebrates with up to 90% of the expressed ORs. It has been assumed that Class I ORs respond to water soluble odorants, whereas the Class II ORs respond to more volatile compounds. In this respect, the frog *Xenopus laevis* possesses two separate olfactory epithelia that it uses under water and on land. Interestingly, Class I OR-expressing cells can only be found in the water compartment, while the Class II ORs are expressed in the compartment that is used in air (Freitag *et al*., 1995).

Besides the classical ORs, other receptor gene families related to olfactory function have bee identified and comprise the trace amine-associated receptors (TAARs) that detect amine-based chemicals such as emitted by decaying food or rotting meat (Liberles and Buck, 2006), and V1R (linked to the G-protein, $G_{\alpha i2}$; Dulac and Axel, 1995) -V2R (linked to the G-protein, $G_{\alpha o}$; Herrada and Dulac, 1997) receptors which in mammals are expressed in the vomeronasal epithelium and partake in the reception of pheromones, and formyl peptide receptors (Liberles *et al*., 2009) which are also found in the innate immune system and may allow organisms to judge whether conspecifics are healthy or infected, though these will not be further discussed in the premises of this research.

1.4. Regulation of OR Gene Expression

Ever since the historical identification of ORs (Buck and Axel, 1991), several puzzling observations regarding their expression patterns were made, for which the functional mechanisms remain largely unexplained. It has been shown that a single neuron is restricted to the expression of only a single functional OR from the entire repertoire (Serizawa *et al*., 2000, 2003), that a single OR is not expressed across the entire extent of

the olfactory epithelium but is rather restricted to a subzone of the epithelium (Ressler *et al*., 1993; Vassar *et al*., 1993), and that these zonal patterns of ORs expression in the main OE and the axonal projections patterns to the OB correlate (Wang *et al*., 1998). Though, in spite of these observations, the specific details of the underlying mechanisms of OR gene expression elude us.

1.3.1. The One Neuron - One Receptor Rule

It has been shown that every single OSN only expresses a single OR gene from the entire genomic repertoire in a mutually exclusive manner (Serizawa *et al*., 2000, 2003; Mombaerts, 2004). Thus the olfactory epithelium may contain up to 1500 individual populations of OSNs, which are defined by the expression of their respective ORs. Evidence for this one neuron – one OR rule comes largely from RNA in situ hybridization studies using OR-specific riboprobes and cell counts in the OE. These experiments have shown that in rodents (possessing ~1300 OR genes) a given OR can be found in about 0.1% of all OSNs, suggesting that there is no or little overlap of OR gene expression across the entire OSN population (Vassar *et al*., 1993, Ressler *et al*., 1993). Similarly, in fish, with an OR repertoire of ~100 genes, a given subfamily of ORs are expressed in 0.5%-2% of all OSNs (Chess *et al*., 1992). Single-cell RT-PCR studies performed by Malnic *et al*. (1999) have also shown that usually only one OR sequence from a single OSN can be amplified. Unfortunately, the method has its flaws since most of the time no OR gene can be amplified at all and is thus one of the weaker evidences of the one neuron-one receptor rule. Observing the axonal projections of OSNs to OB glomeruli seems to present one of the most convincing evidences. Mombaerts *et al*. (1996) have studied the glomerular convergence of OSN axons in the mouse OB through the use of gene-targeted mice expressing various OR genes together with a tau-lacZ or GFP reporter sequence. Thus all the OSNs expressing a given OR can be visualized at once by virtue of the marker expression. They have seen that OSNs expressing the tagged ORs always project their axons to just two topographically fixed glomeruli on each OB, one glomerulus per hemibulb, thus a total of 4 glomeruli per animal. They could also show, that no axons from OSNs expressing other ORs innervate these glomeruli and that glomeruli in the OB specifically represent a single OSN subpopulation that is defined by their OR expression. The numbers also match, as there are usually 2 glomeruli per OR in each OB, a total of

around 3000 glomeruli should be found per OB, which is usually seen in mouse (Bozza *et al*., 2009).

1.3.2. Singular Expression

Most autosomal genes are generally expressed in a biallelic manner, thus from both the maternal and paternal allele. However, a small number of genes are expressed monoallelically such as B- and T-cell receptor genes, natural killer receptor genes, interleukins and some immunoglobulin genes (Pernis *et al*., 1965). Thus, the second allele of these genes is silenced by a specific mechanism called allelic exclusion. A similar phenomenon has been observed for the OR gene family as well, making it the largest family of genes that is monoallelically expressed (Chess *et* al., 1994). Thus, a single OSN only expresses one of the two parental alleles of a given OR gene. However, both alleles are expressed in the entire OE at equal rates and OSNs expressing either allele converge onto the same glomerulus. The phenomenon was first observed by Chess *et al*. (1994), and has been confirmed by various researchers over time (Mombaerts, 1996; Li *et al*., 2004, Ishii *et al*., 2001; Lomvardas, 2006). Li *et al*. have used transgenic mice expressing the M71 OR gene differentially tagged with RFP and GFP in each allele, where it was shown that none of the OSNs appeared yellow, suggesting that a single OSN does not express both alleles of the OR, which supports the concept of allelic exclusion. In mice expressing a single tagged OR, the number of labeled OSNs in homozygous mice is exactly twice the number of cells seen in heterozygous animals, also indirectly supporting monoallelic expression (Mombaerts, 1996). As a direct test for monoallelic expression of OR genes combined DNA/RNA fluorescent in situ hybridization (nuclear FISH) has been performed on OSN nuclei. These experiments have shown that only a single RNA signal indicative of active OR expression can be found in the nucleus. This signal co-localizes with one of the OR alleles that have been detected using DNA probes specific for the OR locus (Ishii *et al*., 2001).

Monogenic expression, the manifestation of the one neuron-one receptor rule, together with monoallelic expression has been combined into a single concept termed singular expression (Vassalli *et al*., 2002). However, this concept does not explain the mechanisms that underlie both of these phenomena. It is not known for certain which one

precedes the other during the decision-making step, whereby an OSN chooses to express a given OR and one allele of it. One possibility is that the cell first chooses the OR gene and then one of the alleles that is expressed. Another possibility could be that the allele of a cluster is chosen before the cell actually decides onto which OR from that cluster is expressed. One final possibility is that the choice mechanism occurs simultaneously for monogenic and monoallelic expressions, thereby counting every allele as an individual. That way, the number of possible candidates for expression would be double the number of expressed OR genes (Fuss and Ray, 2009).

1.3.3. Co-Expression

Intriguingly, certain exceptions to the aforementioned one neuron-one receptor rule have been shown to exist. Fluorescent in-situ hybridization experiments by Sato *et al.* (2007) have shown that three members of the OR103 gene family in zebrafish are coexpressed. Previously, single-cell RT-PCR studies using degenerate primers, followed by double label in-situ hybridization by Rawson *et al.* (2000) had shown the co-expression of I9 and HGL-SL2* rat ORs. A recent research (Tian and Ma, 2008) has shown the coexpression of the MOR256-3 together with other members of a pool of 8 ORs in mice The co-expression frequency of the said genes was as high as ~2% in newborn mice, but dropped to 0.2% in adults. However, in adult mice which had been deprived of sensory input by unilateral naris closure, the same high frequency of 2% co-expression could still be observed, suggesting an activity-dependent mechanism by which OSNs that co-express multiple ORs are eliminated. It is known that neuronal activity is required for the survival of mature OSNs (Watt *et al.*, 2004). Correlating this information with the fact that the frequency with which co-expressed ORs are observed decreases as the mice mature, they investigated the role of an active elimination process based on programmed cell death. Suspecting Bax (a proapoptotic protein) to have a vital role of in the elimination of OSNs with multiple ORs during development, they used Bax-null mice (Tian and Ma, 2008). Interestingly, the number of co-expressing OSNs in adult mice remained at high frequencies.

1.3.4. Zonal Restriction and Relation to Glomerular Projection of OSN Axons

The expression of each OR gene is restricted to a number of defined zones in the OE. These zones were first described in mice and rats as 4 parallel strips lined up anteroposteriorly (Ressler *et al*., 1993; Vassar *et al*., 1993). A comparable arrangement of OSNs expressing a given OR can also be detected in zebrafish, where 10 zebrafish ORs were expressed in at least 3 concentric domains with different diameter, depending on the OR (Weth *et al*., 1996). This zonal restriction mechanism also suggests that, the pool of OR genes available for expression by a given OSN may be restricted to those ORs that are expressed in a specific region of the OE. Further studies on zonal restriction of OR expression have unveiled more information about OR expression patterns and instead of being restricted to the four conventional zones suggested originally, a large number of distinct expression patterns can be found. The expression area of each OR exists in a continuous and overlapping manner spreading out over the OE (Iwema *et al*., 2004; Miyamichi *et al*., 2005). It might even be that each OR has a specific pattern of expression exclusive to it on the epithelium (Fuss and Ray, 2009). The fact that these expression zones are discontinuous on some parts of the OE suggests that the formation of these zones should occur prior to the evagination of the turbinates during early development.

Research on the zonal restriction of ORs has also helped to shed some light onto questions regarding glomerular innervation of the OB as well. Wang *et al*. (1998) have shown that a zone-to-zone correlation exists for the epithelial topography of a given OR and its glomerular positioning on the OB in mice (Figure 1.1). It was initially suggested that sequence similarity or ancestral connections may correlate with zonal segregation (Vassar *et* al., 1993; Kubick *et* al., 1997). However, it was later shown by Miyamichi *et al*. (2005) that members of the same subfamily of OR genes could be expressed all over the epithelium, negating any possibility of a clear-cut rule on the relationship between sequence similarity and zonal patterns. Interestingly, the OR coding sequence does not seem to play as critical a role for zonal restriction as well. Receptor swapping experiments have forced ectopic expression of various OR genes, including those from different classes, in inappropriate zones (Wang *et al*., 1998; Feinstein *et al*., 2004; Bozza *et al*., 2002), and even transgenes lacking an intact coding sequence were shown to express a marker gene in

a zonal pattern close to that of the endogenous OR (Qasba and Reed, 1998; Rothman *et al*., 2005).

1.3.5. Second Choice in Olfactory Sensory Neurons

Although the majority of OSNs express a given OR throughout their lifetime, it has been shown that a certain number of cells may undergo receptor switching (Shykind *et al*., 2004). This receptor switching may occur preferentially in immature OSNs close to the time-point of OR choice. Given the high number of expressed pseudogenes, it is probable that a nascent OSN expresses a truncated OR protein, or a mutant receptor that will not be functional. In such a situation, where an OSN expresses a non-functional OR, the neuron will switch to the expression of another candidate OR gene. By using lineage tracing through a Cre recombinase-based transgene method, Shykind *et al*. have observed the fate of MOR28 expressing OSNs in mice. The experiment clearly displays that immature OSNs expressing the MOR28Δ transgene (replacement of the MOR28 coding sequence with IRES-tauLacZ) switch expression to a different functional OR, with a high bias towards the second allele of the MOR28 gene. In similar experiments, Ishii *et al*. (2001) have substituted the MOR28 gene with a sequence coding for GFP or a pseudogene. Both studies observed that OSNs expressing these mutations make a second choice of OR expression. The arising model suggests that normally an OSN senses the presence of a functional OR protein and terminates the OR choice mechanism by a negative feedback signal (Serizawa *et al*., 2003). Shykind's experiment has also proven that the mechanism by which X-inactivation occurs cannot be used by ORs. In female mammals, the second X chromosome is irreversibly inactivated by being packaged into heterochromatin to prevent over-expression of X-linked genes (Heard *et al*., 1997). The mechanism of X-inactivation was also suggested as a possible model for OR expression; however, Shykind's experiment has proven that the model is not applicable to OR gene expression, since the second allele of an OR is shown to be a possible candidate for receptor switching.

The OR pool available for second choice seems to be restricted to a specific subgroup of ORs instead of the complete OR repertoire (Feinstein *et al*., 2004; Bozza *et al*., 2009). It was primarily suggested that this restriction was zone-based (Serizawa *et al*., 2003), thus that OSNs that fail to express a given OR would express another OR that is

expressed in a similar spatial pattern in the OE and project to a defined region of the OB. Interestingly, for the two different classes of ORs, the class I and Class II ORs, it could be shown, that the set of ORs available for second choice is restricted to the same class of the OR that has been deleted (Bozza *et al*., 2009). Yet, at least for the Class II ORs not all members of the Class II repertoire may be expressed as second choice, suggesting that other distinctions may be present in this large pool of receptors. Apart from class restriction, locus control regions could also play a role in defining the repertoire of ORs that can be expressed by a given OSN including expression through a second choice mechanism (Lewcock and Reed, 2004). However, not all ORs expressed for second choice in the case of the MOR28 deletion experiment were under control of the same locus control region (Serizawa *et al*., 2001).

1.3.6. Suggested Models for OR Expression

Finding out the exact mechanism by which a neuron is able to express a single OR gene from every OSN in a monoallelic way has been a rather elusive challenge. A number of models trying to explain this mechanism have been proposed taking the expression mechanisms of several other multigene families as reference points.

A popular explanation of the singular expression of OR genes was gene translocation, a mechanism very similar in essence to the V(D)J recombination mechanism (Tonegawa, 1983; Jung and Alt, 2004) observed in B- and T-lymphocytes, which results in the expression of a unique surface immunoglobulin (Ig). The nature of this system, whereby it continues recombining until a successful Ig is expressed, also fits the mechanism of receptor switching since ORs also switch alleles if they are not successful. Still, even though the enzymes required for V(D)J recombination, RAG1 and RAG2, are both expressed in the zebrafish olfactory epithelium (Jessen *et al*., 1991), and RAG1 is expressed in mice (Chun *et al*., 1991), the mechanism does not seem to apply for OR gene choice. Mice cloned from mature OSN nuclei show the full repertoire of OR, making irreversible changes in chromosome structure unlikely (Eggan *et al*., 2004; Li *et al*., 2004). Likewise, second choice is not restricted to linked genes from the same cluster or chromosome (Serizawa *et al*., 2000). Gene conversion, as observed in budding yeast mating type switching, does not necessarily cause irreversible DNA modifications (Palmer

and Brayton, 2007). However, copying of a sequence into a remote expression cassette, like with the budding yeast, would result in the presence of a third copy of the target gene locus, which cannot be observed by DNA FISH (Ishii *et al*., 2001; Lomvardas *et al*., 2006).

Another attractive theory on the mechanism for OR expression was the use of a *trans*-acting element that interacts with distant promoter regions across chromosomes. It was believed that the 2.1kb H-region located on mouse chromosome 14 could interact with promoters from different chromosomes (Lomvardas *et al*., 2006). The fact that the H-region could interact with only one promoter at a time would provide a viable explanation for singular expression. However, it was later shown that the H-region is only a long-range *cis*-regulatory element (Fuss *et al*., 2007) similar to a locus control region, operating on the genes from the linked MOR28 cluster. The most prevalent model for OR gene choice involves a negative-feedback regulatory mechanism, which may as well explains the second OR choice in OSNs (Lewcock and Reed, 2004; Serizawa *et al*., 2003). If an OSN senses the presence of an OR protein, this feedback signal may shut down the choice mechanism in OSNs, preventing co-expression of multiple ORs. Similarly, the absence of the signal in OSNs that express a non-functional OR may allow for a second round of the same choice process to prevent the presence of OSNs that would otherwise not express any receptors, or express a non-functional pseudogene. The detailed mechanism and the nature of the feedback signal remain unknown.

1.5. Class Distinction in ORs

As previously mentioned the two classes of ORs do segregate in the phenotypes of their expression, including the underlying control mechanisms. OR genes in vertebrates can be phylogenetically separated into two distinct classes: Class I ORs, which are believed to detect water-soluble odorants, and thus more predominant in fish (Alioto and Ngai, 2005), and Class II ORs, believed to detect airborne odorants and considerably more numerous in mammals and form the majority of the OR gene pool in mouse and other vertebrates (Zhang and Firestein 2002). Even though the anatomical separation of OSNs expressing these classes of genes in amphibians support the odorant-specificities for Class I and Class II OR genes (Freitag *et al*., 1995), the presence of Class I genes in humans, and the small number of pseudogenes present, indicates an evolutionary advantage of keeping

both classes and brings up the possibility of Class I ORs being able to detect airborne odorants, and vice versa (Kratz *et al*., 2002).

In mice, Class I ORs are exclusively expressed in the dorsal OE, which roughly corresponds to the aforementioned Zone I (Vassar *et al*., 1993; Zhang *et al*., 2004; Miyamichi *et al*., 2005; Tsuboi *et al*., 2006). Class II ORs, on the other hand, are much more spread out over the MOE, covering both dorsal and ventral areas which match with Zones I-II-III-IV. The restriction pattern also follows in the OB, where OSNs expressing Class I ORs project their axons to the dorsal part of the OB, and Class II expressing OSNs form glomeruli in both dorsal and ventral regions of the OB (Bozza *et al*., 2009; Tsuboi *et al*., 2006).

It appears that OSNs usually expressing a class I OR are committed by lineage to class I expression (Bozza *et al*., 2009). Deletion of a class I OR consistently results in expression of other class I ORs by these OSNs, whereas deletion of a class II OR only allows for class II ORs as targets for second choice.

It has been shown that the expression of the LIM-homeodomain protein Lhx2 (Hirota and Mombaerts, 2004) is required specifically for the expression of Class II ORs in mice (Hirota *et al*., 2007). The expression of Class I genes, on the other hand, are independent from expression of Lhx2, since the majority of those cells survived when deprived of Lhx2. Two other genes have also been identified which are zone-specific. Olfactory specific medium-chain acyl CoA synthetase (O-MACS) are shown to be specifically expressed in zone 1 of mouse OE, and therefore Class I-specific (Oka *et al*., 2003). Olfactory cell adhesion molecule (OCAM), on the other hand, is expressed in the non-zone I parts of the epithelium, and is therefore expressed by the majority of Class II OR expressing OSNs (Yoshihara *et al*., 1997).

By doing phylogenetic tree analyses on OR genes from zebrafish and two pufferfish species (*Tetraodon nigroviridis and Takifugu rubripes*), and comparing these to the mouse OR gene family, Alioto and Ngai (2005) have come up with the suggestion that the Class II gene family predominant in mammals is the result of a massive expansion of one the of

eight ancestral gene clades they found in fish, particularly Family B (OR101-1). The distinction between Class I and Class II ORs can also be observed anatomically in mice.

2. PURPOSE

In the framework this thesis, I aimed to conduct studies related to the distinction of class I and class II OR expression in zebrafish, including the mechanisms that control their expression. It appears that zebrafish only possesses a single candidate Class II OR, the OR 101-1, which may be related to the phylogenetic origin of the branch of class II ORs that massively expanded in terrestrial vertebrates. As there is a distinct separation of the mechanisms controlling class I and class II OR expression in mammals, it would be interesting to see, whether these differences occur at such an early evolutionary stage. I set out to confirm that OR101-1 indeed is an OR by studying its expression pattern by RT-PCR and in-situ hybridization. To examine whether the single class I OR101-1 is already linked to a unique, class-specific expression control mechanism, I designed two constructs to label OR101-1 expressing OSNs and to study the consequences of an OR101-1 deletion phenotype.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

The Zebrafish (*Danio rerio)* used in this study were obtained from a local pet shop and inbred.

3.1.2. Equipment and Supplies

The equipment and supplies used in this study are listed in Appendix A and Appendix B, respectively.

3.1.3. Buffers and Solutions

Enzymes were only used with the buffers supplied by the manufacturers. All other buffers and solutions used in this study were prepared following the protocols outlined by Sambrook and Russell (1989) unless stated otherwise.

3.2. Methods

3.2.1. Fish Growth and Breeding

All of the fish used in this research were kept and bred following the protocols specified by Brand *et al*. (2002). Briefly, fish were kept at 28.5˚C under a 14h/10h light/dark regime and fed three times daily on flake food and once on live artemia.

3.2.2. Live Tissue Extraction

Fish were euthanized by keeping in ice water for 1 minute, after which they were placed in 1X PBS for the length of the dissection. Dissection was performed under a stereomicroscope at 20X magnification. All the extracted tissues were stored in microtubes and kept on ice until TRIzol® (Invitrogen) was added. First, the head was decapitated using surgical dissection scissors, starting dorsally and moving towards the ventral side and continuing from under the gills. The flower-shaped olfactory epithelial tissue was removed using forceps and stored away. The eyes were harvested by severing the optical nerve with dissection scissors. The jawbones were removed with lateral incisions on the side of the head after which the top of the cranium was removed, revealing the brain and the OB. Abdominal cavity was revealed by a dorsal saggital incision. Liver, heart, eggs and testes were removed and stored separately. The single-nephron kidney was scraped off the dorsal wall of the abdominal cavity gently by the tip of a pair of forceps.

3.2.3. Total RNA Isolation

Total RNA isolation was done using TRIzol® Reagent (Invitrogen), following the manufacturer's protocol directly. Samples were redissolved in DEPC-treated H_2O.

3.2.4. DNase I Treatment and First Strand cDNA Synthesis

DNase I (Promega) treatment of mRNA samples and the subsequent first strand cDNA synthesis was done using RQ1 RNase-Free DNase (Promega) following the manufacturer's protocol directly.

3.2.5. Digoxigenin-labeled Antisense RNA Probe Synthesis

Both linearized vector and PCR products were used as templates for DIG-labeled antisense RNA probe synthesis separately. Plasmids were linearized with restriction enzymes and gel extracted for higher purity. Samples were treated twice with phenol/chloroform/isoamyl alcohol and twice with only chloroform. DNA was precipitated with 10% (v/v) 3M NaOAc and 3X (v/v) 100% EtOH (-20°C) and centrifuged at maximum speed for 30 minutes at 4°C. Pellet was washed with 75% EtOH, centrifuged and resuspended in 20µl H_2O. PCR products were gel extracted to get rid of trace amounts of nonspecific bands. 1µg of transcribed insert was mixed with 2µl of 10x transcription buffer (Roche), 2µl of DIG RNA-labeling mix (Roche), 2µl of T7 RNA Polymerase (Roche) and DEPC-treated H_2O up to a final volume of 20µl. The reaction was incubated at 37°C for 2 hours. 2µl of 0.2M EDTA (pH 8.0), 2.5µl 4M LiCl and 75µl 100% EtOH (-20°C) was added and the mixture was precipitated for 30 minutes at -70°C. The mixture was centrifuged for 15 minutes at 4°C at maximum speed. The pellet was washed with 70% EtOH (-20°C) and centrifuged again for 5 minutes at 4°C at maximum speed. Pellet was dried for 10 minutes and redissolved in 50µl DEPC-treated H_2O. Finally, 1µl of RNase inhibitor (Roche) was added and the sample was frozen in liquid nitrogen. A 5µl aliquot was taken for diagnostic purposes. 10µl formamide and 5µ DEPC-treated H_2O was added to the aliquot and the sample was denatured at 100°C for 5 minutes. After chilling on ice the sample was mixed again and loaded in 1X agarose gel.

3.2.6. Polymerase Chain Reaction

A variety of primers were designed and used for both cloning and diagnostic purposes. For a complete list, see Table 3.1. 50-µl diagnostic PCR reactions containing 1X Taq Buffer (Fermentas), 1.5mM $MgSO_4$ (Fermentas), 0.3mM dNTP (Promega) mix , 0.5µM primers, 50ng of DNA and 1µl of Taq Polymerase were carried out at 2 minutes of initial denaturation of 95 °C, 28 cycles of 95°C for 1 minute, appropriate annealing temperature for 1 minute (with 3 second increments at every cycle), 72 °C for 1 minute and 30 seconds, and a final elongation at 72°C for 10 minutes. The number of cycles was limited to 24 to minimize the possibility of point-mutations if the samples were to be used for cloning purposes. For important and/or hard-to-amplify samples, 1µl of Titanium Taq

(Clontech) and 1X Titanium Taq PCR buffer (Clontech), or 1µl of GoTaq polymerase (Promega) and 1X GoTaq Flexi PCR buffer (Promega) was used instead. For diagnostic colony PCR analyses, colonies were picked using micropipette tips, touched on a backup selection plate and then rubbed into 10µl dH_2O. Bacterial cells were lysed by the initial denaturation step which was extended to 10 minutes to ensure the release of DNA from bacterial cells. The remaining reagents were added to the reaction tubes starting with the first cycle.

Table 3.1. Primers used for cloning and diagnostic purposes.

Primer Name	Primer Sequence (5′ - 3′)
101-1-F	ATGAACACCAGCGGCTCGGT
101-1-R	GTGCCATGCTCATCCTTCTCA
101_PstI_F	ATCTGCAGCGCTCTTGCACTTGCCCTAATTGG
101_SphI_R	TAGCATGCGTACCACAGTATGTTGGGCCATTGG
101_V_Over_F	GTATCACCAGGATGGTGAGCAAGGGCGAGGAGC
101_V_Over_R	CCCTTGCTCACCATCCTGGTGATACACAGCCGTGCAG
101_V_Pst_F	ATCTGCAGGAAGATGCCAGAGTGACAACACC
101_V_EcoR_R	ATGAATTCATTTTACTTGTACAGCTCGTCCATGCCG
101_EcoR_F	CGCTTAGAATGAATTCCATCAATCTGGAAATTGCAATG
101_EcoR_R	TTTCCAGATTGATGGAATTCATTCTAAGCGACAGAAGG
101_Rec_5F	CGCCTGCACAACATCATCTAGTCG
101_Rec_3R	CGCAAAACATTTCCTTCAGCTTTAAGAC
101_V_Xho_R	ATCTCGAGAGCGACAGAAGGAACTGTTTTCC
GFP_F_819	GAGAAGCGCGATCACATGGTCCTG
GFP_F_up	GACAAGCAGAAGAACGGCATCAAGG
GFP_R_5	AACTTGTGGCCGTTTACGTCGC
115_1_3F	CTATACTCTCCTTGTGGAGGGACTG
115_1_5R	CTGTCTTATCTCTTTGGTCTGGAAACC
115_3p_3F	GACTTAAACGAGACATTCAGACACAGC
115_3p_5R	CAGAATTAACTTTTTTTCTCCACCACAG
115_5_3F	GGACAACCTGACATTCACAAATGAC
115_5_5R	GTCAACTGCATTTTTCCACCAGCG
115_6_3F	GTGGGTCCCATGGCCAAACC

Table 3.1. Primers used for cloning and diagnostic purposes (continued).

115_6_5R	GACATTAACCTTTTTGTGCTACGGC
115_7_3F	CAGCATTCTCCTCGTGGAGGGGC
115_7_5R	GCACCAGAGTTTTACAACCTTCTGTC
115_8_3F	GGACAACCTGACATTGACAAACAACG
115_8_5R	GAGCAAAGTTTGCTAGTGTTTTGTCT
115_9_3F	GGACAACCTGACATTCAGACACAGCG
115_9_5R	CCTCTGCCTTATCTCTTTGGTTTG
115_10_3F	GGATAACCAGACATTCAGATCCAGC
115_10_5R	GTCTTATTTCCTTGACCTGCAAACC
115_11_3F	GGACAACCTTACCCTCAGATACAG
115_11_5R	GGGATTCAACCCTGGTGGTAC
115_12_3F	GGATAACCGGACGTTCAGATACAGC
115_12_5R	CCTTGGCTTGTAAGCCATAAATTATTGG
115_14_3F	GGACAACCAGACATTCCAGTACAGC
115_14_5R	CAAGGCTGGTGGAATAACATGAAGG
115_15_3F	GCAGCTAGAAGGACTAAGGGTATC
115_15_5R	GCTCAACACCTGTATCAGAACATGTC
pKOV_Kan_F	GTATTGATGTTGGACGAGTCGGAA
pKOV_Kan_R	ACTCCAACGTCAAAGGGCGA
GAPDH-F	TGGGGTGATGCAGGTGCTAC
GAPDH-R	AAGGAGCCAGGCAGTTGGTG
B-Actin-F	CTGGGATGACATGGAGAAGATCTG
B-Actin-R	CCTTGATGTCACGGACAATTTCTC
M13-F	CGCCAGGGTTTTCCCAGTCACGAC

Table 3.1. Primers used for cloning and diagnostic purposes (continued).

M13_R_highTM	GGAAACAGCTATGACCATGATTA
T7_highTM	TAATACGACTCACTATAGGGCGAATTGG
SP6	ATTTAGGTGACACTATAGAAT
GFP_F_819	GAGAAGCGCGATCACATGGTCCTG
GFP_R_5	AACTTGTGGCCGTTTACGTCGC
XXSB_top	TCGAGGAGGTCTAGAGTCGACGGATCCAGCT
XXBS_bottom	GGATCCGTCGACTCTAGACCTCCTCGAGTAC
zOMP_cDNA_F	GTCCAGCTGACGGAGATGATG
zOMP_cDNA_R	CGTCTTCAAAGGCGAACAGGA

3.2.7. Real-Time Polymerase Chain Reaction

Real-time PCR was performed on te LightCycler® 480 Real-Time PCR System (Roche). Reaction mixtures were composed of LightCycler® 480 SYBR Green I Master premix (Roche), 0.5mM primers and 5µl cDNA in a final volume of 20µl.

PCR reactions were carried out with 10 minutes of initial denaturation at 95°C, 50 cycles of 95°C denaturation for 10 seconds, 60°C annealing for 15 seconds and 72°C elongation for 30 seconds (single acquisition after each cycle). Melting curve analysis was done by heating the samples to 95°C, immediate cooling to 65°C and slow re-heating to 97°C, with 5 acquisitions per degree Celsius.

3.2.8. Gel Extraction of DNA

DNA samples were run on 1% agarose gel in 1X TBE buffer stained with ethidium bromide together with a molecular weight marker until the required DNA bands were clearly distinguishable. Agarose gel fragments containing the right bands were excised with a clean scalpel. QIAquick Gel Extraction Kit (Qiagen) was used following the

manufacturer's protocol to obtain DNA fragments of desired molecular weight. DNA was eluted in 30/50µl of Elution Buffer (depending on the expected concentration). The concentration of the samples were measured by absorbance spectrophotometry at 260nm, using a NanoDrop spectrophotometer. The samples were stored at -20 °C.

3.2.9. DNA Purification

Enzymatic reactions were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's protocol directly. DNA was eluted in 30/50µl of Elution Buffer (depending on the expected concentration). The concentration of the samples were measured by absorbance spectrophotometry at 260nm, using a NanoDrop spectrophotometer. The samples were stored at -20 °C.

3.2.10. Ligation to Vectors

PCR products and gel-extracted digestion products were ligated to the needed vectors in 10-µl reactions containing 1X ligation buffer (New England Biolabs). For pGEM-T Easy Vector (Promega) ligations, 50ng of vector and ~250ng of DNA was used. For pBluescript and pKOV-Kan ligations, samples were run together with 50ng of vector on 1% agarose gels, an amount of sample in 3-Molar-excess to the vector was calculated and the ligation was prepared accordingly. For pGEM-T Easy ligations Promega T4 Ligase and the supplied buffer was used. For other ligations, NEB T4 Ligase and its supplied buffer were used.

3.2.11. Alkaline Phosphatase Treatment

Alkaline phosphatase (calf intestinal) treatment was performed on vectors linearized with a single restriction enzyme prior to ligation. 2µl of CIP (Promega) was added to 50µl of linearized vector and incubated at room temperature for 10 minutes before heat-inactivation at 60°C for 20 minutes and subsequent purification using the QIAquick PCR Purification Kit (Qiagen). DNA was eluted in 30/50µl of Elution Buffer (depending on the expected concentration). The concentration of the samples were

measured by absorbance spectrophotometry at 260nm, using a NanoDrop spectrophotometer. The samples were stored at -20 °C.

3.2.12. Preparation and Transformation of Competent Cells

A single colony of Top10 *Escherichia coli* is inoculated into 100ml LB medium which is grown until the culture has an OD_{550} of 0.48 – 0.60. Culture is chilled on ice for 15 minutes and centrifuged at 4,000 rpm for 10 minutes at 4°C. After the supernatant is decanted, the bacteria is resuspended in 10ml of 0.1 M $CaCl_2$ per 100ml culture and stored on ice for 15 minutes. The culture is re-centrifuged at 4,000 rpm for 10 minutes at 4°C. The supernatant is decanted and the bacteria is resuspended in 4ml of 0.1 $CaCl_2$, 15% glycerol per 100ml culture. 100µl aliquots are prepared and frozen in liquid nitrogen.

50µl of Top10 competent cells in 1.5ml microtubes were briefly thawed on ice. 5µl of each ligation reaction was added to the competent cells and the tubes were gently mixed by flicking. After incubating on ice for 30 minutes, the cells were heat-shocked for 1 minute in a water bath at 42 °C and returned to ice for 5 minutes. All 100µl of cells were plated on agar selection plates containing the required amount of the appropriate antibiotics.

3.2.13. Plasmid Isolation

Colonies proved positive via colony PCR were inoculated in 5 ml of LB containing the appropriate antibiotics and incubated at 37 °C for 14 hours with shaking at 300 rpm. 4 ml of cells were harvested by centrifugation at 15,000 x *g* for 1 minute at room temperature. Plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen). The manufacturer's protocol was followed directly. DNA was eluted in 50µl of Elution Buffer (Qiagen). The concentration of the samples were measured by absorbance spectrophotometry at 260nm, using a NanoDrop spectrophotometer. The samples were stored at -20 °C.

3.2.14. Embryonic In-situ Hybridization

In situ hybridization of zebrafish embryos was performed as described by Thisse and Thisse (2009). Embryos were fixed with 4% paraformaldehyde in PBST overnight at 4°C. After washing twice with 1X PBS, the embryos were transferred to a petri dish and dechorionated using watchmaker forceps. After dechorionation, embryos were transferred to 2ml microtubes, washed with 50% methanol once and with 100% methanol twice, each for 5 minutes. At this stage, the embryos can be stored at -20°C for up to even 6 months.

Fixed embryos were placed in 6-well plates and rehydrated with successive dilutions of methanol in 1X PBS; 75% (vol/vol) methanol, 50% (vol/vol) methanol, 25% (vol/vol) methanol, 5 minutes each. Rehydrated embryos were washed 4 times in 100% PB, 5 minutes each and permeabilized by digestion with proteinase K (10µg/ml, Finnzymes) at room temperature. The duration of proteinase K (Finnzymes) digestion is 10 and 30 minutes for 1-day-old and 2/3-day-old embryos respectively. Digestion was stopped by incubation in 4% (wt/vol) paraformaldehyde in 1X PBS for 20 minutes. Embryos were transferred to 1.5ml microtubes and pre-hybridized with 700µl hybridization buffer (HB) for 4 hours at 70°C. HB was replaced with 200µl of HB containing 30ng of digoxigenin-labeled antisense RNA probe and left to hybridize overnight at 70°C.

All of the solutions used during the wash steps were pre-warmed to 70°C. HB was changed to 2X SSC, washing the embryos with successive dilutions of HB and 2X SSC; 75% HB, 50% HB, 25% HB and finally 100% 2X SSC, 5 minutes each at 70°C. After washing twice in 0.2X SSC for 30 minutes each at 70°C, the buffer was gradually replaced with PBT via washing (10 minutes each) with successive dilutions of 0.2X SSC in PBT at room temperature: 75% 0.2X SSC, 50% 0.2X SSC, 25% 0.2X SSC and finally 100% 1X PBT. Embryos were incubated at room temperature in blocking buffer (Roche Blocking Reagent) for 4 hours. Blocking buffer was replaced with blocking buffer containing anti-DIG antibody solution (Roche Anti-Digoxigenin-AP, Fab freagments) diluted at 1:10000 and embryos were left to gently rotate on a horizontal orbital shaker overnight at 4°C.

Antibody solution was replaced with PBT briefly, and then washed with PBT 6 times at room temperature, 15 minutes per wash, and gently rotating. Buffer was removed

completely and embryos were briefly dried using a corner of a Kimwipe®, taking care not to contact the embryos directly. Embryos were washed with alkaline Tris buffer 3 times at room temperature, 5 minutes per wash. Alkaline Tris buffer was replaced with staining solution (Roche NBT/BCIP Solution) and incubated at room temperature for 24 hours in the dark, gently agitating on a horizontal orbital shaker. When the right amount of staining was reached, the staining was stopped by transferring the embryos to 1.5ml microtubes and replacing the staining solution with 1ml of stop solution. Embryos were washed again 3 times with stop solution at room temperature for 15 minutes each after which they were transferred to 6-well plates containing 100% glycerol, with minimum possible carryover of stop solution. Embryos were gently agitated overnight at room temperature in 100% glycerol, which replaces water in the tissues and increases transparency in the embryos. After incubation, embryos were mounted in 100% glycerol and observed microscopically. All of the buffers used during in situ hybridization were prepared as specified by Thisse and Thisse (2008) except for Roche reagents, which were prepared according to the manufacturer's specifications.

3.2.15. Bacterial Artificial Chromosome Recombineering

Bacterial artificial chromosome (BAC) recombineering was performed as described by Lalioti and Heath (2001). BAC containing *E. coli* cells (DH10B) were grown in 20ml LB with 20µg/ml Chloramphenicol (Cm) overnight at 37 °C. 1ml of the overnight culture was diluted in 100ml LB and grown at 37 °C until 0.5-08 OD. Cells were kept on ice for 30 minutes and then harvested by centrifugation at 4000 rpm for 15 minutes at 4 °C. The pellet was resuspended in 20 ml of 100mM $CaCl_2$ and left on ice for 30 minutes after which the centrifugation step was repeated. Competent BAC cells were resuspended in 2ml of 100mM $CaCl_2$ + 15% glycerol and 100µl aliquots were frozen at -70 °C.

One aliquot of competent BAC cells was co-transformed with 1µl of pKOV-Kan+insert plasmid and 5µl of pDF25 plasmid. The cells are kept on ice for 30 minutes and heat-shocked at 42 °C for 1 minute. 1ml LB is added and the cells are left to grow at 30 °C for 1.30 hours. 100µl of cells is plated on Cm+Kanamycin (Kan) plates and incubated overnight at 30°C. 4-6 colonies are pooled in 1ml LB and vortexed. 100µl is plated on Cm+Kan plates and incubated overnight at 43 °C. Colonies are tested via colony

PCR for cointegration of the insert sequence into the BAC. Competent cells are made from positive cointegrants using the above protocol except that this time the cells are grown at 43 °C with Cm+Kan.

2µl of pDF25 is transformed into the competent cointegrants using the above protocol. 100µl is plated on Cm plates and incubated overnight at 30 °C. 4-6 colonies are pooled into 1ml LB and diluted 1:10. 100µl is plated on Cm and 5% Sucrose plates and incubated overnight at 43 °C. The last step is repeated one more time. Single colonies are picked from the second Cm+sucrose plate and streaked on both Cm+Kan+sucrose and Cm+sucrose plates which are incubated overnight at 30 °C. Colonies which did not grow on Cm+Kan+sucrose plates are checked for correct resolution via colony PCR.

3.2.16. Embryonic Injection of Recombinant DNA

All of the embryonic injection experiments in this research have been performed following the protocols specified by Gilmour *et al*. (2002).

3.2.17. Sequence Analysis

BLAST analyses relating to the OR101-1 sequence was done using the NCBI (National Center for Biotechnology Information) BLAST (blastn, blastp, tblastn, bl2seq) tool for human and zebrafish species. BLAT analyses were performed by using the University of California Santa Cruz (UCSC) Genome Browser BLAT tool for human, zebrafish (Danio rerio), three-spined stickleback (Gasterosteus aculeatus), tetraodon (Tetraodon nigroviridis), fugu (Takifugu rubripes), and medaka (Oryzias latipes) species.

Phylogenetic analyses were done using the MEGA 4: Molecular Evolutionary Genetics Analysis tool (Kumar et al., 2008). Protein sequences from zebrafish and human OR genes were aligned (first separately, then together) using the integrated MEGA alignment algorithm with default parameters. N- and C-terminal tails were trimmed for every sequence. Phylogenetic trees rooted to adenosine A1 (A1R) and β2 adrenergic (β2-AR) receptors were constructed using the neighbor-joining algorithm and JTT-matrix model for distance computation.

The analyses of sequencing results and all other construct designs were done using Vector NTI Advance 11.0 (Invitrogen) software.

4. RESULTS

4.1. Phylogenetic Analysis of OR101-1

I was interested in whether zebrafish ORs can be separated into different members belonging to the different classes that have been described in other vertebrates, namely the class I and class II ORs. Therefore a phylogenetic tree of all known zebrafish ORs was constructed, using the neighbor-joining algorithm. The phylogenetic analysis of 136 zebrafish ORs shows that they fall into 8 distinct branches, correlating to the clades A to H, which have been described by Alioto and Ngai (2005). Niimura and Nei (2005b) have also grouped zebrafish, fugu, Xenopus and chicken ORs in a similar pattern. The phylogenetic tree was rooted to adenosine A1 (A1R) and β2 adrenergic (β2-AR) receptors, two GPCRs, to provide a better evaluation for the relationship of aligned sequences. The tree displays OR101-1 in a branch distinct from the rest of the OR repertoire (Figure 4.1).

To better relate them to the class I and class II ORs, the set of zebrafish ORs was aligned with a repertoire of 347 human Ors (Malnic *et al*., 2004). As expected, human ORs separate into two major branches corresponding to class I and II, respectively (Figure 4.2). When compared to zebrafish ORs, a major branch, identified as clade A, clusters tightly with the human class I ORs. However, most of the zebrafish ORs do not cluster tightly with either of the two major branches, but are somewhat closer related to class I ORs. These results suggest that only a single branch (A) gave rise to the mammalian class I ORs, while the majority of zebrafish receptors is only loosely related to them.

Interestingly, a single zebrafish OR, OR101-1, clusters tightly with the major clade of human class II ORs, suggesting that this receptor bears some evolutionary relationship with the branch of ORs that massively expanded during the phylogeny of higher vertebrates. Thus, the zebrafish OR 101-1 may represent an ancient class II OR or at least may be related to the ancient OR that gave rise to the class II repertoire in higher vertebrates. In search for homologoues genes in other species, the OR101-1 sequence was blasted against sequenced genomes. Genomic sequences of three-spined stickleback (*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridis*), fugu (*Takifugu rubripes*),

and medaka (*Oryzias latipes*) species were scanned (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://genome.ucsc.edu/cgi-bin/hgBlat) for both nucleotide and translated nucleotide sequence similarities to OR101-1. E-value threshold was set as $1 \cdot e^{-30}$ to allow for remote relatives. However, none of the sequenced fish species contained a homologous sequence. The closest OR in the mouse and human genomes are the MOR171-13 and OR06.09.01, which show a 52.1% and 43% sequence identity, respectively.

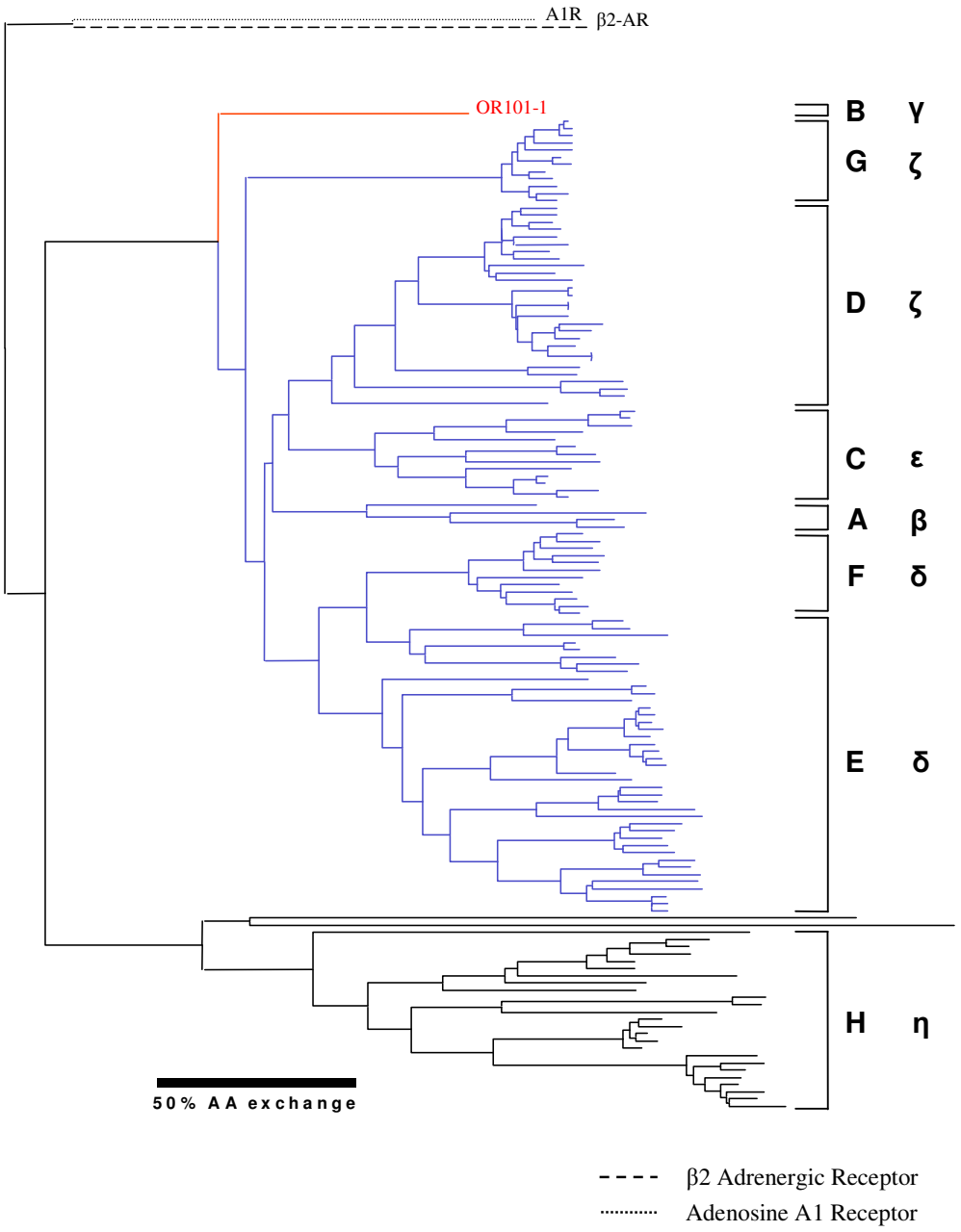


Figure 4.1. Phylogenetic analysis of amino acid sequences from 136 zebrafish OR genes. 8 ancestral clades (A-H) described by Alioto and Ngai (2005) and corresponding groups (greek letters) by Niimura and Nei (2005b) are shown in brackets.

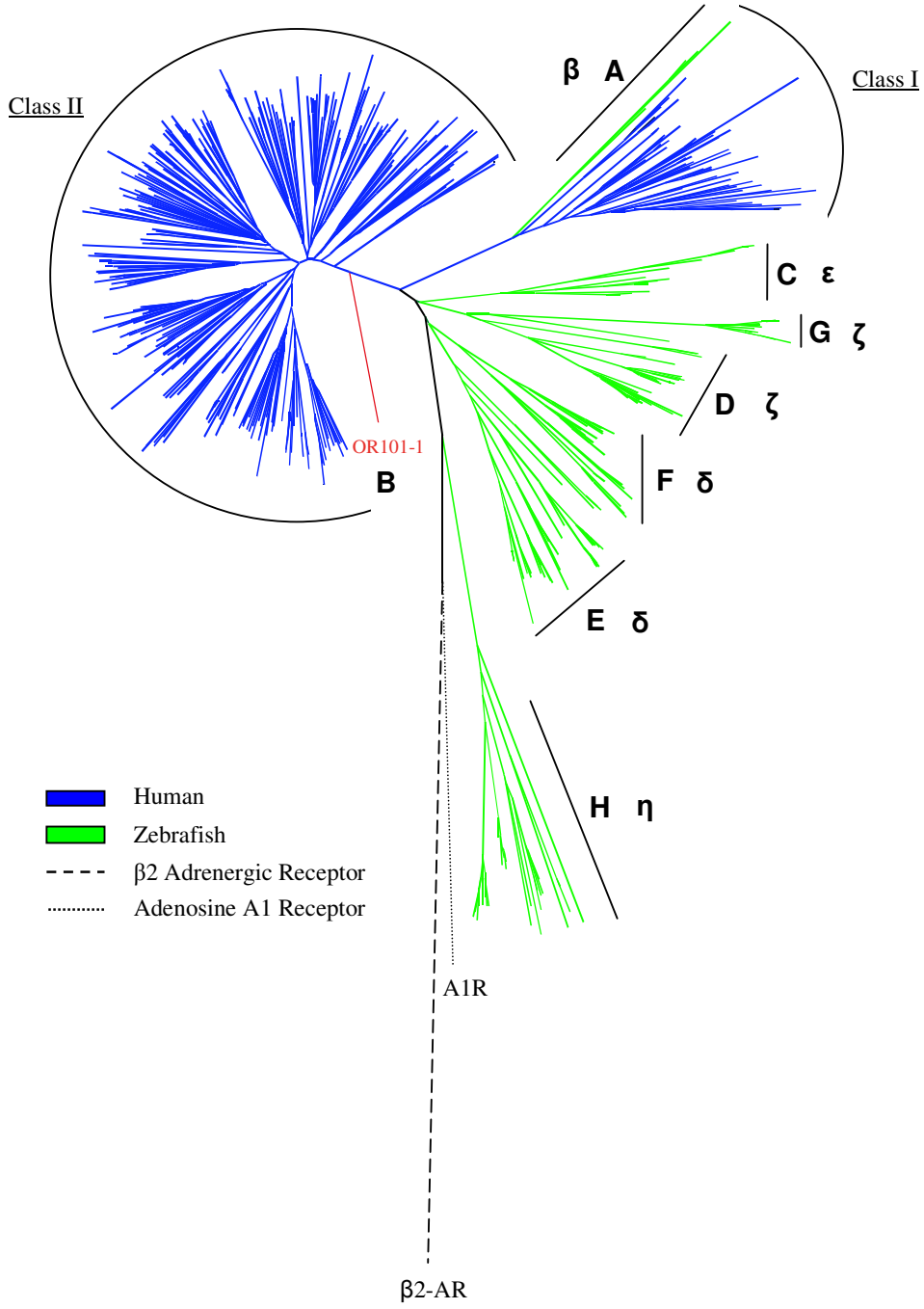


Figure 4.2. Phylogenetic analysis of amino acid sequences from 347 human and 136 zebrafish OR genes. A clear separation between the human Class I and Class II ORs is evident. A single branch of zebrafish ORs, related to branch A, clusters tightly with the human Class I repertoire. Only a single zebrafish gene, OR101-1, shows phylogenetic relationship with the human Class II ORs. 8 ancestral clades (A-H) described by Alioto and Ngai (2005) corresponding groups (greek letters) by Niimura and Nei (2005b).

4.2. Genomic Organization of OR101-1

In the mouse and human genome, class I and class II ORs are clearly separated on the level of their genomic organization. While class II ORs are found on almost every chromosome, class I ORs are contained within a single large cluster on chromosome 7 (Bulger *et al.*, 1999), with no intermingling of class II ORs. Genomic mapping of the OR101-1 receptor in zebrafish using Ensemble (www.ensembl.org) data shows that it is located on zebrafish chromosome 21 (42,178,376 - 42,179,326), on the reverse strand. The coding sequence of OR101-1 consists of a single exon that is 948 basepairs long. The putative class II gene OR101-1, however, is closely situated next to a cluster of class I ORs (branch F), which all belong to the OR115 gene family, located ~8kb downstream of OR101-1. OR115 family has 15 members (1-15) including 2 pseudogenes (OR115-3p and OR115-4p), roughly distributed over 125kb, and located on the forward strand (Figure 4.3). Pairwise similarity comparison (bl2seq) of OR101-1 with members of the OR115 family has resulted in an overall 6% similarity between these genes.

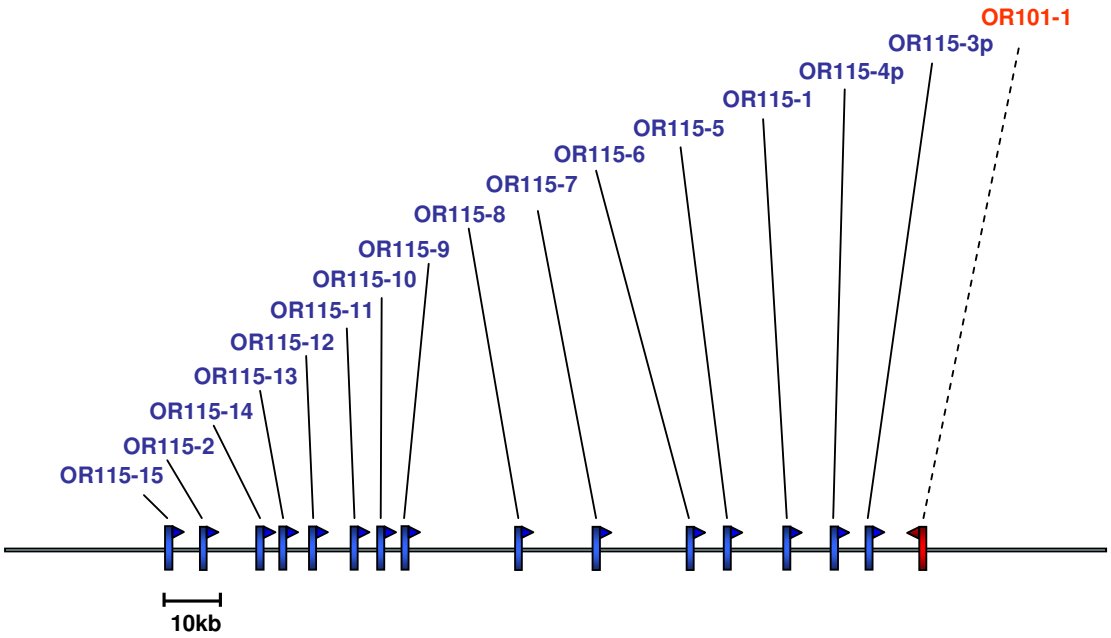


Figure 4.3. Genomic organization of OR101-1 and members of OR115 family on zebrafish chromosome 21. (Region: ~42,050,000 – 42,190,000) Arrows indicate the direction of the open reading frame.

4.3. Expression of OR101-1 in Zebrafish

The repertoire of 136 zebrafish ORs has been identified by genome data mining. Therefore, these sequences may not necessarily represent ORs per se but could bear some similarity to them, yet without fulfilling any olfactory function. Therefore it is important to verify that the OR101-1 is indeed an OR. In order to validate the expression of OR101-1 in zebrafish, OE mRNA was extracted from adult zebrafish and used to make cDNA. 101-1-F and 101-1-R primers (see Table 3.1), flanking a 673bp region inside the OR101-1 gene, were used to amplify the desired sequence from OE cDNA using standard PCR. Primer specificity was tested through zebrafish BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat). No other similar matches were detected. A negative control template (–RT) which did not recieve any reverse transcriptase during cDNA synthesis was used to check for genomic contamination. A strong band of expected size (673bp) could be observed in the OE cDNA well, confirming OR101-1 expression. A weak band could be detected in the –RT reaction, indicative of genomic DNA contamination (Figure 4.4).

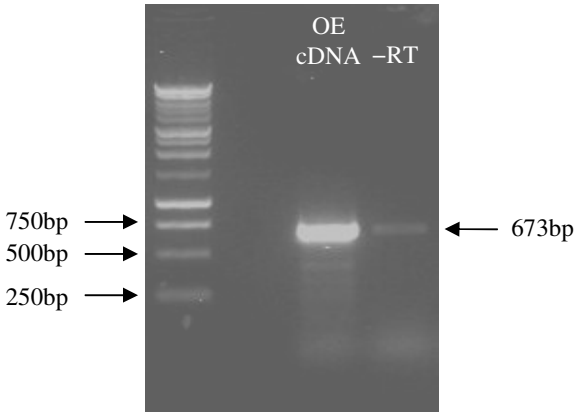


Figure 4.4. Amplification of OR101-1 from adult zebrafish OE cDNA. 28 cycles of amplification was performed on zebrafish OE cDNA using specific primers that amplified a 673bp fragment (arrow). Product size corresponded to the expected value.

To further substantiate the role of OR101-1 as a functional OR, the tissue distribution of its expression was profiled. It was assumed that OR101-1 expression should be exclusive to the nose, and not likely to be expressed in other tissues. For mammalian ORs, however, it has been shown, that specific ORs are also expressed in a variety of tissues, where they may perform other functions, such as controlling renal blood flow in the kidney (Pluznick *et al*., 2009). For this reason, tissue specific PCR amplification was performed on cDNA derived from various zebrafish tissues. OE, eye, brain, gill, kidney, liver, sperm and ovary tissues were extracted from ~30 dissected adult zebrafish. mRNA was extracted and first strand cDNA synthesis was performed on each tissue separately (Figure 4.5).

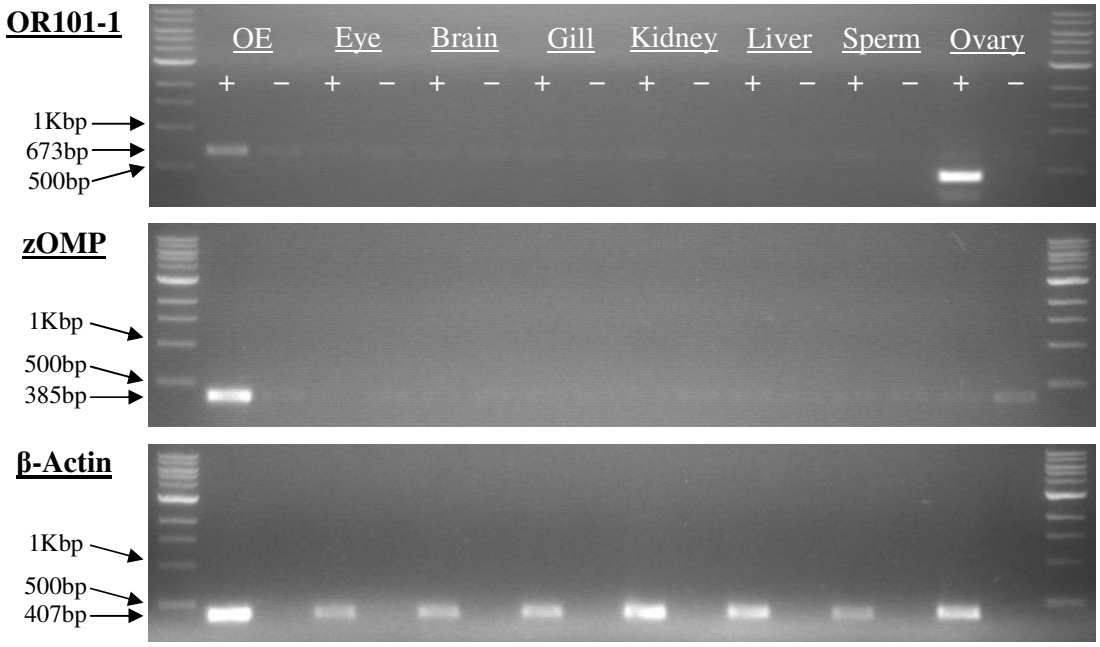


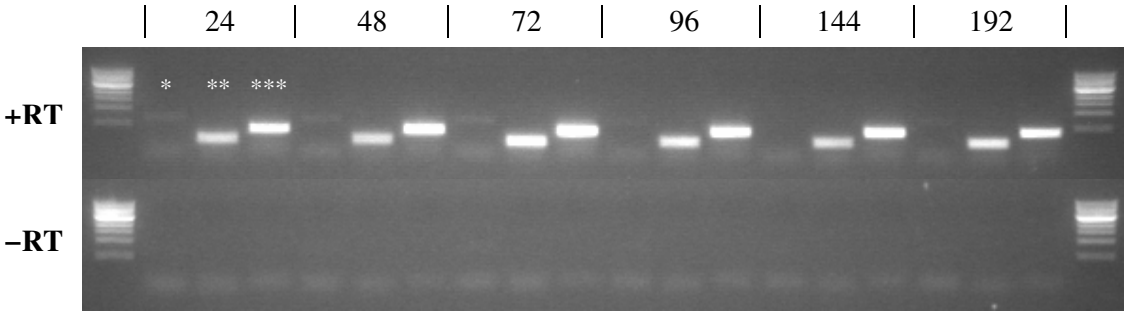
Figure 4.5. Tissue-specific amplification of OR101-1 (top), zOMP (middle) and β-actin (bottom). 28 cycles of amplification was performed on cDNA (+) and –RT controls (–) from 8 zebrafish tissues (OE, Eye, Brain, Gill, Kidney, Liver, Sperm, Ovary; left to right) using transcript-specific primers. All product sizes corresponded to the expected values.

Zebrafish Olfactory Marker Protein (zOMP) an olfactory specific transcript (Celik *et al*., 2002; Sato *et al*., 2005) and β-Actin transcripts were amplified as controls. Negative control (−RT) samples did not receive any reverse transcriptase during cDNA synthesis.

As expected, β-Actin was expressed in all of the tissues, and zOMP could only be observed in the OE sample. In spite of some background amplification in the –RT control and other tissues, the product obtained from OR101-1 in the OE template was discernibly brighter. Importantly, the level of OR101-1 expression in other tissues never exceeded the levels that were seen from genomic contamination, making it likely that OR101-1 is exclusively expressed in the OE. Surprisingly, no genomic contamination was seen for the β-Actin amplification, which would have resulted into a 718bp band, due to the presence of an intron between the primer pair. An unexpected product about 400bp in length was observed in the ovary sample for OR101-1.

4.4. Temporal Expression of OR101-1

We were interested in the temporal profile of onset of OR101-1 expression in the OE. Therefore, zebrafish embryos from different post-fertilization stages were harvested and mRNA was extracted for cDNA synthesis. cDNA from six time points was acquired: 24, 48, 72, 96, 144 and 192 hpf. OR101-1 was amplified from each sample using gene specific primers. Two housekeeping genes, β-Actin and GAPDH, were included as positive controls and as controls for genomic contamination. Negative control (−RT) samples did not receive any reverse transcriptase during cDNA synthesis (Figure 4.6). A weak expression for OR101-1 could be observed which further decreases after 72hpf. Positive control genes were amplified in every sample, matching the expected sizes. No products could be observed in −RT wells.



*OR101-1 (673bp), **GAPDH (220bp), ***B-Actin (407bp)

Figure 4.6. Temporal expression of OR101- 1(*), GAPDH (**) and β-Actin (***). 28 cycles of amplification was performed on zebrafish cDNA from 6 post-fertilization time points (24, 48, 72, 96, 144, 192 hpf, respectively) using transcript-specific primers. All product sizes corresponded to the expected values. Expression of OR101-1 can already be observed as early as 24 hpf.

The RT-PCR experiment shown above does address the relative proportion of OE size and its growth during development. In order to better assess the expression levels of OR101-1 during early embryonic developmental stages with respect to the growth of olfactory tissue, the relative transcript levels of OR101-1 to zOMP was measured by qRT-PCR using the olfactory specific transcript zOMP, which is expressed in every OSN, as a reference (Figure 4.7). A stable $1 \cdot e^{-1}$ relative expression level was observed for all time points except for 48hpf. Melting curve analysis displayed single peaks for all samples except for 48hpf, which showed an extra shoulder, suggesting some amount of contamination or nonspecific amplification (Figure 4.8) and may be the result of an experimental error. As a result, OR101-1 appears to grow at equal rates with the growth of the OE as indicated by zOMP expression.

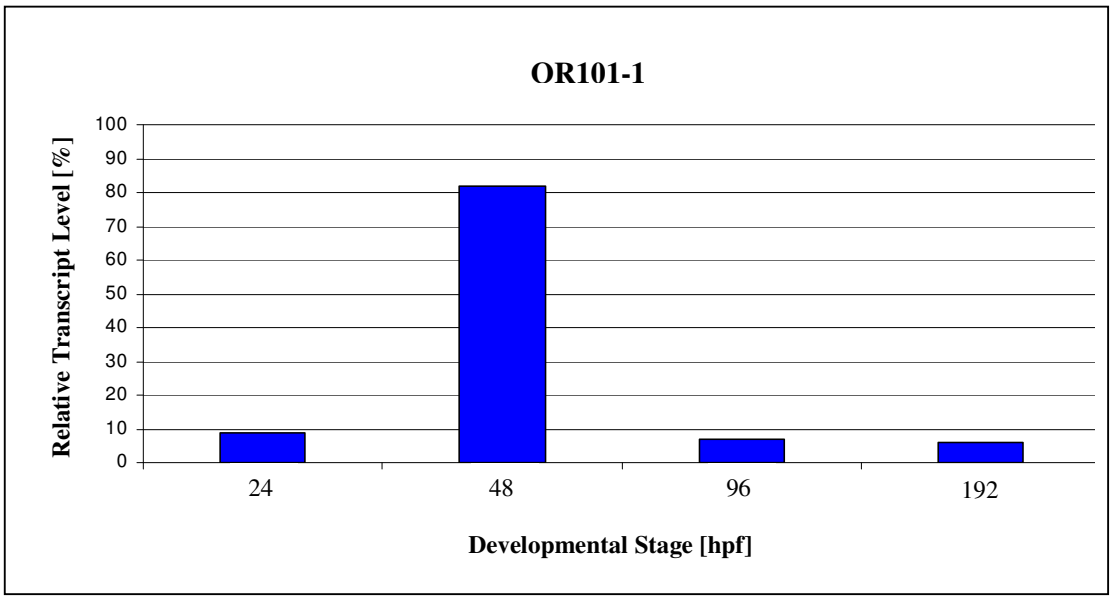


Figure 4.7. Relative transcript levels of OR101-1 in early embryonic development of zebrafish. Transcript levels of OR101-1 were measured at four different time points (24, 48, 96, and 192) and normalized to zOMP levels, which indicates the developmental growth of the OE. OR101-1 expression levels are in a stable ratio to zOMP expression levels for all time points except 48hpf because of experimental errors.

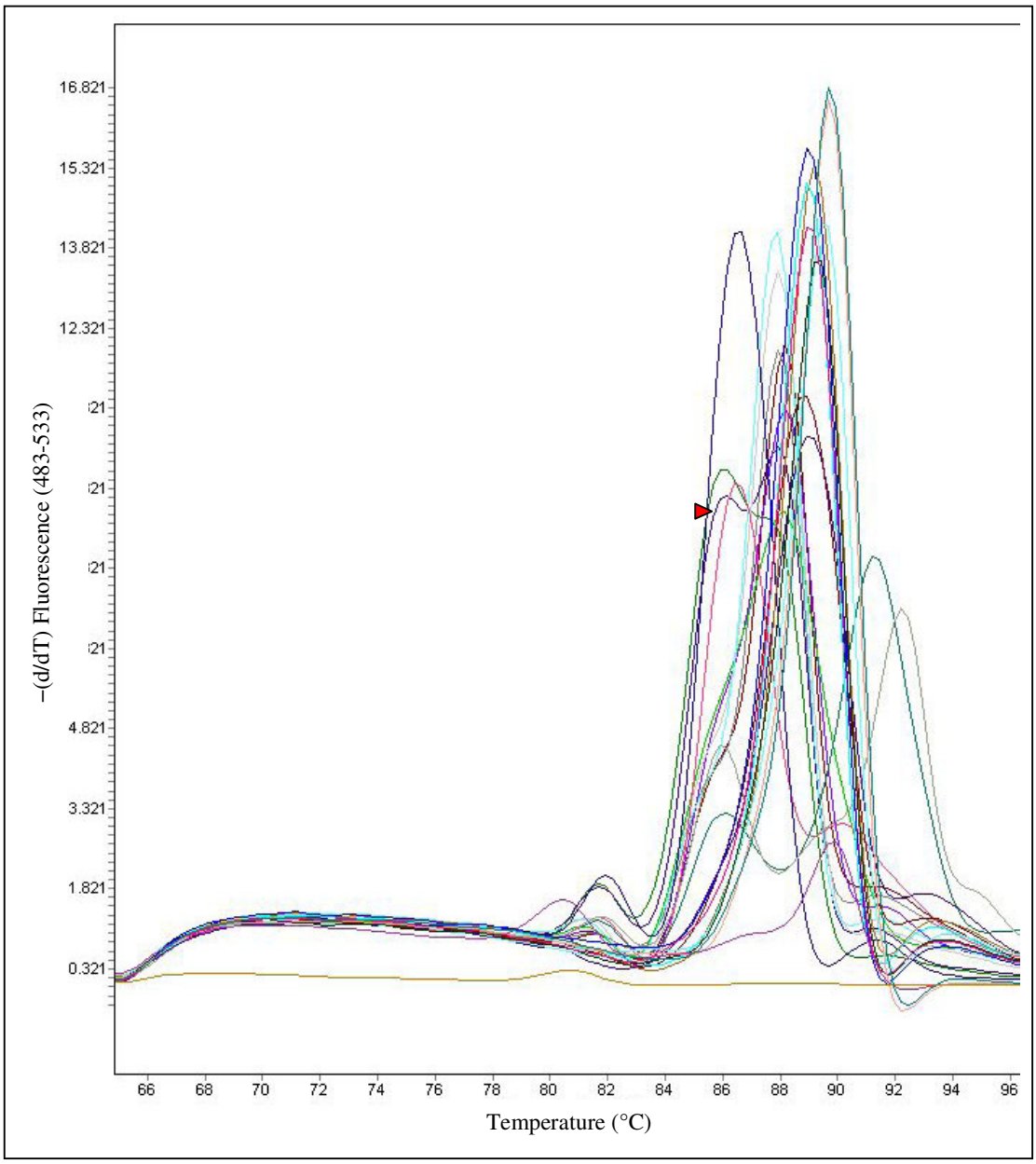


Figure 4.8. Melting curve analysis of RT-PCR reactions. The second peak seen in the 48 hpf sample is shown with the red arrow.

4.5. Embryonic In-situ Hybridization of OR101-1

To further confirm the role of OR101-1 as an olfactory receptor, the expression pattern of OR101-1 was further investigated by in-situ hybridization of zebrafish embryos. Three post-fertilization time points (24, 48, 72 hpf) were taken for the study.

A 673bp fragment of the OR101-1 coding sequence was cloned into pGEM-T Easy vector in reverse orientation to the T7 RNA polymerase promoter in order to transcribe antisense RNA riboprobes *in vitro* using T7 RNA polymerase. The template was amplified using M13-F and 101-1-F primers, to be used for antisense RNA probe synthesis. Prior to probe synthesis, the template was purified by agarose electrophoresis and subsequent gel extraction. 5µl of the synthesized probe was checked by agarose electrophoresis. No degradation was observed (Figure 4.9).

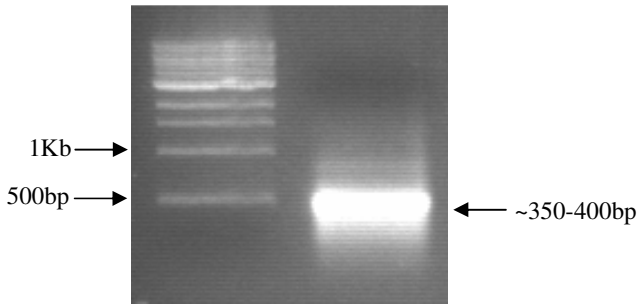


Figure 4.9. Agarose gel electrophoresis of DIG-UTP-labeled OR101-1 antisense RNA probe. A strong band at approximately half the size of the template is visible. The DNA ladder is used only as a qualitative reference, and not for quantitative purposes.

The specificity of the riboprobe was assessed via zebrafish BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat). No similar sequences other than OR101-1 were found. The closest similarity of carried by OR123-1 is 42.7%, below the cutoff for specificity of detection by in situ hybridization, around 80% sequence identity.

After hybridization, the embryos were embedded in 100% glycerol and observed under 10X-20X magnification by a dissection stereomicroscope and at 40X magnification

by an inverted microscope for higher detail (Figure 4.10, 4.11, 4.12). OR101-1 positive OSNs are visible by their dark purple label beginning already from 24hpf, consistent with the results from the temporal series of RT-PCR. The number of positive cells was counted in each nose for every fish individually. A total of 11, 35, and 78 embryos were scored at 24, 48, and 72 hpf, respectively. For all three time points, equal average numbers were found on either of the two OEs per animal (Figure 4.14).

The penetrance of OR101 expression was investigated as the percentage of fish showing at least one positive cell for every post-fertilization stage. An increase in the number of positive fish is observed. At 24 hpf, only 56% of fish are positive for OR101-1, whereas at 48 and 72 hpf the percentages increase to 94.3% and 96.2%, respectively (Figure 4.15). Average number of cells per nose also shows an overall increase (Figure 4.13).

12 members of the neighboring OR115 family were also amplified using the appropriate flanking primers (Table 3.1) and cloned into the pGEM-T vector. These plasmids will also be utilized as templates for the synthesis of specific DIG-UTP-labeled antisense RNA riboprobes. In situ hybridization protocol will be performed for these 12 ORs in order to check for their expression patterns in the zebrafish OE.

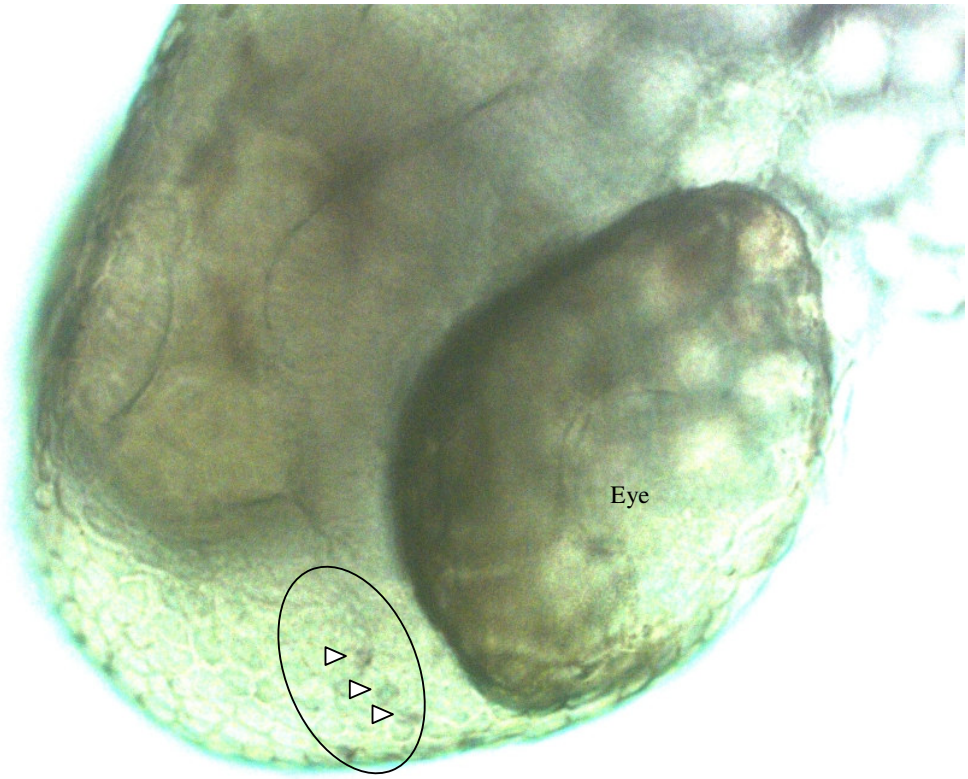


Figure 4.10. In-situ hybridization of 24 hpf zebrafish embryos with DIG-UTP-labeled antisense OR101-1 RNA probe. Embryos were visualized under 40X magnification with an inverted microscope. OR101-1 is expressed in the OE (circle) already at this time point.

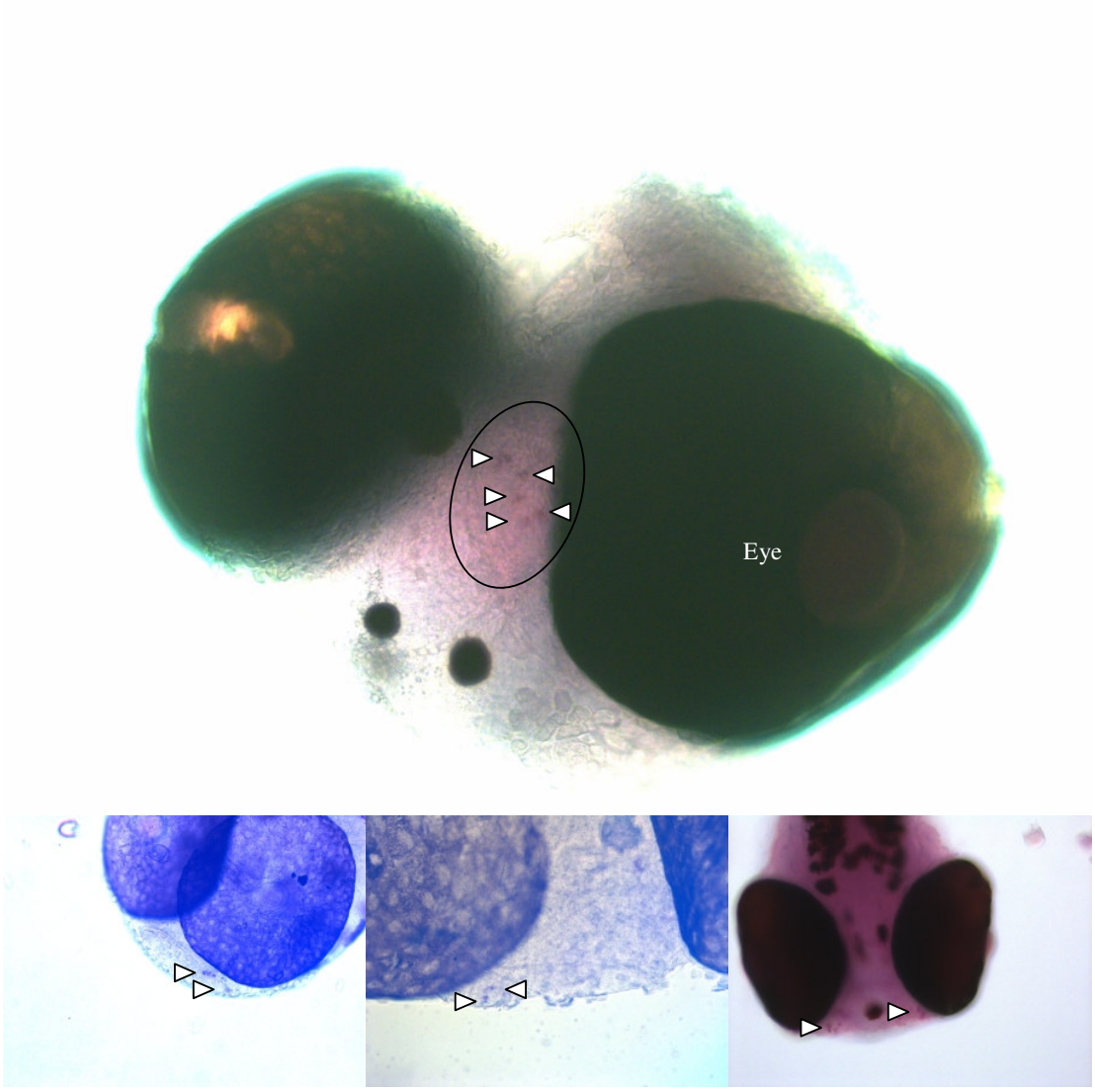


Figure 4.11. In-situ hybridization of 48 hpf zebrafish embryos with DIG-UTP-labeled antisense OR101-1 RNA probe. Embryos were visualized under 40X magnification with an inverted microscope. OR101-1 expressing cells (arrows) are considerably higher in number compared to 24 hpf embryos and appear in clusters.

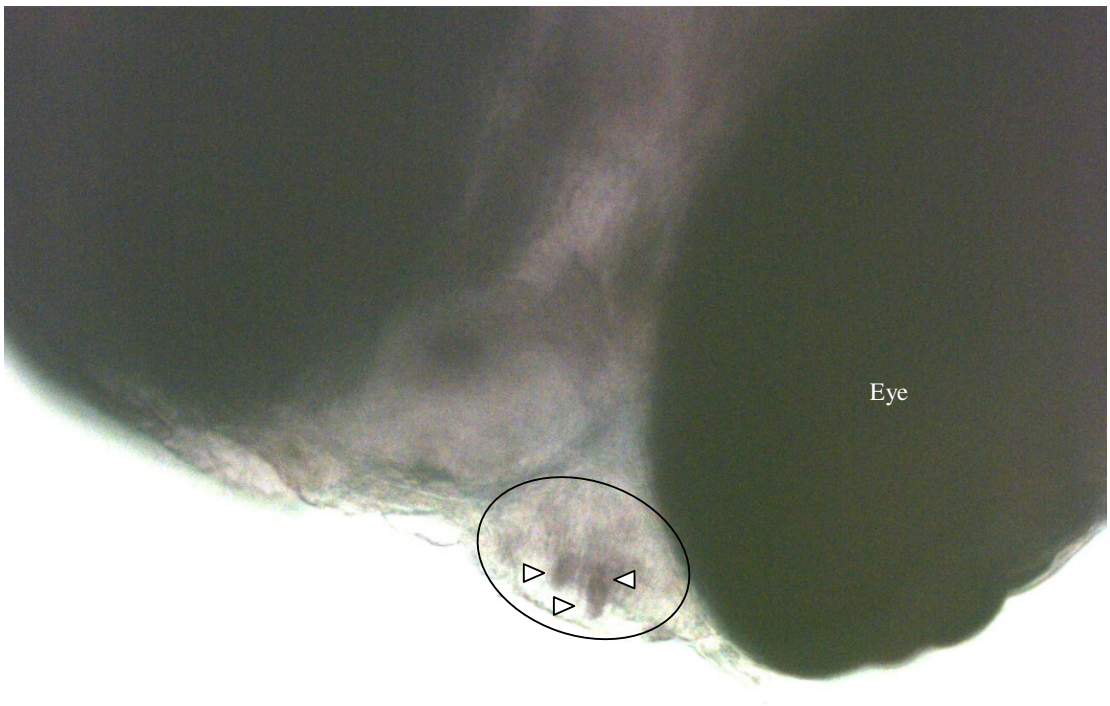


Figure 4.12. In-situ hybridization of 72 hpf zebrafish embryos with DIG-UTP-labeled antisense OR101-1 RNA probe. Embryos were visualized under 40X magnification with an inverted microscope. OR101-1 expressing cells are considerably higher in number compared to 24 hpf embryos. The clusters of cells (arrows) expressing OR101-1 are denser in comparison to 48 hpf embryos.

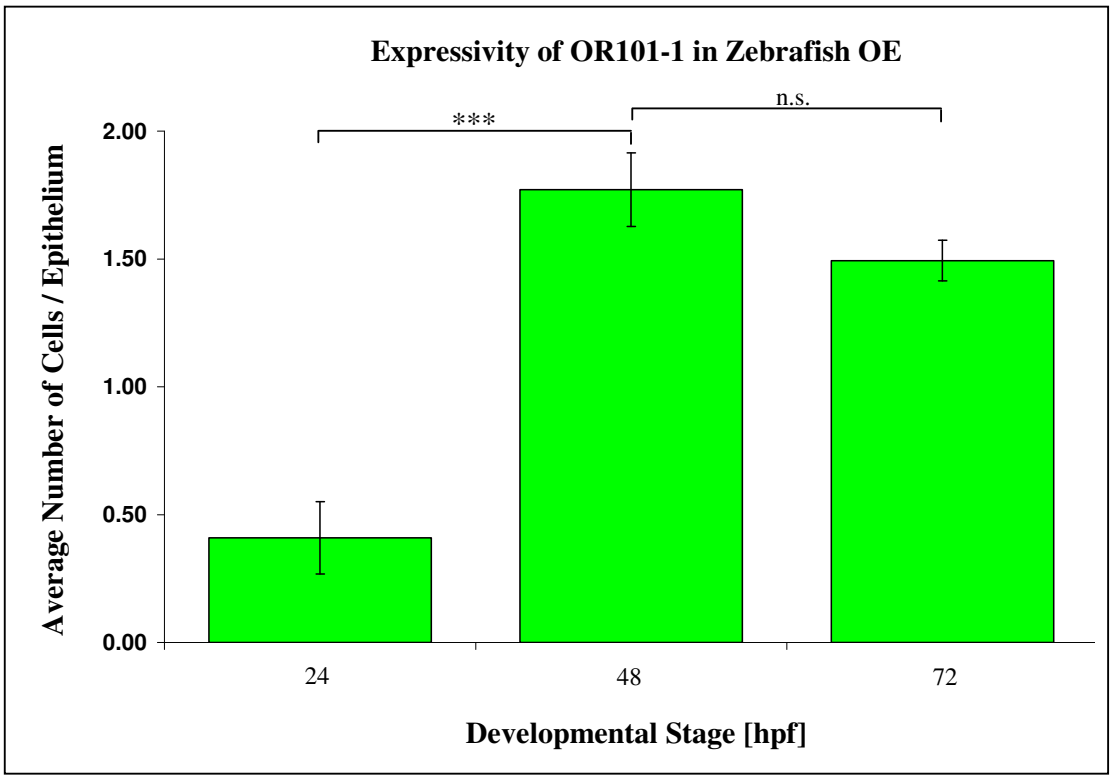


Figure 4.13. The average number of cells expressing OR101-1 in zebrafish OE during early developmental stages. The number of OR101-1-positive cells were counted for three post-fertilization time points: 24, 48, and 72hpf. The average number of positive cells per epithelium substantially increase between 24 and 48h but are stagnating between 48 and 72 hpf. The significance of increases was analyzed using an unpaired Student's t-test (two samples, two tailed), *** $P<0.0001$, n.s. not significant.

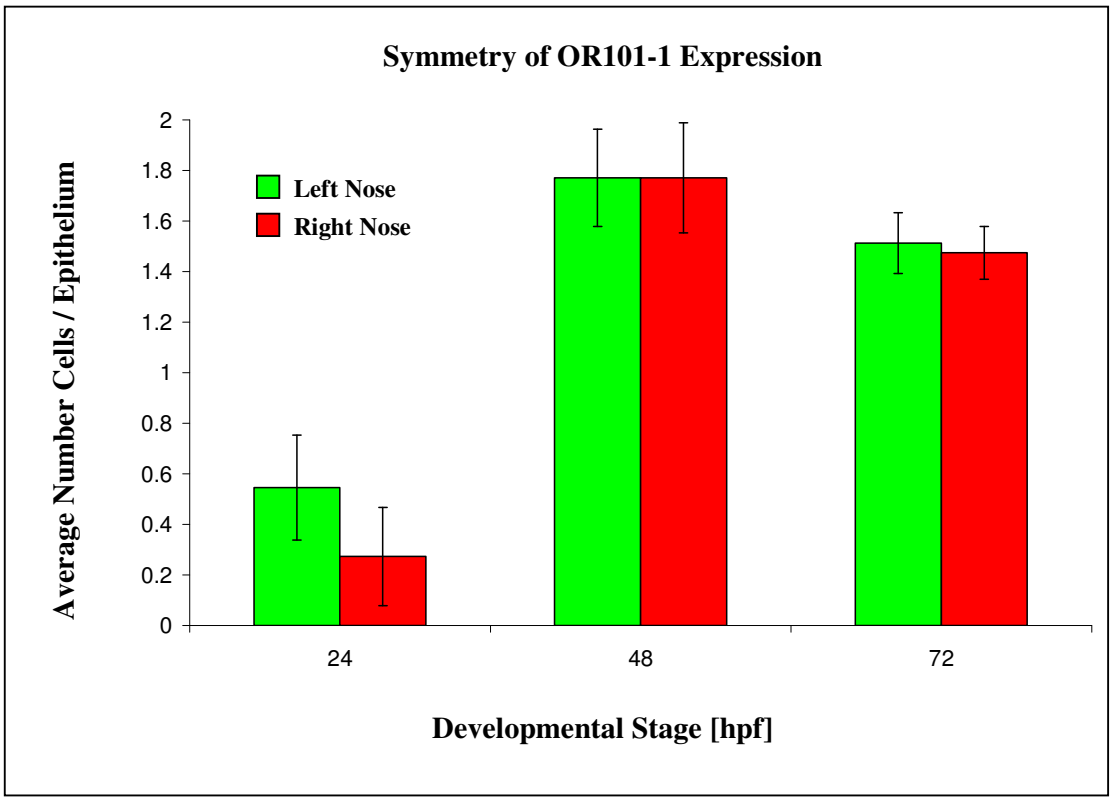


Figure 4.14. The average number of cells expressing OR101-1 per nose epithelium for three post-fertilization time points. The number of OR101-1-positive cells per nose was counted from every embryo and grouped according to post-fertilization stages. In all three time points, symmetry in the number of OR101-1 positive cells is observed.

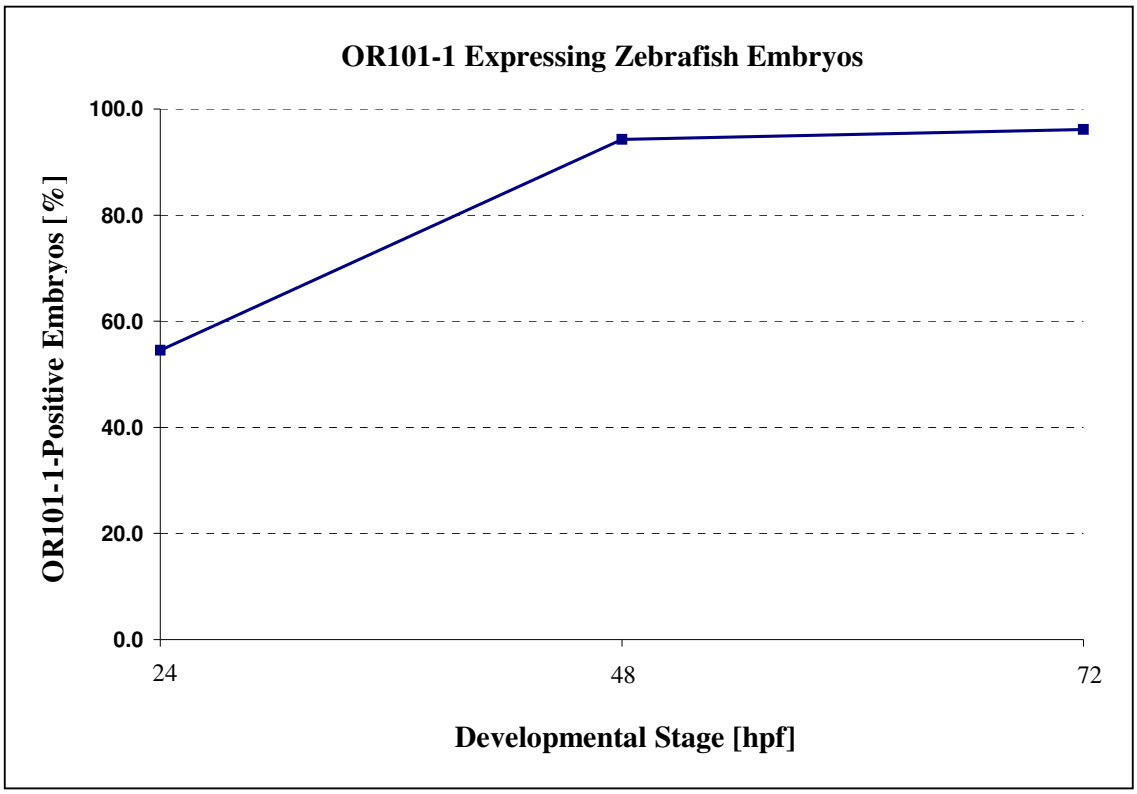


Figure 4.15. Penetrance of OR101-1 expression. Analysis of the number of early developmental zebrafish embryos showing at least one OR101-1 expressing cell in the OE. For each developmental time point, the number of embryos expressing at least one OR101-1-positive cell was divided by the total number of embryos from the same group. Beginning with 48 hpf, the expression of OR101-1 has started in more than 90% of the observed population of embryos.

4.6. Preparation of Deletion and Tag Constructs for BAC Recombineering

The complete progression of the BAC recombineering process is as depicted in Figure 4.16. I intended to create two different transgenic BAC constructs that will visualize OR101-1 expression. The first is a tag construct in which a yellow fluorescent protein sequence (Venus) is fused to the 3′ end the OR101-1 coding sequence via an internal ribosomal entry site (IRES) sequence. The expression of this construct in zebrafish will allow us to visualize OR101-1 expressing OSNs and to track the axonal projection of OR101-1 expressing OSNs from the OE into specific glomeruli on the OB. The second construct is a deletion construct in which the OR101-1 coding sequence is completely replaced by a Venus sequence. Since an OSN attempts to choose a second OR gene when its first promoter of choice fails to express a functional OR (Shykind *et al*., 2004; Serizawa *et al*., 2003), the expression of this construct will help us observe whether and how second choice will occur in OSNs which usually choose to express from the OR101-1 promoter. These studies will be particularly interesting, because second choice appears to be restricted by class (Class I vs. Class II ORs) in the mouse. Because the zebrafish genome contains only a single class II related OR, it would be interesting to see, whether second choice can take place and to learn about the specificity of genes that are targets for second choice. It will also allow us to examine whether the class distinction between class I and class II OR choice mechanisms occurred early during phylogenesis and as early as the occurrence of the first class II OR. The two different constructs were meant to be constructed by modifying bacterial artificial chromosomes (BAC) sequences containing the OR101-1 gene, as no information about the regulatory sequences of the OR gene are available. The mutations were intended to be introduced by the process of BAC recombineering (Lalioti and Heath, 2001). Therefore short recombination (targeting) constructs were designed and created. The arms flanking IRES-Venus in the tag construct, and in the Venus deletion construct act as homology arms during BAC recombineering

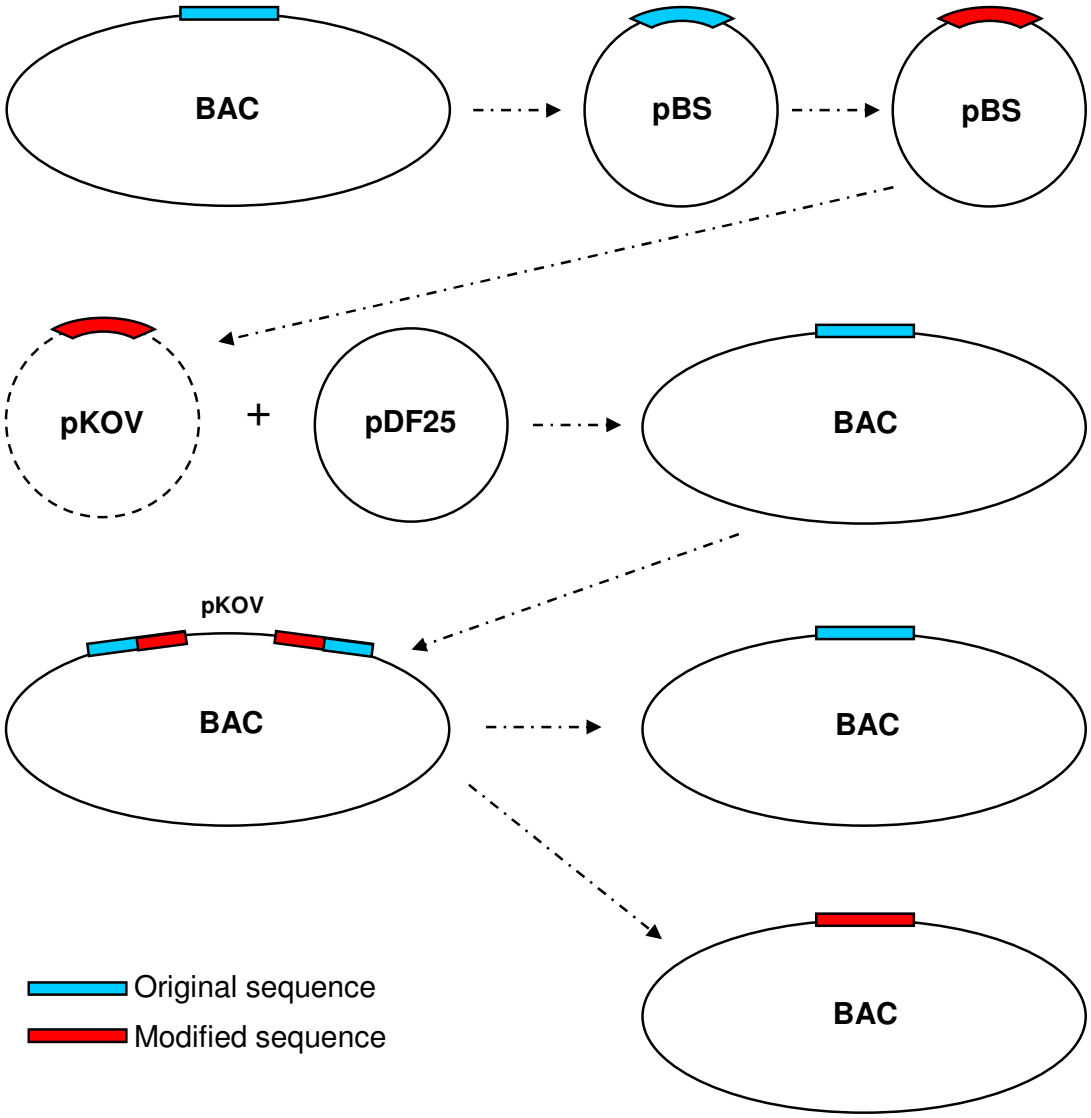


Figure 4.16. Overview of BAC recombineering. Original sequence is cloned from the BAC into pBS where the required modifications are done. The modified sequence is cloned into pKOV, and co-transformed with pDF25 (RecA coding plasmid) into competent cells containing the original BAC plasmid. pKOV plasmid carrying the modified sequence gets cointegrated into the BAC as a whole. Cointegrant cells are transformed with pDF25 again to induce recombination, which has two possible outcomes: BAC containing the original sequence and BAC containing the modified sequence.

4.6.1. Identification of a OR101-1 containing BAC

In order to locate a suitable BAC clone for OR101-1, Zv8 zebrafish Whole Genome Shotgun (WSG) assembly was scanned for OR101-1. Ensembl (www.ensembl.org) search results showed one BAC clone, CH211-218M3, as carrying the OR101-1 coding sequence. However, analysis of the CH211-218M3 sequence revealed the OR101-1 gene to be closer to the 3′ end of the BAC than we preferred, and since no information is available concerning the regulatory regions of OR101-1, we prefer to include as much upstream and downstream sequence in the final construct as possible. Searching the Sanger Zebrafish Genome Fingerprinting Project database for CH211-218M provided us with a number of alternatives, one of which, the DKEY206-L8 BAC, covered the ideal amount of OR101-1 upstream and downstream sequence. However, since DKEY206-L8 itself had not been sequenced yet, we had to construct its sequence manually, using sequences from two neighboring BAC clones: CH211-218M3 and DKEY36I3. The sequences from these two BAC clones were joined together using Vector NTI Advance 11.0 and the end sequences of DKEY206-L8 obtained from Sanger database were located on the complete sequence to identify the exact reach of DKEY206-L8 sequence (Figure 4.17). DKEY-206-L8 BAC clone designed by Keygene was used as a template for every amplification reaction relating to OR101-1.

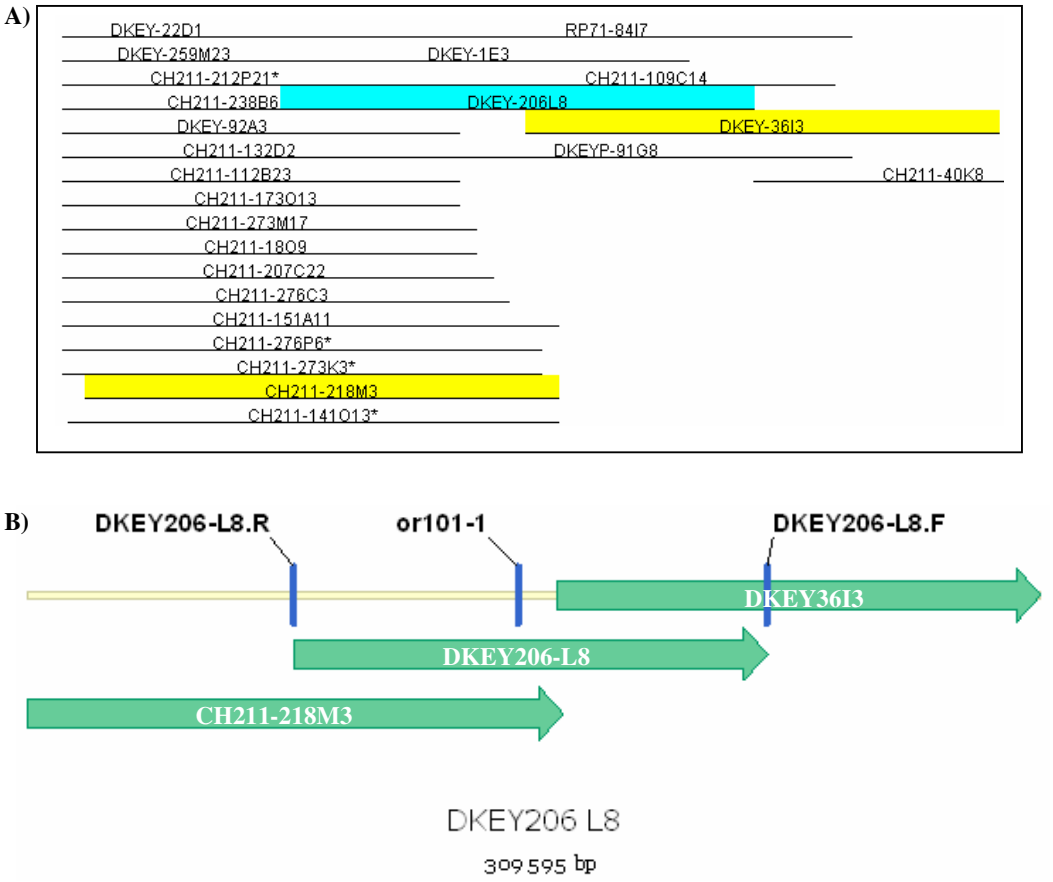


Figure 4.17. Identifying the DKEY-206L8 BAC clone. (A) Sanger WebFPC (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebra/large.shtml) view of related BAC clones. DNA sequences are available for yellow colored BAC clones. (B) VectorNTI Advance 11.0 image displaying the relative genomic coverage of CH211-218M3, DKEY206-L8 and DKEY36I3. OR101-1 coding sequence is located in the center of the DKEY206-L8 BAC clone.

4.6.2. Cloning of recombination constructs

First, a custom polycloning site was created in pBS-KS(-) by annealing XXSB_top and XXSB_bottom primers (see Table 3.1), encoding 5 restriction sites flanked by KpnI and SacI overhangs (killing these sites upon ligation) in the following order: SalI, PstI, EcoRI, SphI, and SalI. The original pBluescript II KS- vector (pBS) was digested using KpnI and SacI restriction enzymes, and the polylinker was ligated into the linearized

vector, creating a modified pBS vector with a custom polycloning site. The modification was confirmed by colony PCR (Figure 4.18; Bayramlı and Fuss, unpublished).

Figure 4.18. Colony PCR analysis of the XXSB polycloning site in pBS-KS−. The introduced polycloning site was amplified from pBS using M13-F and M13_R_highTM primers. The XXSB polycloning site is shorter (164bp) in comparison to the original pBS polycloning site (265bp).

The left homology arm for the tag construct was amplified using 101_PstI_F and 101_EcoR_R primers which introduce new restriction sites. The right homology arm was amplified using 101_EcoR_F and 101_SphI_R primers. Both fragments were cloned into the modified pBS-XXSB vector, using the introduced PstI/EcoRI and EcoRI/SphI sites, respectively. 101-EcoR_F and 101_EcoR_R primers were specifically designed to introduce an extra EcoRI restriction site 3 bases downstream of the OR101-1 stop codon via site-directed mutagenesis. An IRES-Venus sequence was created by modifying an IRES-Tau-Venus-ACNF encoding pBS vector through enzymatic digestion. The IRES-Venus fragment, which was flanked by two EcoRI sites, was cloned into the EcoRI site of the modified pBS vector containing the left and right homology arms (Figure 4.19). 12 colonies were scanned, of which only two colonies, 5 and 12, were positive (Figure 4.20). Diagnostic restriction digest analysis showed that only colony 12 had the construct in the right orientation (Figure 4.21). Plasmid sample from Colony 12 was sent to sequencing. Results were analyzed via Vector NTI Advance 11.0 ContigExpress software and no mutations were found (data not shown). After confirming the validity of the results, the constructs were digested out of the pBS vector using the two flanking SalI sites, and cloned into the SalI site of the recombination vector pKOV-Kan (Lalioti and Heath, 2001) vector in both forward and reverse orientations (Figure 4.22). Plasmids from both orientations were checked by diagnostic restriction digest analysis (Figure 4.23, 4.24).

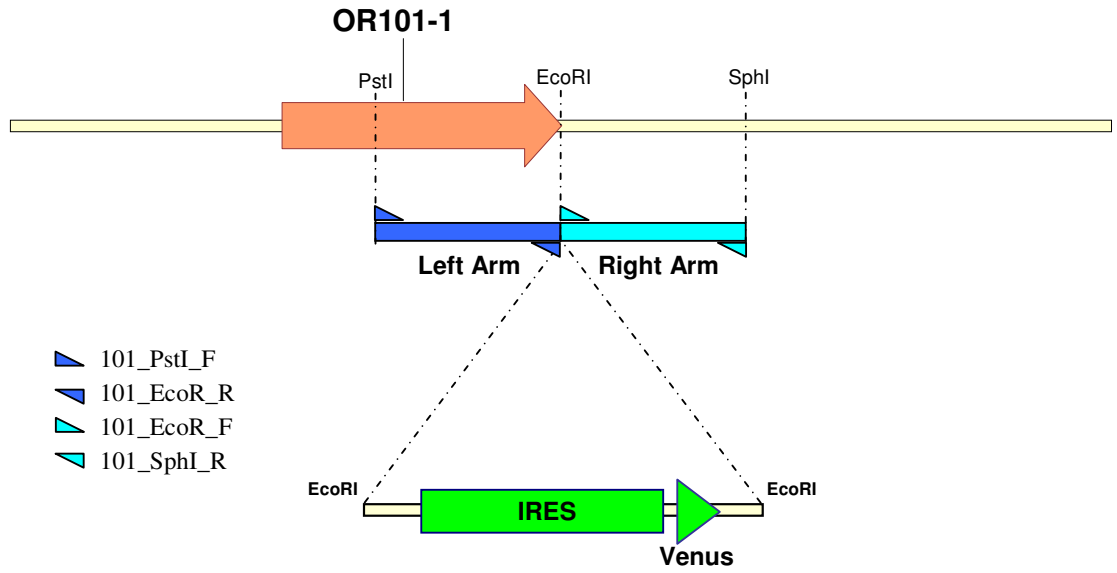


Figure 4.19. Cloning of the OR101-1-IRES-Venus construct. Left and right homology arms (382bp and 533bp, respectively) were cloned into pBS. IRES-Venus (1361bp) was later cloned into the EcoRI restriction site between the homology arms.

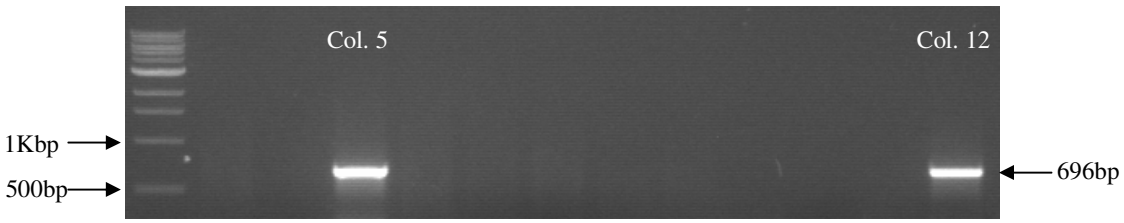


Figure 4.20. Colony PCR analysis of OR101-1-IRES-Venus in pBS. 2 positive colonies (5 and 12) were acquired out of 12 colonies scanned using GFP_F_819 and T7_highTM primers.

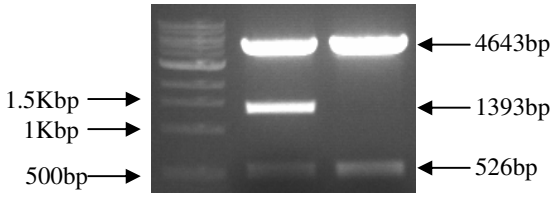


Figure 4.21. Diagnostic digest of OR101-1-IRES-Venus in pBS. Plasmids from colonies 5 and 12 were digested using HindIII. Colony 5 was a mixed colony carrying the IRES-Venus sequence in both forward and reverse orientations. Colony 12 was positive.

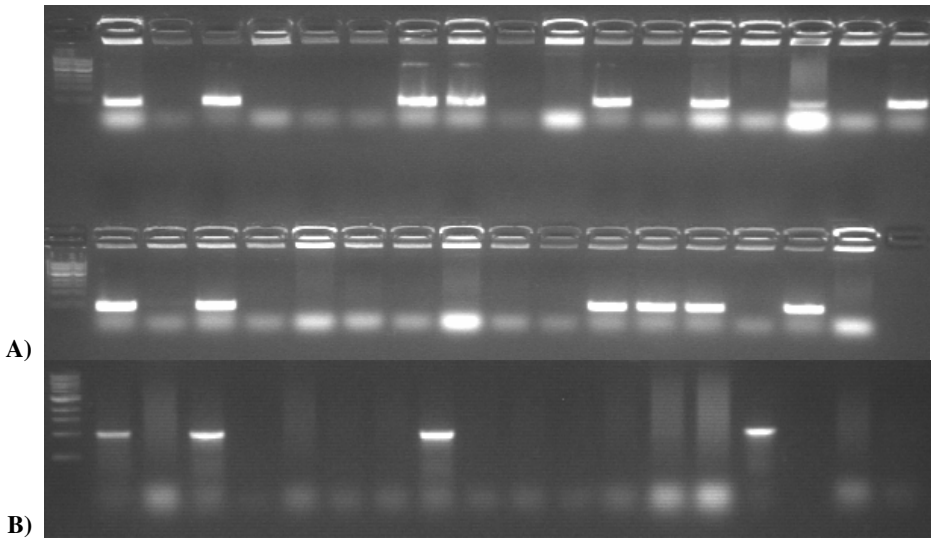


Figure 4.22. Colony PCR analysis of OR101-1-IRES-Venus in pKOV-Kan. (A) 14 positive colonies were acquired out of 36 colonies scanned using pKOV-Kan-F and 101-1-R primers. (B) 4 positive colonies were acquired out of 18 colonies scanned using pKOV-Kan-F and GFP_F_819 primers.

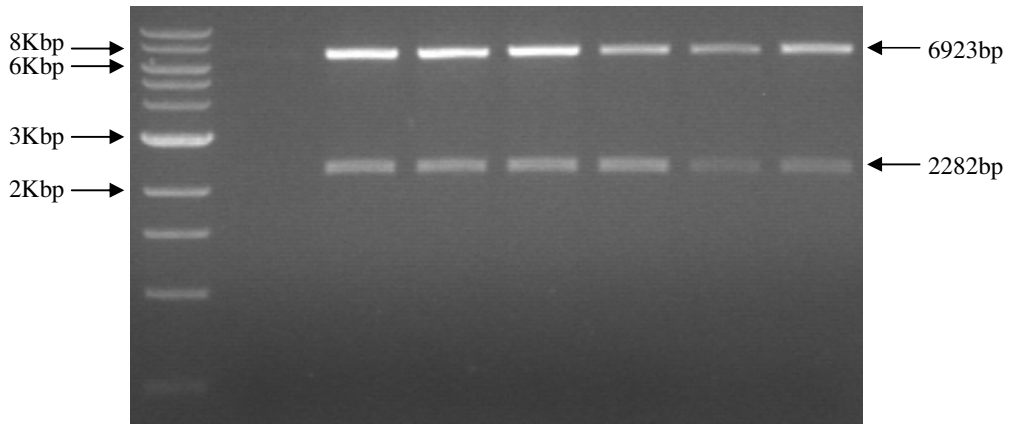


Figure 4.23. Diagnostic digest of OR101-1-IRES-Venus in pKOV-Kan in forward orientation. Plasmids from colonies 1, 3, 13, 17, 31, 32 were digested using SalI, which should release the construct from pKOV-Kan. All 6 colonies turned out positive and in forward orientation.

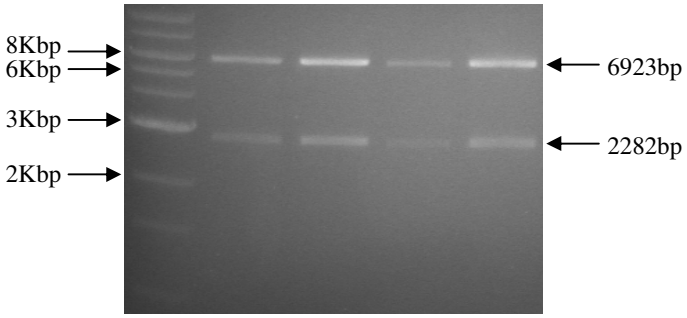


Figure 4.24. Diagnostic digest of OR101-1-IRES-Venus in pKOV-Kan in reverse orientation. Plasmids from colonies 1, 3, 13, 17, 31, 32 were digested using SalI, which should release the construct from pKOV-Kan. All 4 colonies turned out positive and in reverse orientation.

The same downstream homology sequence was also used for the deletion construct (Figure 4.25). The left homology arm was amplified using 101_V_Pst_F and 101_V_Over_R primers. The complete Venus sequence was amplified using 101_V_Over_F and 101_V_Over_R primers. The two amplified products were then used

as templates in an overlap PCR reaction, using 101_V_Pst_F and 101_V_EcoR_R primers (Figure 4.26). The final product (1246bp) was ligated into the PstI-EcoRI site of the pBS vector containing the right homology fragment.

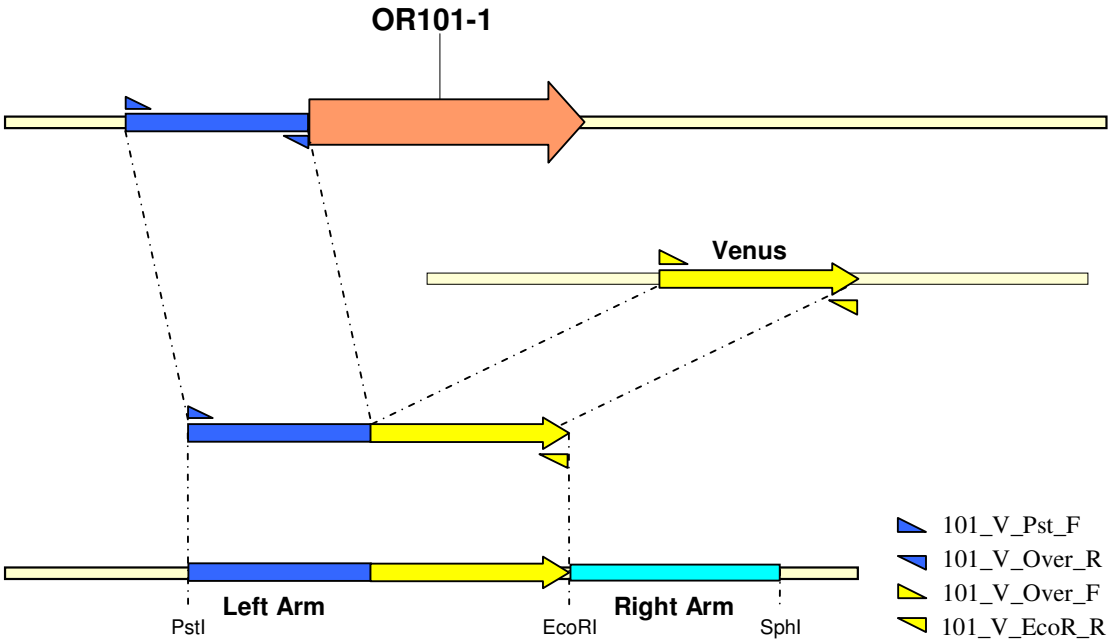


Figure 4.25. Cloning of the Venus-Deletion construct. Venus sequence (720bp) was fused to the left homology arm (526bp) via overlap PCR. The overlap PCR product was cloned into pBS together with the right homology arm (533bp).

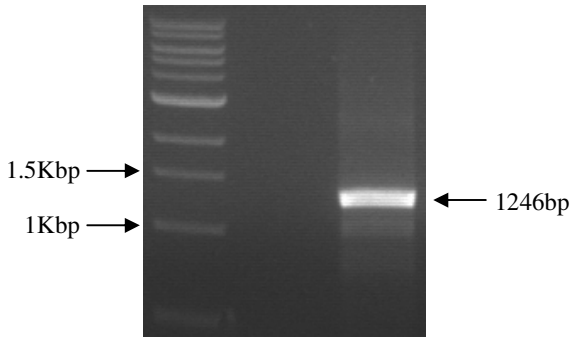


Figure 4.26. Overlap PCR of the left homology arm and Venus coding sequence using V_Pst_F and 101_V_EcoR_R primers.

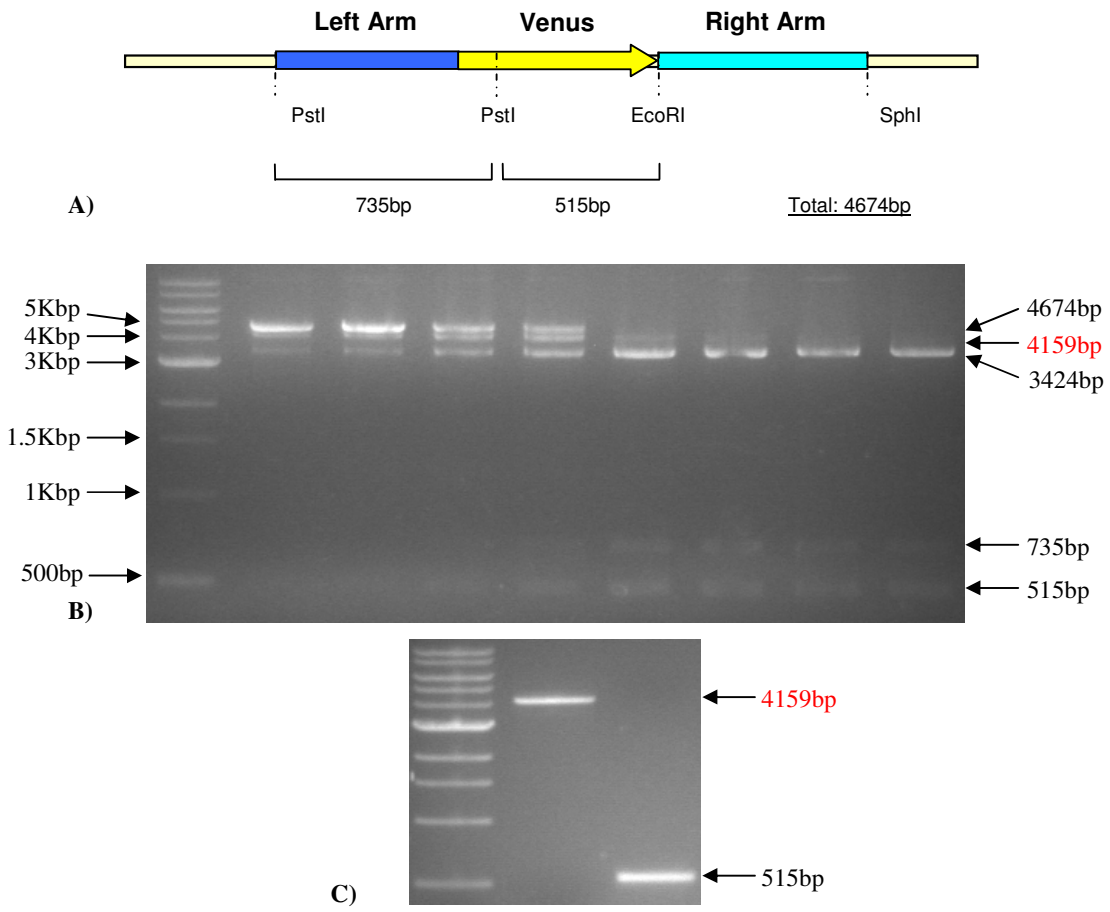


Figure 4.27. Repairing the Venus-Deletion construct. (A) The point mutations are in the 515bp-long PstI-EcoRI fragment of the Venus-Deletion construct. (B) The EcoRI-linearized vector is partially digested using PstI. Aliquots from the inital reaction are taken at 5 minute time points and heat inactivated to stop digestion. (C) The mutation free fragment (515bp) is ligated to the digested vector lacking the corresponding site (4159bp).

Following the final diagnostic restriction enzyme analysis, plasmid from the only positive colony that was found, colony 11, was sent to sequencing. Sequencing results turned out two point mutations (missense and nonsense) in Venus: $298_{T\rightarrow C}$, $472_{C\rightarrow T}$. The mutated region was contained between a PstI and an EcoRI site. Therefore, we attempted to repair the sequence using a mutation-free sequence from a parallel clone for another

gene (Figure 4.27A). Plasmid from colony 11 was first linearized with EcoRI. Then a partial digest was done using PstI. A 10µl aliquot was taken from the digestion reaction and heat inactivated every 5 minutes, while adding 1µl of 1:10 diluted PstI to the reaction every 10 minutes. Aliquots were observed with agarose gel electrophoresis, and the desired digestion condition was marked (Figure 4.27B). PstI digestion was repeated with optimum conditions, and the right band was gel extracted (Figure 4.27C). The mutation-free insert was ligated to the vector lacking the original sequence. The final construct was extracted using SalI and ligated into pKOV-Kan in both forward and reverse orientations (data not shown).

4.7. BAC Recombineering

pKOV-Kan plasmids containing both forward and reverse orientations of the tag and deletion constructs were co-transformed with pDF25 into DKEY-206-L8 containing competent *E. coli*. The pDF25 vector contains a sequence coding for the RecA protein under control of a temperature sensitive origin of replication (Imam *et al*., 2000). After an overnight incubation at 30°C, 4-6 colonies were pooled in 1ml LB and spread on Cm+Kan selection plates and incubated overnight at 43°C. Resulting colonies were scanned by colony PCR for both possible orientations (Figure 4.29) of co-integration using a primer pair consisting of a primer endogenous to the BAC (101_Rec_5F or 101_Rec_3R) and a primer endogenous to the pKOV-Kan construct (pKOV_Kan_F, pKOV_Kan_R, GFP_819 or GFP_R_5). Only 18 co-integrants for the OR101-1-IRES-Venus construct were found, which were all from the second orientation (Figure 4.28). Co-transformation for OR101-1-IRES-Venus reverse and Venus-Deletion was repeated 3 times each, where the transformed cells were plated in 1:1000, 1:10,000 and 1:20,000 dilutions. For the Venus deletion constructs an alternate RecA incubation method was also utilized, where instead of an overnight incubation on agar plates, the cells were incubated in LB medium for 6 hours at 30°C. However those cultures also failed to give any colonies on the Cm+Kan plates after 43°C overnight incubation.

According to Lalioti and Heath (2001), any descendant of a particular co-integrant *E. coli* colony will always resolve in only one of the two possible resolution outcomes (Figure 4.30). Therefore 6 out of 18 positive co-integrant colonies were made competent

and were re-transformed with pDF25. Transformed cells were plated on Cm plates and incubated overnight at 30°C for RecA induction. Positive colonies were pooled and re-plated on subsequent selection plates. 4 colonies from each co-integrant were streaked separately on Cm+Kan+sucrose and Cm+sucrose plates. Correctly resolved colonies were expected to grow on Cm+sucrose plates, but not on Cm+Kan+sucrose plates, since the KanR encoding pKOV-Kan is supposed to have resolved out. However, all the colonies tested grew on both selection plates, suggesting a flaw in the stringency of previous selection plates. Selection procedure was repeated two more times; however, no colonies were observed after the first sucrose selection step both times.

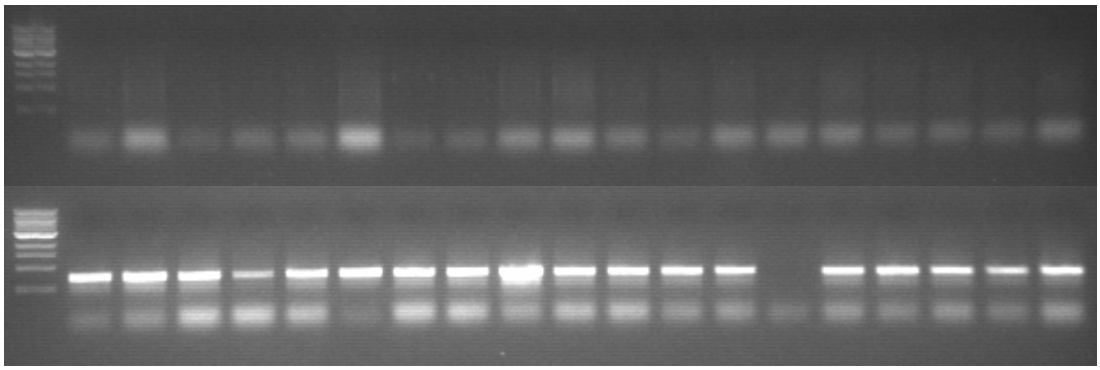


Figure 4.28. Colony PCR results for OR101-1-IRES-Venus co-integration into DKEY-206-L8. 19 colonies were checked using both pKOV_Kan_F / 101_Rec_3R (top) and GFP_F_819 / 101_Rec_3R (bottom). 10 colonies were found to be co-integrants for the tag construct in the second orientation.

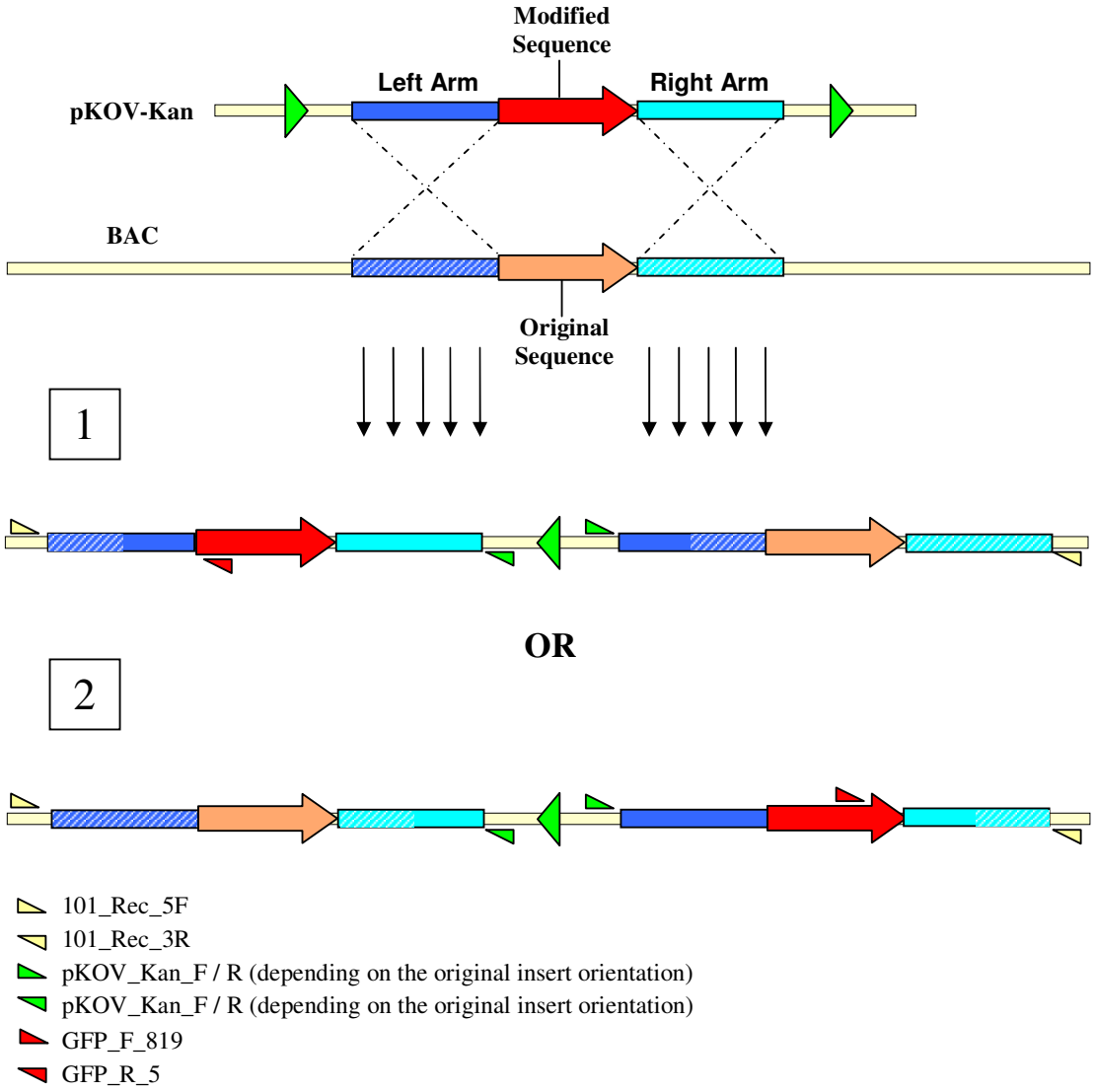


Figure 4.29. Co-integration step of the recombineering process. The modified sequence co-integrates into the BAC carrying the complete pKOV-Kan vector inside. The co-integration procedure can result in two possible orientations. Colony PCR is performed using one BAC-specific primer and one primer endogenous to the pKOV-Kan vector to ensure correct co-integration.

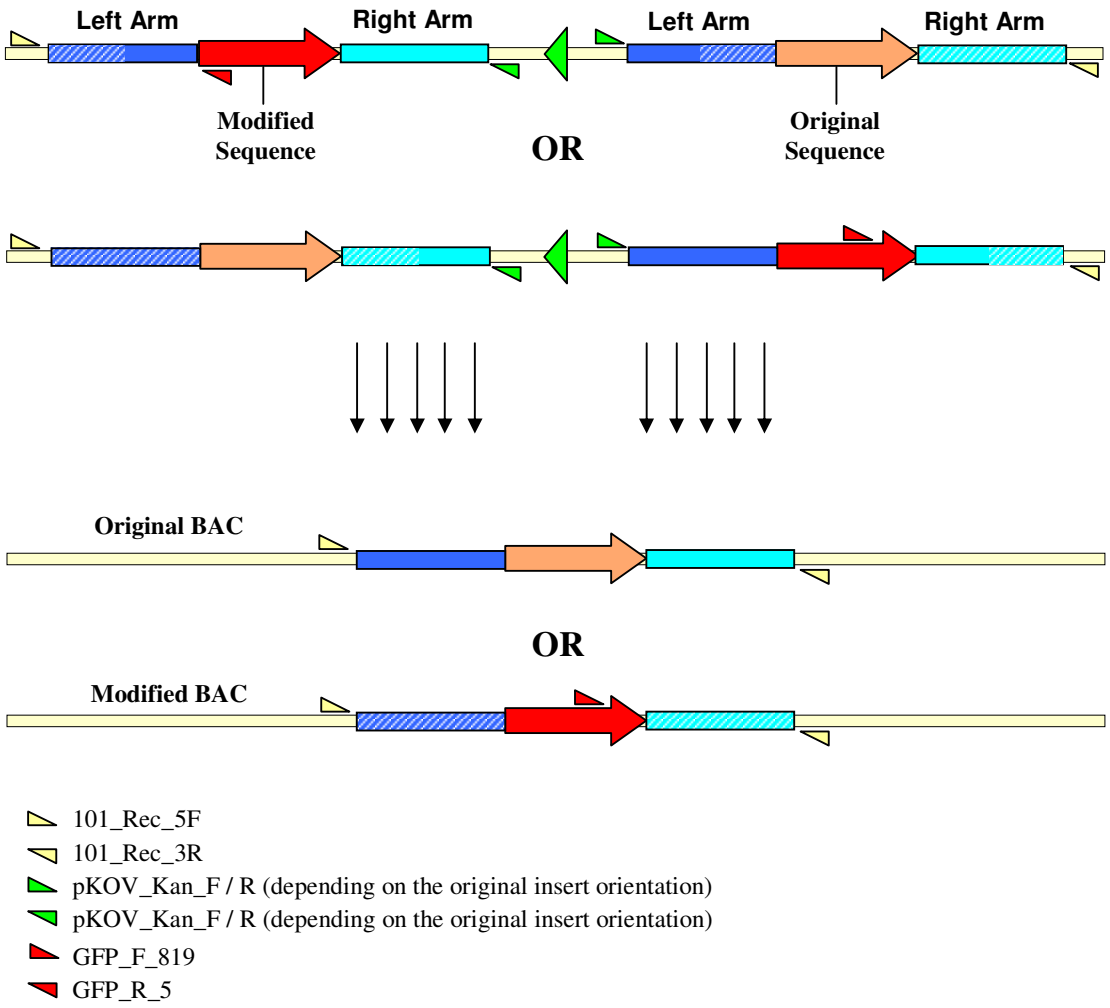


Figure 4.30. Resolution step of the recombineering process. After induction with RecA, homology arms recombine among each other which can result in two possible sequences. The introduced sequence can recombine out the exact way it co-integrated in the first place, resulting in the original BAC clone and the original pKOV-Kan construct, or the recombination can move out the original sequence, leaving the modified sequence in the BAC clone instead.

4.8. Expression of Transgene DNA in Zebrafish OSNs

In order to visualize transgene expression in OSNs and as a practice for plasmid injection into zebrafish oocytes, a construct containing a sequence coding for enhanced yellow fluorescent protein (EYFP) followed by a polyA tail downstream of the zOMP promoter sequence (pOMP; Celik *et al*., 2002) was used for injection. The construct was linearized by releasing it from the parent vector pBS-KS(-) by PvuI digestion and purified by gel extraction.

Fertilized zebrafish eggs were collected directly after spawning. ~50 eggs were injected with 50ng/µl of pOMP-EYFP DNA each. Embryos were then placed in E3 medium for further development. Embryos were checked for EYFP expression under a fluorescent stereomicroscope the next day. Of the 10 surviving embryos, 3 embryos were observed that expressed EYFP in their OSNs (Figure 4.31).

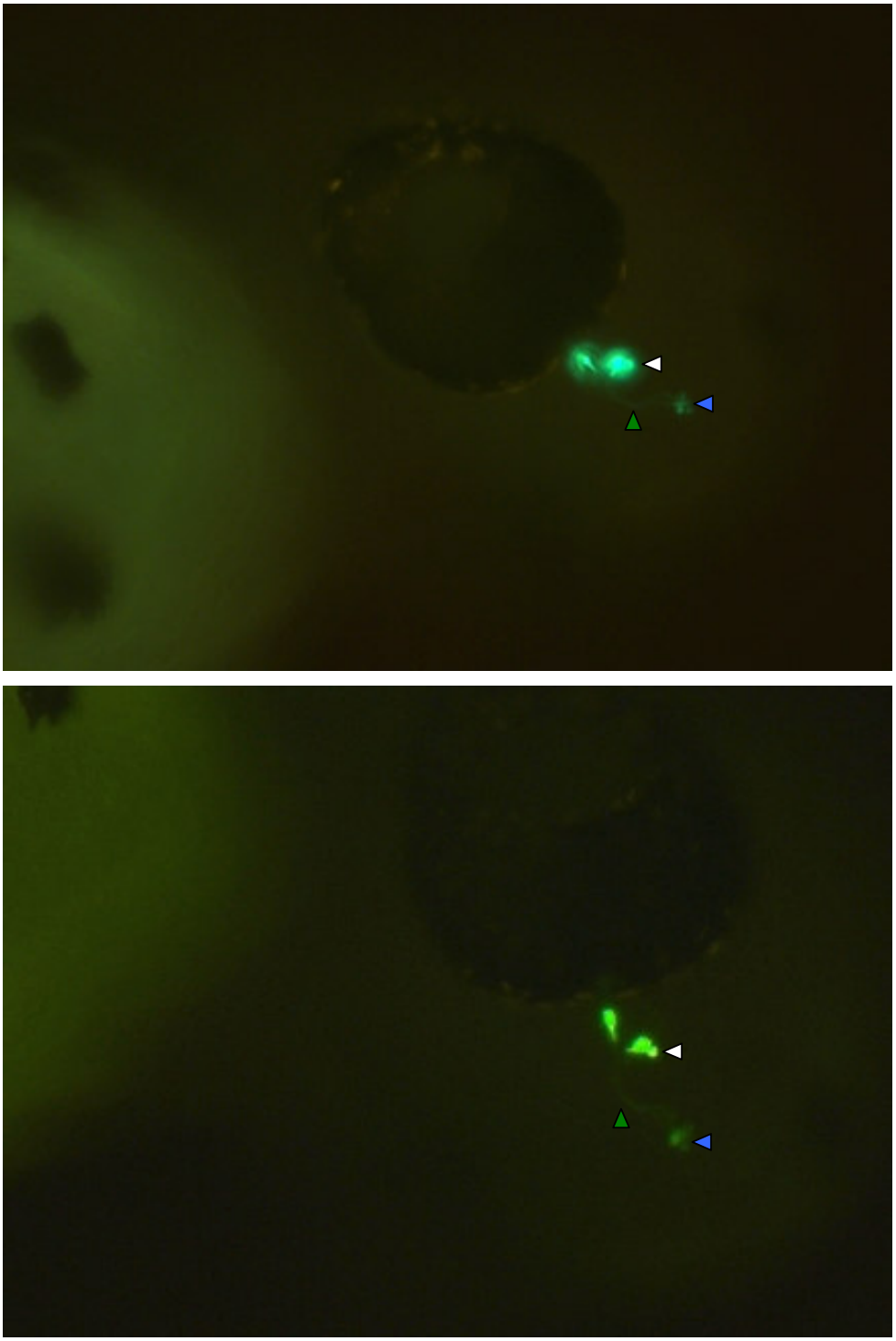


Figure 4.31. Transgene expression of EYFP in zebrafish nose. Three OSNs with EYFP expression can be observed (white arrow). The EYFP expression continues on throughout the axons (green arrow) until reaching the OB (blue arrow).

5. DISCUSSION

ORs in mammals are classified under two major families, Class I and Class II, which have distinct mechanisms of gene regulation. It is also suggested that this class distinction plays a role in certain aspects of OR second choice mechanism of the mammalian olfactory gene regulatory system. This phenomenon is believed to be non-existent in fish which express only Class I ORs. The zebrafish model organism, for that matter, provides us with the perfect opportunity to study class distinction: a single Class II OR. Observing the reaction of naturally OR101-1 expressing cells to the second choice mechanism will provide us with more information concerning the absolute nature of the class distinction phenomenon, and whether or not this system works in a similar way in fish as well.

As a first step, we analyzed the phylogeny of OR101-1 to ensure that the gene is of Class II ancestry. OR101-1 amino acid sequence was compared to the zebrafish OR repertoire provided by Alioto and Ngai (2005). The alignment of 136 intact zebrafish OR genes was rooted to two fundamental GPCR sequences, adenosine A1 receptor and β2 adrenergic receptor. The tree displays OR101-1 in a remote branch, separated from any other cluster of OR genes but close to the human branch of class II ORs. The 8 zebrafish OR clades defined Alioto and Ngai can also be clearly seen in their appropriate sub-branches. Our version of the zebrafish OR phylogenetic tree perfectly matches the analysis done by Alioto and Ngai (2005). Following this data, we compared the same zebrafish OR sequences to the human OR database provided by Malnic *et al*. (2004). As expected, the Class I human ORs were grouped together with all of the zebrafish ORs except OR101-1 which was located on the major branch of Class II human ORs. OR101-1 was compared to the members of OR115 family, the closest OR cluster to OR101-1 on chromosome 21. No significant similarity was found. Three-spined stickleback (*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridis*), fugu (*Takifugu rubripes*), and medaka (*oryzias latipes*) species were also scanned for an OR homologous to OR101-1, but no similar genes could be found. All of these results suggest that OR101-1 might very well be the only example of a Class II OR seen in fish yet.

In order to validate that OR101-1 is a functional OR protein, we checked its expression via PCR amplification of zebrafish cDNA samples. Primarily, OE cDNA was amplified using OR101-1-specific primers and was shown to be expressed in the zebrafish nose. With the purpose of proving that OR101-1 expression is limited only to the OE, we compared its expression in 8 different zebrafish tissues (OE, eye, brain, gill, liver, kidney, sperm and ovary) together with zOMP, which is an OSN-specific marker, and β-Actin. Although some amount of background amplification is observed in all samples due to genomic contamination, OR101-1 was clearly amplified only in the OE sample. The product acquired from the ovary sample did not match the expected product length. The unexpected band is most probably the result of a non-specific amplification.

OR101-1 expression levels during early development were checked by PCR analysis of embryonic cDNA from 6 developmental time points (24, 48, 72, 96, 144 and 192 hpf). Positive controls were brightly amplified in each time point; however, OR101-1 had a faintly visible product until 72 hpf, after which the brightness of the band decreased even further before finally disappearing at 192 hpf. The result of this observation is most probably due to the relative growth of the zebrafish body to the OE, and the corresponding amounts of cDNA. The body grows at a much faster rate in comparison to the OE, and since equal amounts of total cDNA is used in every reaction, the amount of OR101-1 cDNA in each sample will be getting lower, resulting in a decrease in the amount of product amplified. Standard RT-PCR amplification did not reveal any observable fluctuation in the expression levels of OR101-1. For a more precise analysis, qRT-PCR was performed on 24, 48, 96 and 192 hpf cDNA and compared to the expression levels of zOMP. All time points except 48 hpf showed an expression level in a constant ratio to zOMP. The melting curve analysis displayed an extra shoulder in the 48 hpf reaction which might be due to non-specific amplification. Therefore, the results from the 48 hpf sample were discarded. The expressivity of zOMP during early developmental stages of zebrafish has been previously shown by Celik *et al.* (2002). The number of OSNs that express zOMP almost quadruples between 24 and 48 hpf, after which the rate of increase slows down. Correlating our findings with the presented material, we can say that OR101-1 expression follows a similar increase during the early stages of embryonic development.

In-situ hybridization of OR101-1 mRNA with DIG-labeled antisense RNA probes was performed to visualize the expression of OR101-1 in embryos. 24, 48 and 92 hpf embryos were checked for OR101-1 expression. Positive cells could easily be identified on the nose epithelia of embryos beginning with 24 hpf. Further statistical analyses of positive cells also show a significant increase in the average number of cells expressing OR101-1 between 24 and 48 hpf. Symmetrical expression of OR101-1 between each epithelium is also observed. However, although the obtained data seems accurate and in accordance with previous findings, a higher number of specimens would definitely allow for a more precise statistical analysis. It is also possible to increase the amount of probe used during hybridization since almost no background staining was observed. Increasing the probe concentration might allow us to stain more cells that express OR101-1. If we compare the expressivity and penetrance values we have obtained to those observed by Argo *et al*. (2003), we can see a symmetry between the expression patterns of OR101-1 and 2 OR genes they have tested, namely ZOR2, ZOR5 until 72 hpf. Argo *et al*. have divided the 10 OR genes they have analyzed into three groups depending on their expression dynamics. However, although OR101-1 shows a similarity in expression to ZOR2 and ZOR5 until 72 hpf, it would be wrong to assume a similar growth in OR101-1 expression at later time points since it is hard to deduce such an outcome from samples limited to only 72 hpf.

BAC recombineering provides us with an easy way to modify zebrafish sequences outside of the organism and create transgenic lines by injection of the modified clone into single-cell zebrafish embryos. Another advantage of using a BAC is that since it encompasses such a large region of the zebrafish genome, any possible upstream or downstream promoter/enhancer sites will automatically be included in the expression of the modified sequence. The modifications on the BAC clone are achieved via RecA induced recombination between the original sequence in the BAC and the modified construct in the pKOV-Kan plasmid. The aim of the OR101-1-IRES-Venus tag construct we designed is to help visualize the natural epithelial expression pattern and glomerular projection of OR101-1 expressing OSNs, whereas the Venus-Deletion construct will enable us to observe the response of OSNs which choose to express a deleted OR protein. This experiment has two possible outcomes: First, the cell abides by the class-restriction rule in OR second choice, and fails to choose another OR, since OR101-1 is the only Class II OR in the zebrafish OR repertoire. Second, the OSNs that attempted to express OR101-1

make random second choices among the closest Class I OR genes, in which case the Venus protein initially expressed by the OR101-1 promoter will allow us to observe the different axonal projections of these OSNs. Sato *et al*. (2007) have shown in zebrafish that the second OR choice is restricted to OR genes within the same cluster/family, which means that OR115 members could also possibly be potential candidates for second choice in the case of OR101-1 deletion.

Recombineering procedure progressed well for the OR101-1-IRES-Venus forward construct until the final selection step. The most probable results for that might be a problem with the levansucrase (SacB) selection method. The SacB gene that is carried into the BAC together with pKOV-Kan causes cells to die on growth media containing sucrose. After the resolution step, we screened for colonies lacking the pKOV-Kan sequence, and thus SacB, by growing the cells on sucrose medium. However, the SacB gene can acquire mutations frequently. If the last selection steps were flawed due to a non-functional SacB gene, this would allow cells that are still in co-integrant formation to pass through until the last differential selection plates where they would grow on both Cm+sucrose and Cm+Kan+sucrose. We have not been able to acquire any co-integrants for OR101-1-IRES-Venus reverse construct and both orientations of the Venus-Deletion construct. Most probably amount of pDF25 transformed and the amount of time required for RecA incubation needs to be optimized better. Lalioti and Heath (2001) state that the details of the procedure vary slightly for different BACs, therefore this method needs to be further optimized specifically for DKEY-206-L8 BAC.

The results of the embryonic injection of a transgene construct were as expected. About 20% of the embryos had managed to survive the injection and the penetrance of transgene expression was around 5% in total. However, even though the injection was successful, the success rate was unpredictable with repeated tries. The quality of the injections is highly dependent on the quality of the microinjection needles. A low quality needle can easily rupture the cell, or result in the injection of inconsistent amounts of DNA into the oocyte, which can result in a lack of expression or death.

In this study, I primarily aimed to learn more about the phylogenetic background of OR101-1 and its expression pattern in the zebrafish OE. The phylogenetic analyses we

performed suggest a Class II nature for OR101-1. OR101-1 was closely clustered together with the other Class II human ORs. The suggestion Alioto and Ngai (2005) have made concerning the expansion of an ancestral family of fish ORs related to OR101-1 into the current mammalian Class II OR repertoire seems plausible. However, the fact that no homologues for OR101-1 were found in the close relatives of zebrafish (three-spined stickleback, tetraodon, fugu, and medaka) suggests a possibility that the evolution of OR101-1 and mammalian Class II ORs occurred separately. RT-PCR and qRT-PCR analyses on temporal zebrafish cDNA samples displayed an early developmental expression pattern in the OE, which was further corroborated with in situ hybridization. qRT-PCR analysis of OR101-1 transcript levels in comparison to zOMP showed a parallel growth in expression (Celik *et al.*, 2002), supporting our previous findings. Statistical analysis of in situ hybridization results showed a discernible increase in the expression levels OR101-1 transcript over a period of 72 hours post-fertilization. However, for a more thorough conclusion on the complete expression pattern of OR101-1, in situ hybridization should be performed on later stages of development as well. BAC recombineering protocols need to be further optimized in order to achieve complete recombineering of the reporter and deletion constructs. The introduction of these transgene constructs will definitely be interesting since they will allow us to observe the expression of OR101-1 in the natural state of zebrafish and in a state of forced second choice. The deletion construct will hopefully answer some of our questions concerning the second choice mechanism in zebrafish. Since the OR115 family members are possible candidates for second choice, they also need to be studied by in situ hybridization and possibly a different reporter transgene construct. Olfaction research is still in its infancy, and we have much to learn. Hopefully the continuation of these studies will give us more insight into the workings of OR gene regulation in vertebrates.

APPENDIX A

Equipment

Autoclave sterilizers:	Astell Scientific Ltd., UK
Centrifuges:	Eppendorf, Germany (Centrifuge 5424, 5417R)
Cold room:	Birikim Elektrik Soğutma, Turkey
Electronic balances:	Sartorius, Germany (TE412)
Electrophoresis equipment:	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Fluorescence Stereomicroscope:	Leica Microsystems, USA (MZ16 FA)
Freezers:	Arçelik, Turkey
	Thermo Electron Corp., USA (Thermo Forma 723)
Gel documentation system:	Bio-Rad Labs, USA (Gel Doc XR)
Heating block:	Fisher Scientific, France (Dry-bath Incubator)
Heating magnetic stirrer:	IKA, China (RCT Basic)
Incubator:	Weiss Gallenkamp, UK (Incubator Plus Series)
	Nüve, Turkey (EN400)
Incubator shaker:	Thermo Electron Corp., USA (Forma Orbital Shaker)
Inverted Microscope:	Zeiss, USA (Axio Observer.Z1)
Laboratory bottles:	Isolab, Germany
Micropipettes:	Gilson, USA (Pipetman)
	Eppendorf, Germany (Research)
Microwave oven:	Vestel, Turkey
Microinjector:	Eppendorf, Germany (FemtoJet express)
pH meter:	WTW, Germany (pH330i)
Refrigerators:	Arçelik, Turkey
Software:	Invitrogen Corp., USA (Vector NTI 11.0)
Spectrophotometer:	NanoDrop Technologies, USA (ND-1000)
Stereo Microscope:	Olympus, USA (SZ61)

Thermal cycler:	Bio-Rad Labs, USA (C1000™ Thermal Cycler)
	Roche Apllied Science, USA (LightCycler® 480)
Vortex mixer:	Scientific Industries, USA (Vortex Genie 2)
Water bath:	Grant Instruments, UK (JB Aqua 12)

APPENDIX B

Supplies

Disposable Labware:

14 ml PP Tubes:

Greiner Bio-One, Belgium (187261)

CELLSTAR® 6-well plates:

Greiner Bio-One, Belgium (657160)

CELLSTAR® PP-Test tubes, 15ml:

Greiner Bio-One, Belgium (186161)

CELLSTAR® PP-Test tubes, 50ml:

Greiner Bio-One, Belgium (227261)

Fisherbrand® Micrscope Cover Glass:

Fisher Scientific, UK (12-545-F).

Fisherbrand® Superfrost®/Plus Microscope Slides:

Fisher Scientific, UK (12-550-15).

Filter Tips:

Greiner Bio-One, Belgium (771288, 772288, 740288)

Microtubes:

Citotest, China (34730015)

PCR Tube Strips:

Bio-Rad, U.S.A. (TBS0201)

Chemical Supplies:

1kb DNA Ladder:

New England Biolabs, U.S.A. (N3232L)

10X Titanium™ Taq PCR Buffer:

Clontech, U.S.A. (639142).

5X GoTaq® Colorless Flexi Buffer:

Promega, U.S.A (M890A).

Anti-Dioxigenin-AP-Fab Fragments:

Roche, Germany (11 093 274 910).

Alkaline Phosphotase, Calf Intestine (CIP):

New England Biolabs, U.S.A (M0290S).

BamHI:

Promega, U.S.A (R602A).

Blocking Reagent:

Roche, Germany (1 093 657).

Bovine Serum Albumin:

Promega, U.S.A (R396E).

Calcium chloride dihydrate for molecular biology:

Sigma-Aldrich., U.S.A. (C3306-500G).

DIG RNA Labeling Mix 10X:

Roche, Germany (11 277 073 910).

EcoRV:

Promega, U.S.A (R635A).

EcoRI:

New England Biolabs, U.S.A (R0101 S).

Ethanol Absolute Chromasolv®:

Sigma-Aldrich, U.S.A. (34870).

Ethidium Bromide Solution 10 mg/ml.

Sigma Life Sciences, U.S.A. (E1510-1ML).

Ethylenediaminetetraacetic acid (EDTA) disodium salt:

Sigma-Aldrich., U.S.A. (E5134-1KG).

Formamide, deionized, minimum 99.5% (GC):

Sigma-Aldrich, U.S.A. (F9037).

Glycerol, for molecular biology:

Sigma-Aldrich, U.S.A. (G5516-500ML).

GoTaq® Flexi DNA Polymerase:

Promega, U.S.A (M830B).

HindIII:

Promega, U.S.A (R604A).

Hydrochloric Acid 37%:

Riedel-de Haen®, Germany (07102).

Kpn I:

Promega, U.S.A (634A).

LB Agar:

Sigma Life Sciences, U.S.A. (SL08394).

LB Broth EZMix™ Powder:

Sigma-Aldrich, U.S.A. (L7658-1KG).

Lithium Chloride:

Merck, Germany (1056790100).

Magnesium Chloride, 25mM:

Promega, U.S.A (A351H).

Magnesium Sulfate Anhydrous:

Sigma-Aldrich, U.S.A. (M7506)

NBT/BCIP Stock Solution:

Roche, Germany (1 681 451).

NcoI:

Promega, U.S.A (R651F).

NotI:

New England Biolabs, U.S.A (R0189 L)

Paraformaldehyde:

Sigma-Aldrich, U.S.A. (P6148-1KG).

pGEM®-T Easy Vector Systems:

Promega, U.S.A (A137A).

Phenol:Chloroform:Isoamyl alcohol:

Sigma-Aldrich, U.S.A. (P2069).

Potassium Acetete, for molecular biology:

Sigma-Aldrich, U.S.A. (P119-100G).

Potassium Chloride:

Sigma-Aldrich, U.S.A. (P9541-500G).

Proteinase K:

Finnzymes, Finland (F202-L).

PstI:

New England Biolabs, U.S.A (R140 L).

RQI RNase-Free DNase:

Promega, U.S.A (M610A).

RQI RNase-Free DNase Buffer:

Promega, U.S.A (M198A).

RQI RNase-Free DNase Stop Solution:

Promega, U.S.A (M199A).

SacI:

Promega, U.S.A (R606A).

SalI:

New England Biolabs, U.S.A (R0138 L).

SeaKem® LE Agarose

Cambrex Bio Science, U.S.A (50004).

Sodium Acetate:

Sigma-Aldrich, U.S.A. (S8625)

Sodium bicarbonate, for molecular biology:

Sigma Life Sciences, U.S.A. (S7277-250G).

Sodium chloride, SigmaUltra:

Sigma-Aldrich, U.S.A. (S7653-1KG).

Sodium citrate tribasic dihydrate:

Sigma-Aldrich, U.S.A. (C8532-1KG).

Sodium hydroxide, SigmaUltra:

Sigma-Aldrich, U.S.A. (S8045-1KG).

SpeI:

Promega, U.S.A (R659D).

SphI:

Promega, U.S.A (R626A).

Sucrose, minimum 99.5% (GC):

Sigma., U.S.A. (S0389-500G).

T4 DNA Ligase:

Promega, U.S.A (M180A).

T7 RNA Polymerase:

Roche, Germany (10 881 767 001).

Titanium™ Taq DNA Polymerase

Clontech, U.S.A. (639208)

Trizma® Base:

Sigma-Aldrich, U.S.A. (T6066).

Tween® 20:

Sigma-Aldrich, U.S.A. (27,434-8).

XhoI:

New England Biolabs, U.S.A (R0146 S).

Yeast t-RNA:

Roche, Germany (10 109 223 001).

Commercial Kits:

DIG Nucleic Acid Detection Kit:

Roche, Germany (1 175 041).

Plasmid Midi Kit (100):

Qiagen®, U.S.A. (12145).

QIAprep® Spin Miniprep Kit (250):

Qiagen®, U.S.A. (27106).

QIAquick® PCR Purification Kit (250)

Qiagen®, U.S.A. (28106)

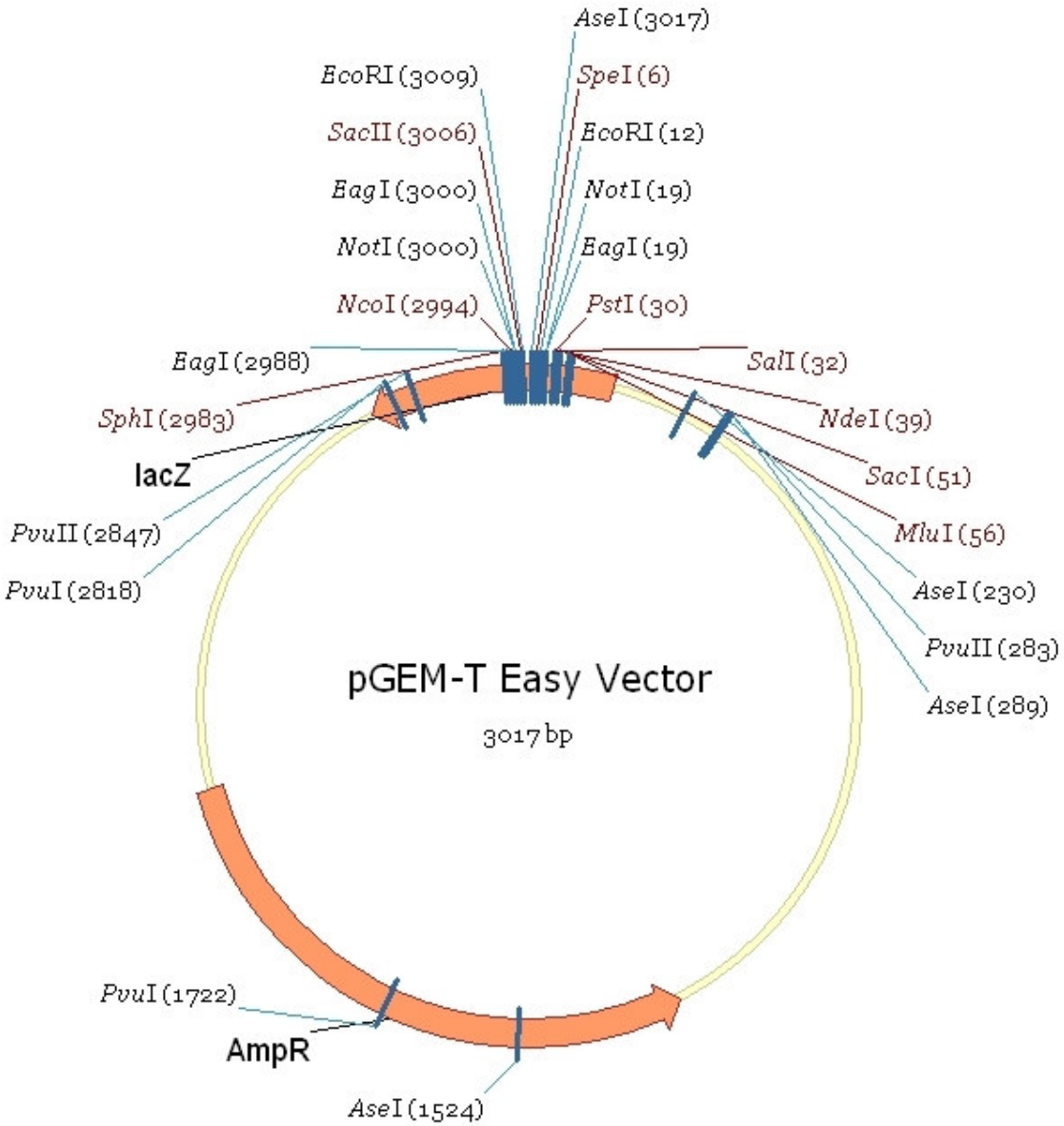
SuperScript® First-Strand Synthesis System for RT-PCR:

Invitrogen, U.S.A. (11904-018)

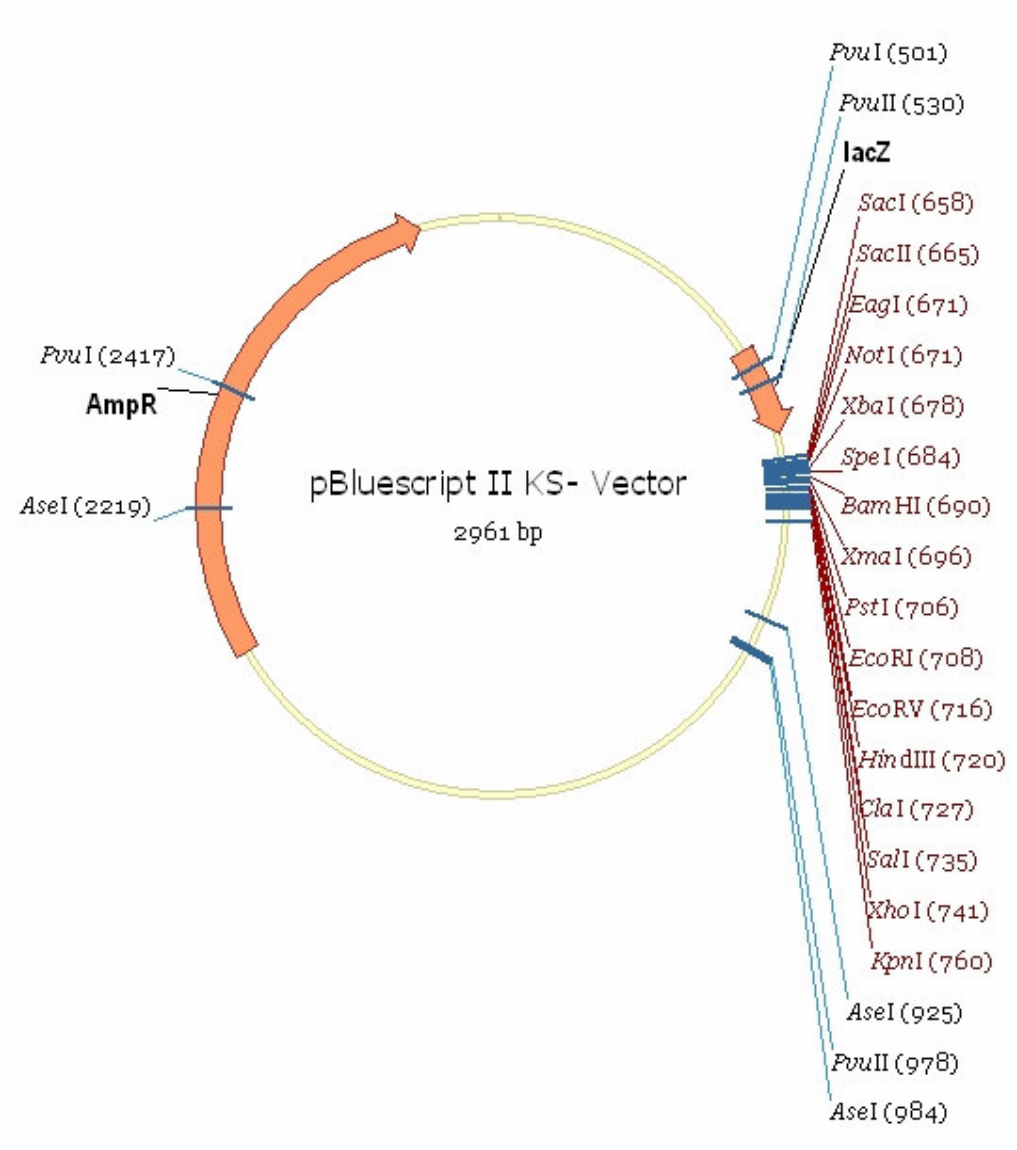
APPENDIX C

Vector Maps

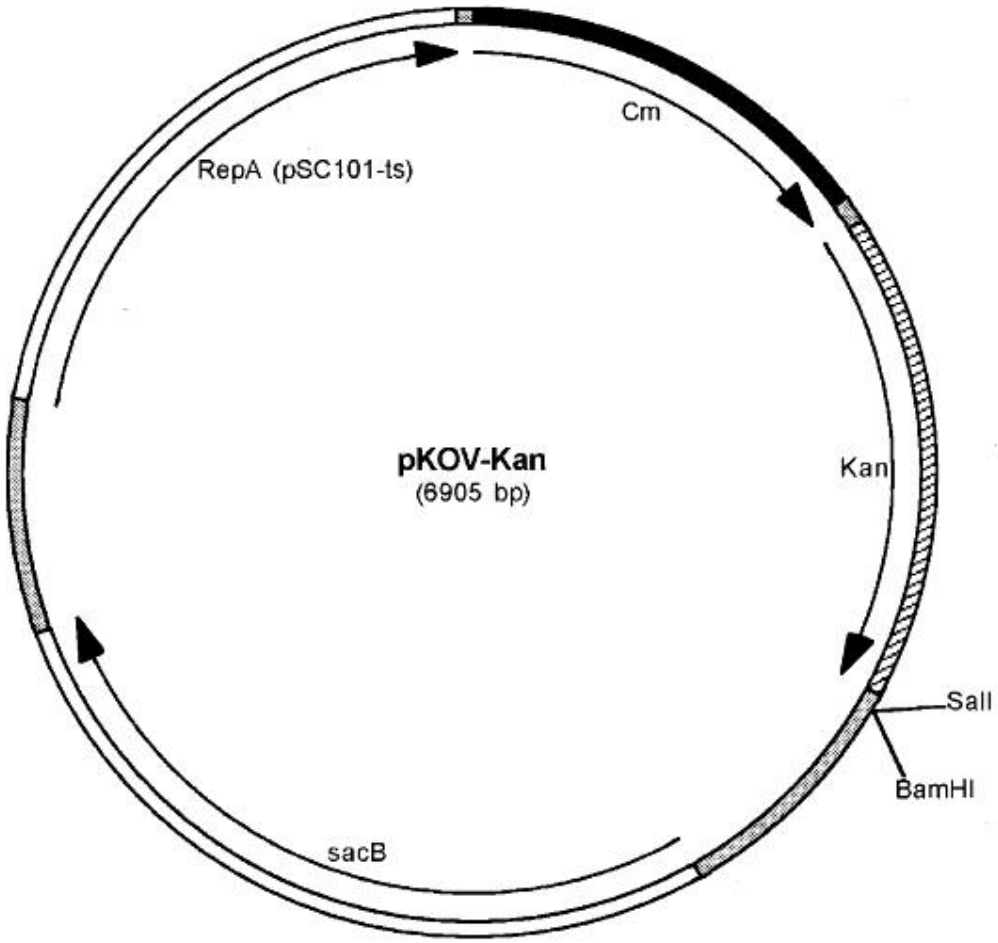
pGEM-T Easy Vector



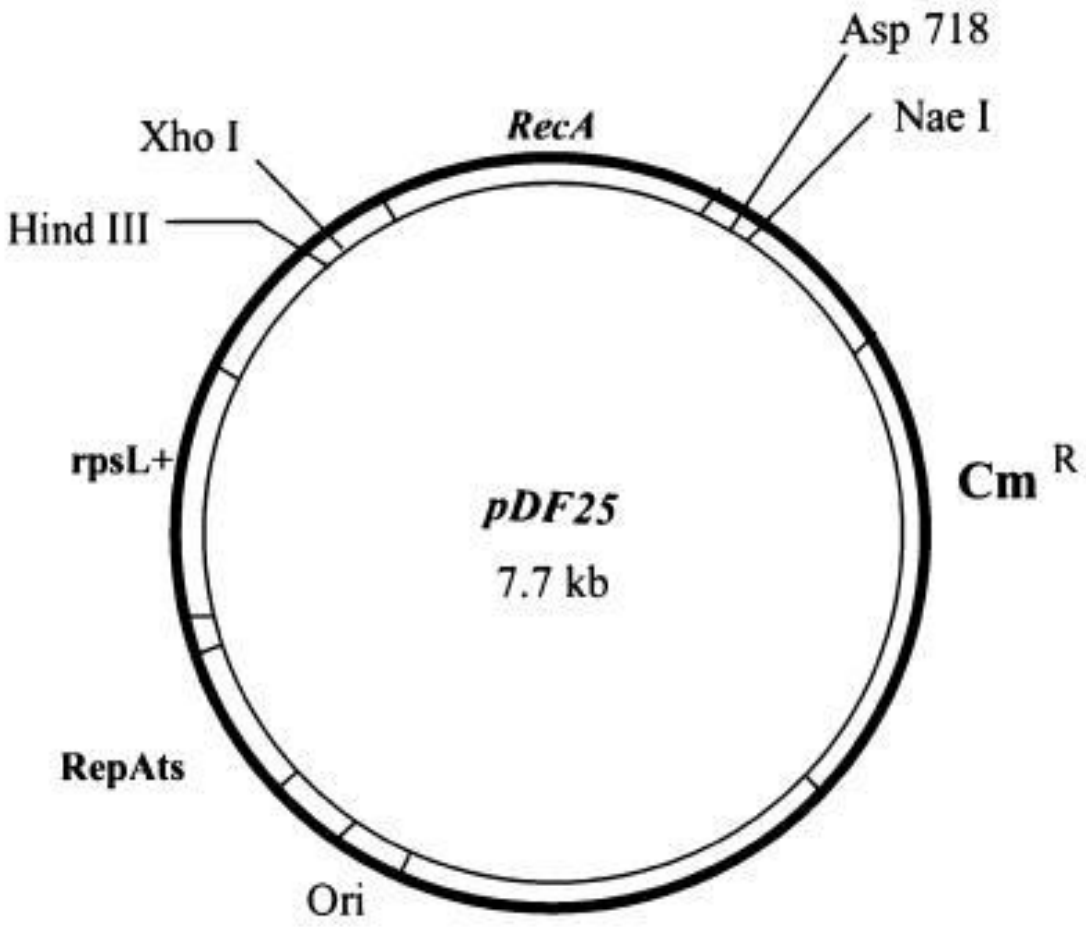
pBluescript II KS- Vector



pKOV-Kan Vector



pDF25 Vector



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