REGULATION OF OLFACTORY RECEPTOR GENE CHOICE BY NEGATIVE REGULATORY ELEMENTS

by

Gizem Sancer

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ABSTRACT

REGULATION OF OLFACTORY RECEPTOR GENE CHOICE BY NEGATIVE REGULATORY ELEMENTS

In the olfactory system, each olfactory sensory neuron (OSN) typically expresses only one allele of a single olfactory receptor (OR) gene from a vast genomic repertoire. The mechanisms of OR gene choice, however, are not well understood and may include epigenetic modification as well as locus-specific control by cis-regulatory sequences. It has been shown that both, proximal promoter regions and long-range interacting elements contribute to OR gene choice. Here, critical elements regulating expression of a model zebrafish OR gene, OR101-1, were identified and tested functionally using a transient transgenic approach. In particular, the negative regulatory effect of a specific 500 bp sequence (500i), located 2 kb upstream of the OR101-1 gene have been investigated. Using a promoter bashing approach, it was shown that transgenic constructs that included this 500 bp sequence were less effective, both in the number of embryos and OSNs per embryo that expressed a fluorescent reporter transgene. To further analyze the regulatory influence of this sequence, new transgenic constructs were generated by cloning 500i upstream of a strong OR gene promoter and scored for transgene expression in early embryos. Inclusion of the sequence reduced transgene efficiency by 50%. Next, the role of two candidate transcription factor binding sites located within 500i were investigated using site-directed mutagenesis. Deletion of either one or both binding sites from transgenic constructs was sufficient to rescue high levels of transgene expression, suggesting that these sites, and transcription factors binding to these sites, may be responsible for the observed repressive effect. To further elucidate a biological function for this type of regulation, it was tested whether the sequence could act as insulator to shield the OR101-1 promoter from the influence of a nearby enhancer. Indeed, transgenic constructs that included 500i interspersed between a strong enhancer and the OR101-1 promoter resulted in a reduction of transgene-expressing OSNs. Mutation of the suspected transcription factor binding sites partially rescued the enhancer effect. These findings are significant since they functionally demonstrate, for the first time, that OR gene expression is also under control by repressive regulators.

ÖZET

KOKU RESEPTÖR GENLERİNİN SEÇİMİNİN NEGATİF DÜZENLEYİCİ ELEMENTLERLE DÜZENLENMESİ

Koku alma sisteminde, her bir koku duyu nöronu tipik olarak çok geniş genomik repertuvardan sadece bir tek koku reseptör geninin bir tek alelini ifade eder. Ancak, koku reseptörü gen seçim mekanizması, çok iyi anlaşılamamıştır ve epigenetik değişiklerle kontrol edilebilecği gibi cis-düzenleyici diziler tarafından bölgesel olarak da kontrol edilebilir. Hem proksimal promotör bölgelerinin hem de uzun mesafeli etkileşen elementlerin koku reseptörü gen seçimine katkıda bulunduğu görülmüştür. Bu çalışmada, gen ifadesi üzerinde önemli rol oynayan elementler geçici transgenik yaklaşım kullanılarak zebrabalığı koku reseptörü modeli, OR101-1, üzerinde tanımlandı ve bu elementler işlevsel olarak test edildi. Özellikle, OR101-1 geninin 2 kb yukarısında bulunan 500 bp dizisinin (500i) negatif düzenleyici etkisi araştırıldı. 'Promotor bashing' yaklaşımı kullanılarak, 500 bp dizisini içeren transgenik yapıların hem floresan ropörtör transgenini anlatan embiriyo sayısında hem de embriyo başına düşen koku duyu nöron sayısında daha az etkili olduğu gösterildi. Düzenleyici etkiyi daha derinden incelemek için, güçlü bir koku reseptörü promotörünün yukarısına 500i bölgesi klonlanarak yeni transgenik yapılar oluşturuldu ve erken embriryolarda transgen anlatımı skorlandı. Bölgenin dahil edilmesi transgen anlatımını %50 kadar düşürdü. Daha sonra 500i bölgesinin içinde bulunan iki aday transkripsiyon faktör bağlanma yeri, bölgesel yönlendirilmiş mutasyon kullanarak incelendi. Transgenik yapılardan yerlerinden birinin veya ikisinin silinmesi yüksek seviyedeki transgen anlatımını kurtarmak için yeterli oldu ve bu yerlerin ve bu yerlere bağlanan transkripsiyon faktörlerinin gözlemlenen baskılayıcı etkiden sorumlu olabileceğini gösterdi. Bu tür düzenlemenin biyolojik işlevini daha fazla aydınlatmak amacıyla, dizinin OR101-1 promotörünü yakındaki artırıcıların etkisinden korumak için yalıtıcı olarak davranıp davranmadığı test edildi. Gerçekten de 500i'nin güçlü artırıcı ile OR101-1 promötörünü ayırması transgen anlatan koku duyu hücrelerinin sayısında azalmayla sonuçlandı. Şüphelenilen transkripsiyon faktör bağlanma yerinin mutasyonu, artırıcının etkisini kısmi olarak kurtardı. Bu buluşlar ilk defa koku reseptörü geninin anlatımının baskılayıcı düzenleyicilerin etkisi altında olduğunu işlevsel olarak gösterdiği için önem arzetmektedir.

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LIST OF ACRONYMS / ABBREVIATIONS

AOB	Accesory Olfactory Bulb
ATF5	Activating transcription factor 5
bp	Base Pair
BSA	Bovine Setum Albumin
cAMP	Cyclic Adenosinemonophosphate
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
GFP	Green Fluorescent Protein
GPCR	G-protein-coupled Receptor
ISH	In situ hybridization
kb	Kilobase Pair
LCR	Locus Control Region
LSD	Lysine Demethylase
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
mRNA	Messenger Ribonucleic Acid
mOB	Main Olfactory Bulb
OB	Olfactory Bulb
OE	Olfactory Epithelium
OMP	Olfactory Marker Protein
OR	Odorant Receptor
OSN	Olfactory Sensory Neuron
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid

RP58	Repressor Protein with a predicted molecular mass of 58 kDa
TAAR	Trans Amine Associated Receptor
UPR	Unfolded Protein Response
UTR	Untranslated Region
VNO	Vomeronasal Organ
VR	Vomeronasal Organ
YFP	Yellow Fluorescent Protein

1. INTRODUCTION

1.1. The Olfactory System

Chemosensation is one of the evolutionarily most ancient sensory abilities. In its most simplistic form, it requires the activation of a chemoreceptor by a specific ligand and the transduction of the signal to a behavioral output. Even bacteria and rather primitive animals that do not possess a centralized nervous system can use chemoreception to locate food and mating partners. In higher organisms, chemoreceptors are organized in special sensory organs. In vertebrates, olfactory sensory neurons (OSNs) of the nose detect chemicals through olfactory receptors (ORs) and relay chemosensory information to the brain.

In the vertebrate (and in some invertebrate) olfactory systems, the expression of chemosensory receptors is highly coordinated. Typically, individual OSNs only express a single chemoreceptor gene from a much larger repertoire. However, the mechanisms that control expression of chemoreceptor genes are not fully understood. Here, experiments addressing the control of OR gene expression by regulatory factors that interact with the promoter of a specific model OR in zebrafish are presented.

1.1.1. Anatomy and the function of the Olfactory System

In vertebrates, a number of functionally and morphologically distinct chemosensory subsystems can be distinguished. The main olfactory epithelium (MOE) of the nose provides the organism with a conscious sense of smell perception, whereas the vomeronasal organ (VNO) is specialized in the (unconscious) detection of intra- and interspecies signals, such as pheromones and kairomones (Firestein, 2001).In addition to the MOE and VNO additional subsystems have been identified with the septal organ and Grueneberg ganglion in various species (Liu *et al.*, 2009). The different subsystems differ

in terms of the morphology of their chemosensory neurons, the type of the chemosensory receptor that they express, and the target regions that they innervate in the brain (Ma, 2009).

In the MOE, ORs are located on the cilia of OSNs and interact with odorant molecules from the environment. Each OSN typically expresses only a single member of a large and diverse family of OR genes and projects an axon to a specific glomerulus in the main olfactory bulb (mOB) of the forebrain (Mombaerts *et al.*, 1996; Ressler *et al.*, 1994; Vassar *et al.*, 1994). Olfactory glomeruli receive axonal input from a population of OSNs that express the same type of OR and function as the first relay station to transmit olfactory information to higher brain centers, where the incoming information is processed into smell perception (Mombaerts *et al.*, 1996; Zou and Buck, 2006).

In contrast, chemosensory neurons of the VNO bear cilia, express V1R- and V2R-type chemosensory receptors, and project axons to the posterior section of the OB, the accessory olfactory bulb (Dulac and Axel, 1995; Dulac and Torello, 2003; Mombaerts, 2004a). The main and accessory olfactory system also differ in their downstream connections to higher brain centers (Mucignat-Caretta *et al.*, 2012) and VNO signals are believed to be processed subconsciously (Firestein, 2001).

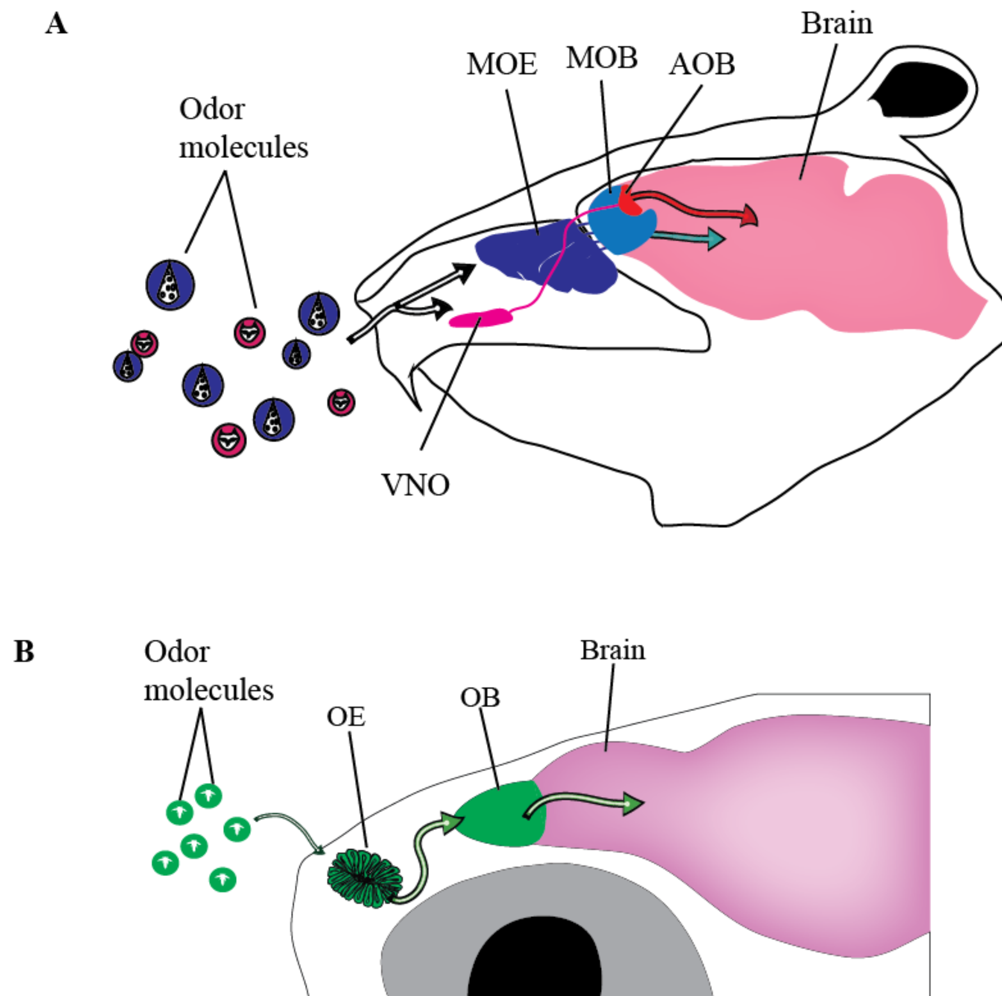

Figure 1.1. The anatomy of the olfactory system in mouse (A) and zebrafish (B). A. The mouse olfactory system has two olfactory organs: VNO and MOE which send chemical information from the nose to the brain via AOB and mOB respectively. B. The zebrafish olfactory system has a single olfactory organ that combines the various mammalian subsystems.

Neurons of the MOE mainly express two classes of chemosensory receptors, the classical olfactory receptors (ORs) and the trace amine-associated receptors (TAARs) (Buck and Axel, 1991; Liberles and Buck, 2006). While ORs are very divers and span a wide receptive range of different chemical classes, TAARs are more specialized for the detection of the volatile amines, which are often associated with predators or decaying prey (Ihara *et al.*, 2013).

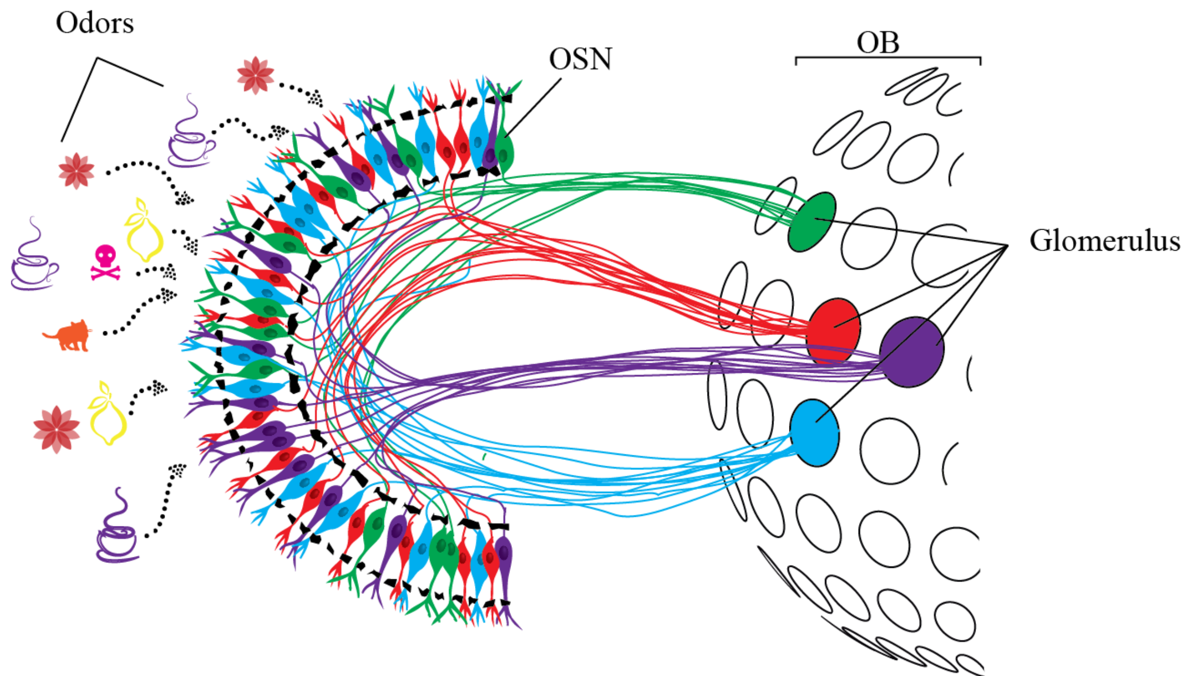

Figure 1.2. Convergence of the axons from OSN. Each OSN express only one type of olfactory receptor. Each glomerulus receives axons from sensory neurons expressing the same odorant receptor.

Neurons of the VNO, on the other hand, express either V1R or V2R vomeronasal receptors, some of which have been shown to respond specifically to components released by conspecifics and predators (Ben-Shaul *et al.*, 2010; Dulac and Axel, 1995). However, not all vertebrates possess a VNO and among those that have a VNO the complexity of the VNO in terms of the number of receptors that are expressed differ widely. For example, rats have at least 107 intact V1R whereas dogs have only 8 (Young *et al.*, 2005).

1.2.2. Chemosensory receptors

The olfactory system is able to detect a virtually unlimited number of chemicals by the vast array of chemoreceptors that are encoded by several evolutionary-related gene families. The first family of chemosensory receptors that was identified was the OR family of odorant receptors expressed in the MOE (Buck and Axel, 1991). OR genes constitute a large multigene family of olfactory-specific G protein-coupled receptors (GPCRs), which

is the largest gene family in most vertebrates. In rats and mice, 1200 – 1500 OR genes can be found in the genome and two third of these genes are functional (Mombaerts, 2004b). OR proteins show a seven transmembrane domain structure typical of GPCRs and are classified as family A rhodopsin-type GPCRs (Buck and Axel, 1991; Jacoby *et al.*, 2006).. OR genes of this class have no introns inside their approximately 1kb large coding region and the coding region (Young and Trask, 2002). They are predominantly expressed in sensory neurons of the MOE but expression in other tissues, such as kidney, sperm, gut and lung, has also been described (Feldmesser *et al.*, 2006; Nei *et al.*, 2008; Pluznick *et al.*, 2009). Recently, another family of chemosensory receptors was found in the MOE as the second class of chemosensory receptors (Liberles and Buck, 2006). These trace amine associated receptors (TAAR) recognize mono- and di-amine compounds and share functional and molecular properties with the classical ORs, such as gene structure and expression profiles. Interestingly, ORs and TAARs are not co-expressed by the same OSN but form separate subsystems in the MOE (Liberles and Buck, 2006).

Vomeronasal receptors form two different superfamilies of GPCRs, the V1R and V2R classes of receptors (Dulac and Axel, 1995). V1R genes have a intronless gene structure similar to OR and TAAR receptor genes and are expressed in vomeronasal neurons that arelocated apically in the VNO (Mombaerts, 2004a). V2R genes on the other hand, have a more complex structure, containing multiple exons and are expressed in basally located vomeronasal neurons. The repertoire of both receptor families is considerably smaller than the repertoire of OR genes. The mouse VNO expresses about 100 V1R and nearly 200 V2R genes (Shi and Zhang, 2007; Young *et al.*, 2005; Zhang *et al.*, 2004). Different from the OR and TAAR families that are expressed in the MOE and utilize the $G_{\alpha olf}$ and CNG signal transduction components, vomeronasal receptors transmit their signals through $G_{\alpha o}$ (V2Rs) and $G_{\alpha i}$ (V1Rs) pathways that include the activation of the TrpC2 transduction channel (Belluscio *et al.*, 1998; Dulac and Torello, 2003; Lin *et al.*, 2000; Mombaerts, 2004a; Zheng *et al.*, 2000).

1.2.3. Odorant Receptors

The OR protein located on the cilia of OSNs is the first component in the transmission of signals from the environment to the nervous system. They were first discovered in the mouse in the grounbreaking studies by Linda Buck and Richard Axel in 1991 (Buck and Axel, 1991). Surprisingly at that time, these classical ORs constituted the largest gene family in vertebrate (and some invertebrate) genomes. ORs are members of the rhodopsin class of GPCRs (Class A) with a predicted a seven transmembrane domain topology (Fredriksson *et al.*, 2003). Even though the overall OR protein structure is similar to rhodopsin, sequence alignment analysis showed that ORs have shorter transmembrane and larger loop regions (Crasto, 2009; Launay *et al.*, 2012). The ligand-binding pocket is believed to be formed by the transmembrane helices: one binding site may be formed by TM3,TM5 and TM 6, while another pocket may be formed by TM3-TM7 (Emes *et al.*, 2004; Liu *et al.*, 2003). The sequences of OR transmembrane domains are hypervariable, a structural feature that allows the group of ORs to recognize a diverse and vast set of odorant ligands (Olender *et al.*, 2004; Pilpel and Lancet, 1999). Binding of the ligand to the receptor causes a conformational change in the protein that activates a heterotrimeric G protein that contains the OSN-specific Gaolf subunit (Turin, 1996).

OR genes are typically found to be organized into clusters in the genome and are rarely interrupt by non-OR genes (Rouquier *et al.*, 1998; Sullivan *et al.*, 1996).A notable exception is the Class I OR cluster on mouse chromosome 7, which is interrupted by the beta-globin locus(Bulger *et al.*, 1999). OR genes which share the same expression pattern are believed to be linked at the same locus (Glusman *et al.*, 2000; Young and Trask, 2002; Zhang and Firestein, 2002) but an increasing number of exceptions are being reported. OR genes have a rather simple gene structure: the coding sequence is about 1kb in length, does not contain exons, and one to three upstream introns in the 5'-UTR can be found (Reiner Hoppe *et al.*, 2003; P Mombaerts, 1999; Sosinsky *et al.*, 2000; Volz *et al.*, 2003; Young *et al.*, 2003).

Not all ORs of the large genomic repertoire are functional and the number of OR pseudogenes varies significantly between species. For example in the human genome up to 72% of OR genes are pseudogenes (Zozulya *et al.*, 2001), while in the mouse only around 3% of genes are pseudogenes (Zhang and Firestein, 2002). The overall number of intact OR genes in fish species is much smaller compared to rodents, with 40-140 OR genes depending on the species (Alioto and Ngai, 2005; Niimura and Nei, 2005). However, within the teleost lineage, the zebrafish has the largest functional repertoire with at least 136 intact OR genes (Alioto and Ngai, 2005; Niimura and Nei, 2005). Even though the number of functional genes differs widely among different species, there is no apparent correlation between the percentage of pseudogenes and the ability to discriminate odors (Zhang and Firestein, 2002). Yet, gains and losses of OR genes and subfamilies are believed to represent different evolutionary adaptations to changes in habitats and environments (Young and Trask, 2002).

The OR repertoire can be grouped into subfamilies based on amino acid similarity. Members of a given subfamily are typically clustered in the genome and can often be found in the same transcriptional orientation, a feature which reflects the evolutionary origin of family members by gene duplication events and which may be important for the transcriptional regulation of OR genes (Barth *et al.*, 1997; Dugas and Ngai, 2001). A major division of OR genes into two different classes can be made for most higher vertebrates. Class I receptors are believed to be evolutionary more ancient, to detect water-soluble odorants, and are often called fish-like receptors (Alioto and Ngai, 2005; Freitag *et al.*, 1998). Nevertheless, class I ORs are also present in mammals and may mediate innate aversive responses to certain odorants (Kobayakawa *et al.*, 2007). They constitute about 10% of the repertoire in the mouse, comprise a lower fraction of pseudogenes, and are genomically linked (Zhang and Firestein, 2002; Zhang and Firestein, 2009). In the zebrafish, virtually all receptors are belong to the Class I clade and only one OR with sequence similarity to class II ORs has been identified (Alioto and Ngai, 2005; Niimura and Nei, 2005). Class II OR genes dominate the mammalian OR repertoire, comprising 90% of all OR genes, and are thought to detect volatile compounds (Zhang and Firestein, 2002). Eventually, class II ORs are rare or absent in teleosts (Meyerhof and Korsching, 2009). Even though they were suggested to detect volatile odorants, Class II OR genes

make up a significant portion of the OR repertoire in marine mammals, suggesting that this class might also be important for the survival in an aquatic environment, most likely because social cues are emitted as airborne odorants (Hayden *et al.*, 2010; Meredith *et al.*, 2011).

1.2.4. The olfactory system of the zebrafish

The zebrafish olfactory system, while generally organized as described above, is also characterized by fish-specific peculiarities (Figure 1.1.B): First, the zebrafish possesses only a single olfactory epithelium, which houses the various morphologically distinct chemoreceptor cell types of the rodent MOE and VNO. Because of its natural aquatic environment, fish-specific odorant molecules have to be water-soluble. Behaviorally relevant odorants have been described with amino and nucleic acids, which serve as food cues (Laberge and Hara, 2001) and fish-specific pheromones, such as steroids, prostaglandins and bile acids (Døving *et al.*, 1980; Sorensen *et al.*, 2005). In the fish, smelling is not linked to inhalation as it is in terrestrial animals, but instead beating cilia of respiratory cells surrounding the inner sensory region of the OE generate a water current along the OE even when the fish is stationary. However, despite these anatomical differences, the neural basis of odorant sensing in fish is similar to higher vertebrates (Cande *et al.*, 2013; Taniguchi *et al.*, 2011). OSNs in the OE express chemosensory receptors and propagate stimulus information through the olfactory nerve to the OB (Figure 1.1b) where OSN axons second order neurons and interneurons form glomeruli. Each OB of the zebrafish contains around 140 identifiable glomeruli, which can be classified into two groups, which can be identified by their immunoreactivity to various antigens (Braubach *et al.*, 2012). It is likely that the one neuron-one receptor rule is also valid in the zebrafish, even though it has not been formally proven. Sato *et al.* (2007) investigated the co-expression of nine receptors from two OR subfamilies from zebrafish chromosome 15 and provide evidence among this limited set of receptors that the majority of these ORs may not be coexpressed.

Another peculiarity of the zebrafish nose is that the single OE contains four morphologically distinct chemoreceptor cell types: ciliated OSNs, microvillus cells, crypt cells and kappe neurons (Ahuja *et al.*, 2014; Hamdani and Døving, 2007; Hansen and Zeiske, 1998). Even though ciliated and microvillus cells are common among vertebrates where they segregate between the MOE and the VNO, respectively, crypt cell and kappe neurons may be teleost-specific chemosensory cells (Hansen and Zeiske, 1998; Schmachtenberg, 2006; Vielma *et al.*, 2008).

The four cell types differ in their morphology and spatial location within the OE (Figure 1.3). Ciliated cells are located more basally, have long dendrites and cilia, while microvillus cells are located more apically, have shorter and thicker dendrites and bear short microvilli at their apical tip. The shapes of the crypt and kappe neurons are similar to each other but distinct from ciliated and microvillous cells. Both, crypt and kappe neurons are located most apically in the OE, but crypt cells have a plum-shaped and roundish cell bodies with buried cilia whereas kappe neurons appear more pear shaped (Ahuja *et al.*, 2013, 2014; Hansen and Finger, 2000). In addition to these neuronal cell types, non-neuronal basal cells, most likely responsible for regeneration of olfactory sensory neurons and a specific type of glia cells, the sustentacular cell can be found (Cancalon, 1982; Hasnsen and Zeiske, 1998) (Figure 1.3).

The different neuronal subpopulations are also characterized by expression of different chemoreceptor classes and molecular markers. Ciliated OSNs express OR and TAAR genes whereas microvillus cells express VR genes (Mombaerts *et al.*, 1996; Wagner *et al.*, 2006; Sato *et al.*, 2005; Yoshihara, 2009). For crypt neurons a single V1R-related gene, ORA4, has been identified (Oka *et al.*, 2012). No receptors are currently known for kappe neurons.

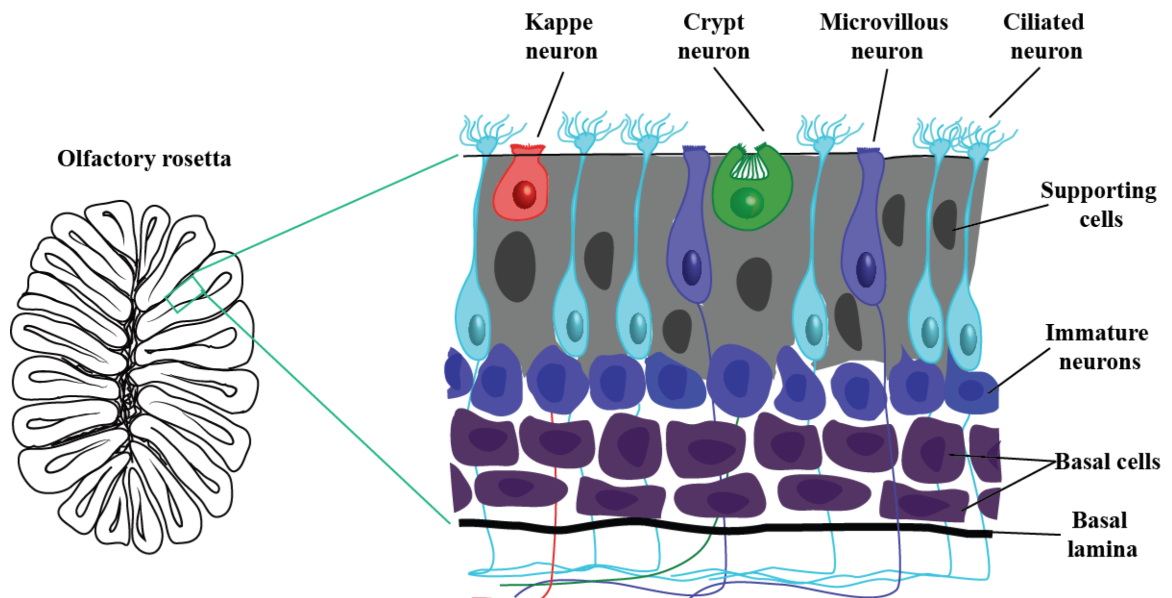

Figure 1.3. The olfactory rosetta of the zebrafish (left) and the organization of the olfactory epithelium in zebrafish.

1.3. Properties of OR expression

Each individual OSN express only one OR gene from the entire genomic repertoire (Bozza *et al.*, 2002; Malnic *et al.*, 1999; Mombaerts, 2004b). This hypothesis is referred to as the 'one receptor-one neuron' rule. The rule is hard to proof formally because of the large number of chemosensory receptor genes but it is supported by several experimental approaches. Single cell RT-PCR from OSN amplifies only a single OR transcript per cell (Malnic *et al.*, 1999) and multi-color in situ hybridization studies did not reveal coexpressio in most cases. After an OR has been selected for exprerssion by an OSN it is important to maintain expression of the chosen OR for the survival of the OSN. So far, a number of mechanisms have been suggested, which support the hypothesis and act on various steps of OR expression to ensure singular expression.

1.3.4. Monoallelic and monogenic expression of OR

One of the apparently random events that contribute singular expression of OR genes is monoallelic expression. Each OSN only expresses the maternal or the paternal allele of an OR but never both (Chess *et al.*, 1994; Gimelbrant *et al.*, 2007; Ishii *et al.*, 2001). Monoallelic expression is believed to be an early step during OR gene choice because the distinction of OR alleles is established during early OSN development manifested by asynchronous replication of OR genes in OSN precursors (Chess *et al.*, 1994; Kitsberg *et al.*, 1993; Mostoslavsky *et al.*, 2001; Simon *et al.*, 1999). Asynchronous replication is established by marking the genes epigenetically during early development to inactivate one of the homologues. The mechanism of monoallelic expression of OR genes is still elusive, but it is bears similarity to X-inactivation and monoallelic expression of immuneglobulins (Gimelbrant *et al.*, 2007; Goldmit and Bergman, 2004; Magklara and Lomvardas, 2013). But unlike X-inactivation, selection of an OR allele is not absolute. If no functional protein can be translated from the active allele, the other allele can be activated (Feinstein *et al.*, 2004; Lewcock and Reed, 2004; Serizawa *et al.*, 2003; Shykind *et al.*, 2004). However, it should be noted that under such conditions it is more likely another OR gene that is expressed rather than the second allele of the first OR locus, suggesting that monoallelic expression and monogenic expression are mechanistically coupled. Overall, monoallelic expression is as important for proper function of OSN as monogenic expression. Without this restriction, two polymorphic alleles might be expressed by the same OSN and the consequence would be the same as expressing two different ORs in the same cell. Since even a single amino acid change might affect the affinity of the binding pocket for its ligand, it might affect the function of the OSN (L. B. Buck, 2000).

Another critical feature that ensures singularity of OR gene expression is termed monogenic expression. A given OSN only express allele of one OR from the entire repertoire (Malnic *et al.*, 1999). However, evidence for this rule is largely circumstancial. In the rat, the pattern of the OR gene expression was examined by in situ hybridization. The number of OSN was determined to be around 20 million cells as established by OMP expression, while the number of OR genes is 1200 (Meisami, 1989; Nimura and Nei, 2007;

Youngentob *et al.*, 1997). Thus, each OR should be represented by 0.1% of the all neuron in the OE and for several OR genes this percentage was found (Iwema and Schwob, 2003; Kubick *et al.*, 1997; Ressler *et al.*, 1993; Royal and Key, 1999; Strotmann *et al.*, 1994). Monogenic expression was also confirmed by double in situ hybridization. The results showed that the number of cells that are labeled with promiscuous family-specific probes is equal to the sum of cells labeled with gene-specific probes (Kubick *et al.*, 1997). When single cell RT-PCR was applied to individual OSN, in half of the analyzed OSN no OR sequence could be amplifies, while in the remaining 50% only a single OR gene could be detected (Malnic *et al.*, 1999).

Additional evidence for monogenic expression comes from physiological experiments. OSN that express a particular OR should detect only odorants that bind to this receptor. Experiments show that for most OSNs, the odorant profile is specific and match with the OR that is expressed by the OSN (Kajiya *et al.*, 2001; Touhara, 2007). Switching the expressed OR also changes the odorant response profile of that OSN. For example changing mouse M71 gene expression to rat I7 results in a change in responsiveness of the neuron from acetophenon and benzaldehyde to octonal and aliphatic aldehydes, in line with the known response profiles of the two ORs (Bozza *et al.*, 2002).

1.3.5. Gene switching and Negative feedback mechanism

After initial selection of the OR by an OSN, the cell expresses the same OR throughout its life and somehow has to prevent expression of other OR genes. In some cases an OSN might express more than one OR gene and those cells are eliminated by apoptosis, either because they cannot guide their axons to the correct glomerulus or because of an activity-dependent feedback mechanism (Feinstein *et al.*, 2004; Feinstein and Mombaerts, 2004; Tian and Ma, 2008; Zhao and Reed, 2001; Zheng *et al.*, 2000; Zou *et al.*, 2004). However, for some ORs switching of OR expression has been observed and a cell would sequentially express different OR genes. This is particularly true if the first choice is a OR pseudogene. To ensure survival, the OSN can switch to expression of

another functional gene (Lewcock and Reed, 2004; Serizawa *et al.*, 2003; Shykind *et al.*, 2004). This switching process does not seem random because the second choice of the OSN was generally from the same class, class I or class II ORs, as the non-functional receptor chosen first (Bozza *et al.*, 2009). The non-randomness appears to be cell type-specific, as different sets of ORs have been uncovered as second choice for OSNs that express the same OR gene from the MOE and the septal organ (Fuss *et al.*, 2012). In addition, early switching of OR expression has also been demonstrated for OSNs that express a functional OR gene by lineage tracing experiments (Shykind *et al.*, 2004). About 10% of cells that expressed the MOR28 gene at an early stage of ontogenetic development stably expressed a different OR gene later in life. The significance of this observation are not fully understood but they could reflect low level expression of many OR genes during early stages of OR gene choice.

Regardless, OSN that express an OR gene from which a functional OR protein can be translated, at some point stick with their choice. A negative feedback mechanism has been proposed to exist that ensures stable OR expression (Serizawa *et al.*, 2003). The negative feedback mechanism is supposed to lock in OR expression and to prevent switching of expression to another OR gene. Expression of an OR protein may initiate a signal that cause a shut down of the choice machinery. In accordance with this model, switching can only be seen in immature OSN (Shykind *et al.*, 2004). Recent studies have shed significant light onto this elusive feedback mechanism as well as OR gene choice. It involves activity of a specific lysine demethylase, LSD1, which removes repressive lysine 9 methyl marks from histone H3 on the chosen OR allele (Lyons *et al.*, 2013). Initially, all OR loci are hypermethylated and reside in constitutive heterochromatin. LSD1 activity would then release one OR locus from repression in a random manner. One the OR protein is synthesized, it triggers a signal cascade in the endoplasmic reticulum that uses components of the unfolded protein response (UPR), activating the protein kinase Perk. Perk then phosphorylates eIF2a, which is followed by the translation of the nuclear form of activating transcription factor 5 (ATF5). ATF5 expression stimulates transcription of the olfactory-specific adenylyl cyclase 3 which somehow cause shut-down of LSD activity, thereby preventing the escape of further OR loci from repression (Dalton *et al.*, 2013).

Thus, in this model, OR gene choice and negative feedback signaling are tightly linked processes that converge on the activity of the key molecule LSD1.

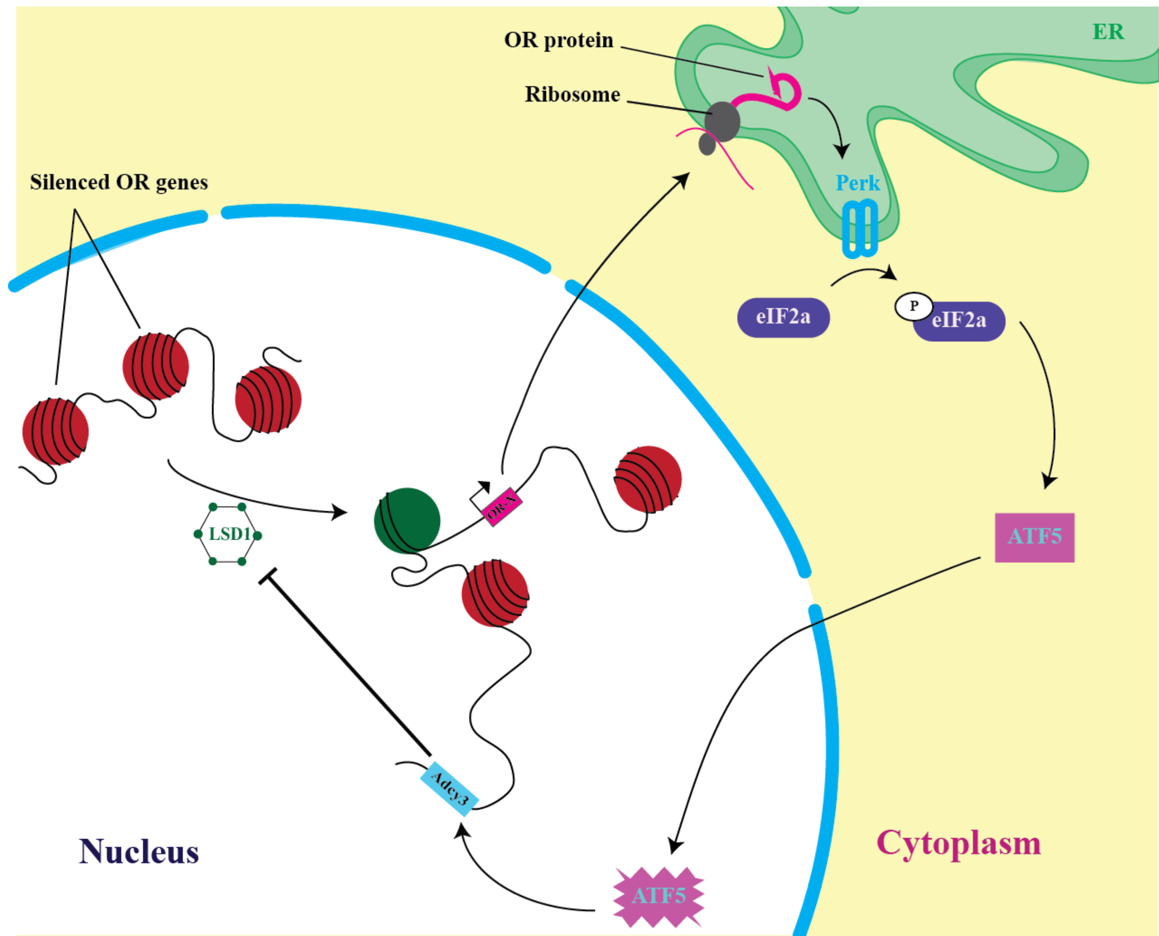

Figure 1.4. Feedback loop generated by OR expression. One of the OR allele is activated by LSD1 by changing its methylation status. The expression of the OR gene from ER initiates the Perk signaling and phosphorylates eIF2a. eIF2a activates ATF5 which cause transcription of Adcy3 that negatively regulates LSD1 expression.

1.3.6. Zonal expression / frequency of the expression

The OR gene choice mechanism described above would result in equal expression of all OR gene loci. The LSD1 would, in a probabilistic fashion activate all OR genes with equal likelihood. However, the probability of OR gene choice is not the same for all OR genes and OSNs. Instead, the expression of individual OR genes is restricted specific domains also referred to as 'zones'. The first observations of zonal expression suggested that there are 4 distinct domains in rat and mouse that are organized as strips with dorso-ventral arrangement (Ressler *et al.*, 1993; Vassar *et al.*, 1993). However, more recent data showed that there are restricted but overlapping expression domain for each OR and these domains are continuous along the dorso-ventral dimension of the OE (Iwema and Schwob, 2003; Miyamichi *et al.*, 2005; Akio Tsuboi *et al.*, 2006). In zebrafish a correlate of the zonal expression exists. Three overlapping concentric expression domains were described for six different ORs, similar to zonal expression of the mammalian olfactory system (Weth *et al.*, 1996). Thus, LSD1 activity must be guided to specific subsets of ORs in different spatial domains of the OE to ensure zonal expression.

The factors that underlie zone-specific expression are not well characterized. Transgenic promoter analysis showed that the zonal OR expression pattern depends on sequences within the OR promoter (Vassalli *et al.*, 2002). Therefore, it is reasonable to speculate that localized transcriptional regulators or gradients of transcriptional regulator expression may establish expression of OR subsets in a zonal fashion. However, zone-specific transcription factor await identification. Yet, various proteins have been identified which show zonal expression although the expression patterns did not match exactly with the definition of OR boundaries. One of these proteins is the olfactory cell adhesion molecule OCAM, which is expressed exclusively in the dorsal OE (Yoshihara *et al.*, 1997). Another study showed that neuropilin 2 is expressed in a graded pattern (Norlin *et al.*, 2001). Since OR expression is overlapping and continuous expression, neuropilin 2 may have a role in patterning OR expression (Miyamichi *et al.*, 2005).

The clearest distinction can be made between the two classes of ORs. Class I OR are exclusively expressed in the dorsal OE while Class II OR can be found in all OE domains (Miyamichi *et al.*, 2005; Tsuboi *et al.*, 2006; Zhang *et al.*, 2004; Bozza *et al.*, 2009). Some studies suggest that the dorsal zone is different in terms of cell type and cell lineage and that it OR choice from either Class I or ClassII may be lineage-specific as well (Bozza *et al.*, 2009).

Another curious observation is that the number of cells expressing a given OR within a zone are not identical. For instance, the MOR28 gene is expressed in 10% of cells within the ventral OE (Shykind *et al.*, 2009), although about 200 ORs are expressed in overlapping domains. Thus, specific factors associated with individual OR loci affect the frequency of expression as well as the spatial expression pattern.

1.4. Properties of OR promoters

1.4.4. Transcriptional regulation

As mentioned above, the stochastic expression mechanism guided by LSD1 cannot explain numerical and spatial differences in OR expression. Thus, additional regulatory mechanisms, most likely linked directly to individual OR loci must be in place to account for these differences. One such mechanism suggests that OR gene choice is guided by local sequence elements. OR genes are arrange in clusters in the genome, and it is possible that sequences within the cluster affect OR expression (Mombaerts, 2004a; Niimura and Nei, 2005). According to this model, locus control regions (LCRs) would regulate OR gene expression from clustered ORs via cis-acting elements. One such element, the H region of the mouse, locates 75kb upstream of the MOR28 gene cluster (Serizawa *et al.*, 2003). This element spans 2.1 kb of genomic DNA and functions as an enhancer of OR gene choice in transgenic animals (Serizawa *et al.*, 2003). Deletion of the H region negatively affects the number of neurons transcribing OR genes from the downstream cluster of 7 ORs (Fuss *et al.*, 2007; Nishizumi *et al.*, 2007). Therefore, H is a cis-acting element that regulates OR

gene expression. Interestingly, 3 of the seven genes of the MOR28 cluster are expressed in the ventral OE, while the remaining 4 genes are expressed in the dorsal OE. Thus, H might affect the frequency of OR gene choice from the MOR28 cluster, it does not direct spatial expression.

Another enhancer has been identified with the P element located inside the P2 cluster on chromosome 7 (Khan *et al.*, 2011). Similar to H, the P element is required for expression of nearby genes and knock-out of P abolishes expression of 10 genes from the total of 24 genes of the P2 cluster. Similar cis-acting enhancers have been functionally identified in and around OR gene clusters in zebrafish (Bessa *et al.*, 2009; Nishizumi *et al.*, 2007). The E-15-1 and E-15-2 region from zebrafish chromosome 15 affect OR expression from the cluster but their sequences do not show significant sequence similarity with the mammalian H region (Nishizumi *et al.*, 2007). Yet, function of these enhancers appears to be conserved across species, as the presence of the mouse H enhancer close to a proximal OR promoter in zebrafish dramatically increases in the number of OSN expressing this OR. These results suggest that the LCR mechanism is conserved throughout evolution and controls the OR gene choice probability across species (Nishizumi *et al.*, 2007; Taştekin, 2012).

The transcriptional start sites of OR genes can be found a few hundred to several thousand bases upstream of the translational start, depending on the ORs mRNA structure (Glusman *et al.*, 2000; Michaloski *et al.*, 2006; Volz *et al.*, 2003). Commonly an AT-rich region is also found within the first 50 bp, the region where TATA boxes would be expected (Hoppe *et al.*, 2006; Michaloski *et al.*, 2006). However, only 50% of OR genes have clear TATA boxes (Hoppe *et al.*, 2000; Lane *et al.*, 2001; Sosinsky *et al.*, 2000). Additional cis-acting elements have been identified close to OR TSSs (Rothman *et al.*, 2005; Vassalli *et al.*, 2002). In the mouse, binding sites for the transcription factor Olf1/Ebf-1 can be found within short proximity of the TSS of OR genes (Hoppe *et al.*, 2003; Michaloski *et al.*, 2006). The significance of some of these transcription factor-binding sites has been validated by transgenic studies. For example, short transgenic constructs, ranging between 300 to 7.000 bp can efficiently and faithfully drive expression

of reporter genes in OR-specific patterns. Transgenic expressions of M4, MOR23, M71, P3 and M72 genes are obtained in short transgenic constructs with 6.7 kb, 405 bp, 161bp, 306bp and 298 bp upstream respectively. These results suggest that critical regulatory elements are present in these sequences that act in short-range on OR promoters (Plessy *et al.*, 2012; Qasba and Reed, 1998; Rothman *et al.*, 2005; Vassalli *et al.*, 2011a, 2002).

Bioinformatics and experimental approaches were used to identify regulatory elements within OR promoters and highlighted conserved sequence motifs that are critical for OR expression. The best characterized regulatory elements are the homeodomain binding sites and binding sites for Olf1/Ebf1 (O/E) transcription factors. The conservation of these factors suggests that these common promoter elements play an important role for OR gene expression (Michaloski *et al.*, 2006). O/E-like sites are generally located within 200 bp upstream of the TSS, whereas the homeodomain-like sites, which can be recognized by Lhx2 and Emx2 transcription factors (Hirota and Mombaerts, 2004; Kolterud *et al.*, 2004; McIntyre *et al.*, 2008) are found further upstream (Michaloski *et al.* 2006).

Interestingly, unique signatures of transcription factor binding sites are present in the promoters of genes from the mouse OR37 subfamily. This OR gene family comprises eight members which share a unique spatial expression pattern in the OE that is distinct from most other OR genes. The genes are divided into two clusters of five genes, which share 90% sequence identity and three genes, which are less similar, with 60% sequence identity. A comparative analysis revealed highly conserved sequence motifs near the TSS for genes from cluster. These identical sequences were categorized into common blocks: blocks I and IV are A-T rich, while block II and V are G-A rich sequences. In addition, a conserved TCCCA motif was found in block III and VI. Analysis of putative regulatory element for OR 37 reveals potential binding sites for the homeobox gene S8, for the zinc-finger transcription factor IK2 and for the NFY-type transcription factor (Hoppe *et al.*, 2000).

Even though homeodomain sites and O/E-like sites occur in virtually every OR promoter, these sites may not necessarily impart detailed instructions on spatial expression patterns (Glusman *et al.*, 2000; Hoppe *et al.*, 2003; Sosinsky *et al.*, 2000; Vassalli *et al.*, 2002). O/E binding sites are present in many olfactory-specific genes such as GnaI (formerly known as Golf), adenylyl cyclase III (AcIII), olfactory cyclic nucleotide gated channel (Cnga2), and olfactory marker protein (Omp) (Kudrycki *et al.*, 1993; M. M. Wang *et al.*, 1993) in addition to OR genes (Glusman *et al.*, 2000; Hoppe *et al.*, 2003; Sosinsky *et al.*, 2000; Vassalli *et al.*, 2002). Thus, they might more generally direct expression to olfactory tissue, yet, their involvement in regulating the frequency of OR choice cannot be ruled out.

Homeodomain-like and O/E-like sites in OR promoters appear to work together. The expression of OR promoter transgenes is severely reduced when both sites are mutated or deleted from transgenic constructs or in the genome (Rothman *et al.*, 2005). The targeted mutation of these site in the mouse M71 gene causes a 3-fold reduction in the frequency of M71-expressing neurons (Rothman *et al.*, 2005). Therefore changes in the homeodomain-like site and the O/E-like site can control the probability of OR gene choice rather than the level of expression at the individual cell level.

Consistent with the bioinformatic data, two homeodomain transcription factors, Lhx2 and Emx2, were shown to be critical for OR gene expression. Emx2 is a homeodomain transcription factors captured by yeast one hybrid assays using the promoter region of M71 and mOR262-2 (Hirota and Mombaerts, 2004; Hoppe *et al.*, 2003). Other transcription factor found in these assays were Lhx2, Cart1 (Alx1), Dlx5, Dlx3, Prrx1 (Prx1), Prrx2 (Prx2), Alx3, Pitx1 (Ptx1) and Barx1 (Hirota and Mombaerts, 2004; Hoppe *et al.*, 2003). In mice Emx2 deficiency selectively affects OR expression and a specific subset of ORs was not expressed (McIntyre *et al.*, 2008). These data support that Emx2 is a homeobox transcription factor that binds to specific OR gene promoters and might be necessary for OR gene choice of these receptors.

Yet, a large fraction of ORs were not affected by Emx2 deficiency (McIntyre *et al.*, 2008). Another factor shown to bind to homeodomain-like sites is Lhx2. Lhx2 is abundantly expressed in OSNs and it was captured by yeast-one hybrid assays using M71 OR promoter fragments (Hirota and Mombaerts, 2004). Lhx2 binds to the homeodomain site in the M71 promoter but also to sites in the H-element. The number of OR genes expressed in Lhx2 deficient mice is very low and developmental abnormalities during the OSN maturation process cannot be ruled out as nearly no fully mature OSNs were found in Lhx2 mutant mice. These results suggest that Lhx2 is important for later stages of OSN development, but could also imply that OR expression, guided by Lhx2 activity, is required for OSN maturation (Hirota and Mombaerts, 2004; Hirota *et al.*, 2007; Kolterud *et al.*, 2004). Different from Emx2, Lhx2 deficiency has a differential impact on Class I and Class II OR genes. Knock-out of the Lhx2 gene in the mouse results in a loss of expression of all ClassII OR genes while Class I genes are not significantly affected (Hirota *et al.*, 2007). These results indicates that Class I and Class II OR gene expression is regulated by different elements and transcription factors. Interestingly, very similar sequence motifs for homeodomain-like sites are found within all OR promoter, suggesting the presence of co-activational factors.

So far, only one study suggested negative regulation by the transcription on OR promoters. Mouse VRs and ORs promoter sequences contain Broad-Complex (BR-C), tramtrack (ttk), and brick a brack (bab) domain Zinc Finger (BTB-ZF) motifs, which are known transcriptional repressors (Michaloski *et al.*, 2011; Zollman *et al.*, 1994) . Bioinformatic analysis of OR and VR gene promoters showed presence of the BTB-ZF motifs with slightly different consensus sequences close to the TSS. Two of these motifs share the consensus sequence CNTCTGG which is present in 49% of VR genes and 40% of the 198 OR genes analyzed. This particular consensus sequence is similar to binding sites for the RP58 transcriptional repressor (CATCTGG), a member of the BTB-ZF family (Aoki *et al.*, 1998). RP58 has been shown to induce heterochromatin-mediated gene inactivation. It is associated with condensed chromatin regions in the nucleus of IMR32 (human neuroblastoma) cells and mediates sequence-specific translational repression (Aoki *et al.*, 1998). While RP58 was not expressed in the mouse OE, the zbtb7 genes, which bind similar sequences are expressed in high levels (Michaloski *et al.*, 2011). Zbtb7b is known

for its repressive function during T-cell maturation in the immune system, where it acts as a repressor of the CD8 cell lineage and promoter of the CD4 cell fate (Bilic and Ellmeier, 2007). Yet, no functional evidence for a similar role of RP58 or zbtb7 in the olfactory system has been shown.

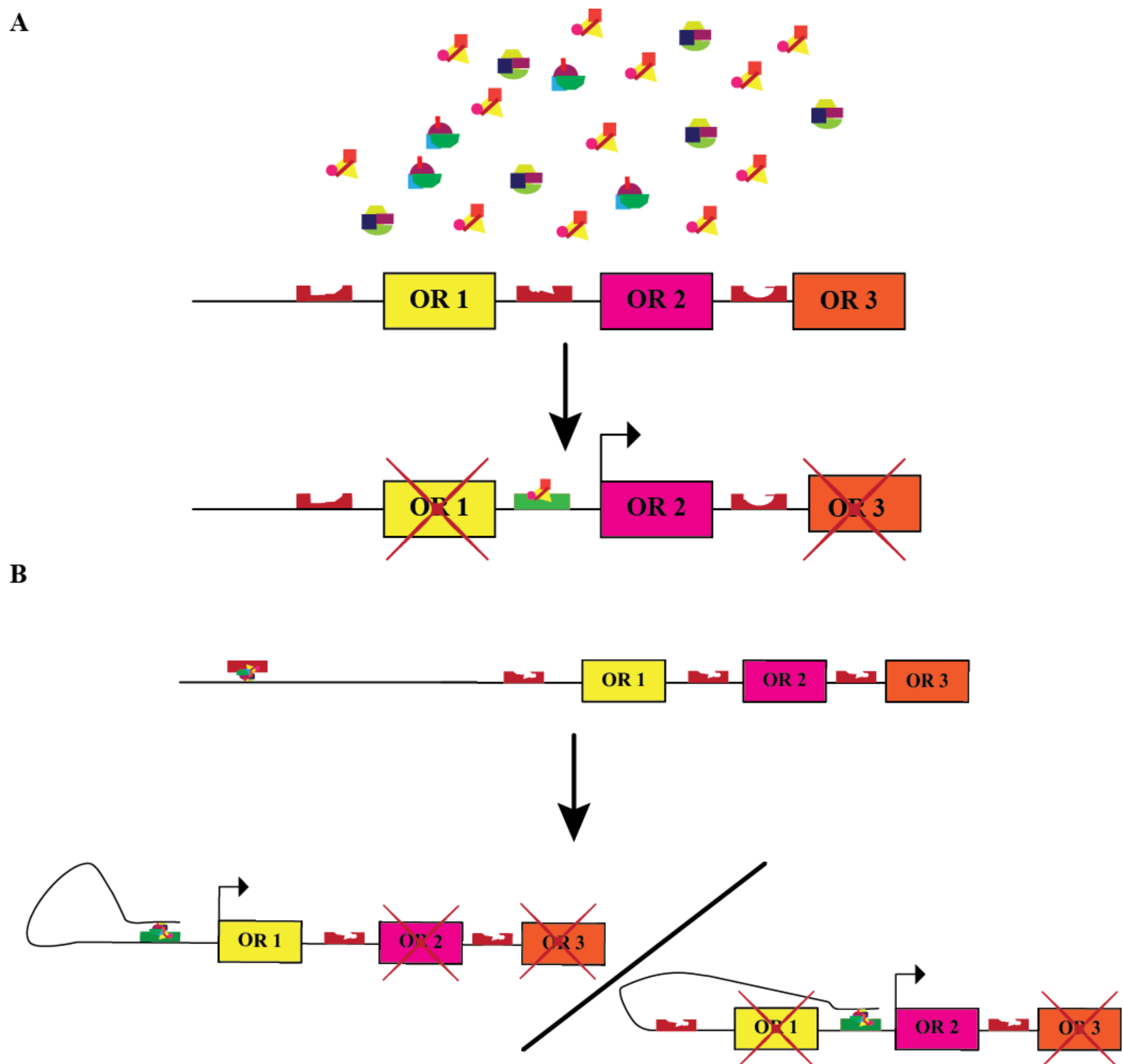

Figure 1.5. Suggested translational regulation mechanisms. A. Short range control: Binding of the transcription factor that to binding sites on the upstream of OR genes may activate their expression. B. Locus Control Region: These regions regulate the expression from a distance by interacting regulatory proteins.

1.4.5. Epigenetic Regulation

Recent studies have shown an important epigenetic regulation of OR expression, especially on the selection of the allele. OR genes, like the other genes in the genome, are subject to chromatin-mediated activation or silencing. Genome-wide chromatin analysis of the OR repertoire shows that OR genes have H3K9me3 and H4K20me3 marks, which are associated with constitutive heterochromatin, while the expressed OR has H3K4me3 marks (Armelin-Correa *et al.*, 2014; Magklara *et al.*, 2011). Passive OR genes are not only silenced around their promoter region but large heterochromatin blocks cover entire OR genomic clusters (Armelin-Correa *et al.*, 2014; Magklara *et al.*, 2011). This pattern is observed in immature OSNs before onset of OR transcription, which precludes interactions with transcription factor synthesized in OSN (Magklara *et al.*, 2011).

Similarly, distinct nuclear localization of OR genes seem to have a significant role in expression. The spatial distribution of OR genes in the nucleus is unique: inactive genes appear in the center rather than periphery of the nucleus. The lamin-b receptor seems to have a role in the segregation of active and passive alleles in the nucleus and disruption of lamin-b receptor expression causes alterations in monogenic OR expression (Clowney *et al.*, 2012). Recent studies showed that constitutive heterochromatin and facultative heterochromatin are located in different nuclear domains. The facultative heterochromatin domains are localized around constituve one. However active allele is associated with facultative heterochromatin which can be regulated transcriptionally whereas passive OR allele colocalize with constitutive domain. However true mechanism behind this feature is unknown (Armelin-Correa *et al.*, 2014).

Thus, OR expression and OR gene choice appear to be under different layers of control, invoking classical and epigenetic regulatory mechanisms. The interplay of these different levels of regulation has not been worked out, but the global epigenetic mechanisms cannot fully explain the distinct expression patterns that are observed for different groups of OR genes. Thus, it is quite likely that epigenetic mechanisms are

guided by classical transcription factors that appear to have a more direct role in establishing OR-specific expression patterns.

1.5. The zebrafish OR101-1 gene

1.5.4. Properties of the OR101-1 gene

The zebrafish OR101-1 receptor gene resides on the reverse strand of chromosome 21 (position 42,178,376 -42,179,326 in Zv9) of the zebrafish genome. The coding sequence of OR101-1 is located within a single exon and spans 948 bps (www.ensemble.org). The gene locates near a cluster of the OR115 gene family in reverse transcriptional orientation. The OR115 gene family has 15 members, which are distributed over 125 kilo base (kb) the first gene of the cluster is located 8kb downstream of OR101-1. Interestingly, OR101-1 is the only zebrafish OR that shows strong sequence similarity to mammalian class II ORs, while all other of the 179 zebrafish ORs are more related to the class I group. A pairwise similarity comparison between OR115 genes and OR101 reveals only 6% similar (Tınaztepe, 2009).

Because OR 101-1 is the only OR gene that shows significant sequence similarity to Class II ORs (Alioto and Ngai, 2005; Niimura and Nei, 2005), it might represent an OR that is closely related to the common ancestor of the Class II repertoire. Comparison with other species also revealed that the closest OR to OR101-1is the MOR171-13 from mouse and OR06.09.01 from human, which showed a 52% and 43% sequence identity respectively (Tınaztepe, 2009).

1.5.5. Expression and regulation of OR101-1 in zebrafish OE

The transcript prediction for OR101-1 gene was done by transcriptome analysis and 5'-RACE by our lab. Both analyses showed similar data with same 5' upstream structure. The transcript of the OR101-1 gene has a non-coding exon, an intron and the coding sequence is within second exon which is around ~950bp.

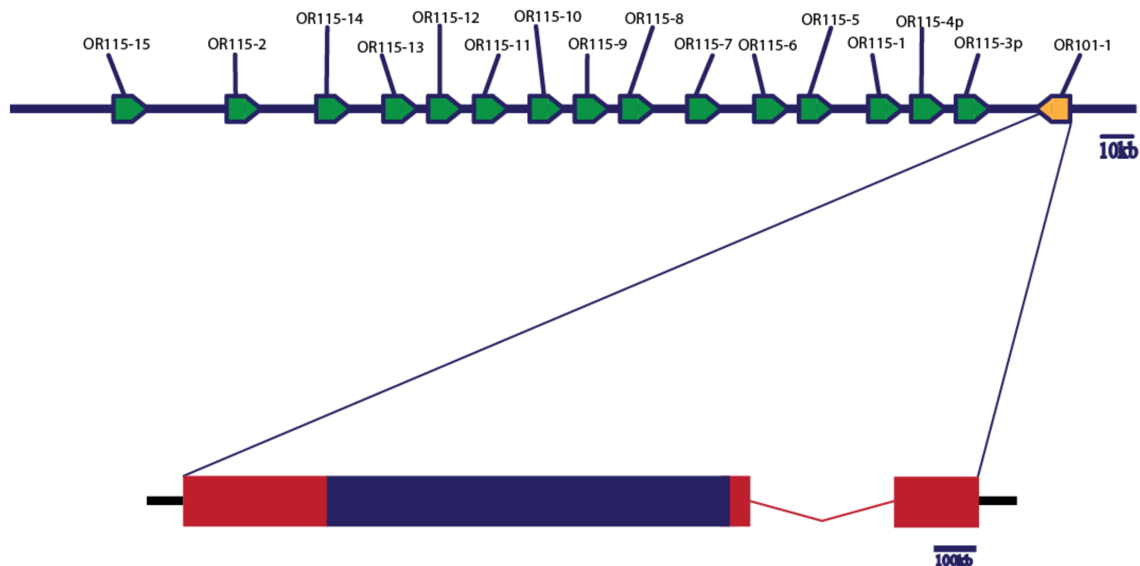

Figure 1.6. OR-101-1 gene location in the chromosome and predicted transcripts of the gene. OR101-1 gene is located with the OR115 cluster but it is transcribed from reverse direction.

The expression of OR101-1 gene in the zebrafish OE has been confirmed multiple times by in situ hybridization in our laboratory and there is no or little expression in tissues other than the OE.

The OR101-1 gene is associated with the OR115 gene cluster. Therefore, it is possible that a specific LCR regulates expression of the genes at the cluster level. However, it has been shown that short proximal sequences surrounding the TSS of OR101-

1 can drive transgene expression in OSNs, suggesting that the gene is not under the control of a cluster regulator.

Thus, the OR101-1 promoter appears to be rather compact, similar to the mouse M71 OR. Regulatory sequences can locate in close distance to the transcription start site of OR genes in mouse (Rothman *et al.*, 2005; Vassalli *et al.*, 2002) and zebrafish (Mori *et al.*, 2000). To identify such sequences a systematic promoter bashing approach was applied to the OR101-1 promoter (Söğünmez, 2012). For this purpose, first the 3.5kb of genomic sequence upstream of the OR101-1 gene were analyzed.

Surprisingly, the experiments showed that construct with up to 2 kb of genomic sequence upstream of the OR101-1 show higher transgene efficiency compared to longer constructs. Constructs longer than 2 kb were expressed less efficiently. Further analysis shows that 500 bp of sequence, located between 2 kb and 2.5 kb upstream of OR101-1 establishes the observed inhibitory effect. When this sequence is deleted, the efficiency of the transgene increases accordingly. Cloning this 500 bp of sequence in front of as shorter but efficient promoter construct causes a significant drop in transgene efficiency. The partial removal of the 5'- or 3'-half of the sequence had only partial effects. Therefore, it is likely that this 500 bp sequence contains important transcription factor binding sites that negatively regulate the OR101-1 expression.

Bioinformatic analysis revealed that the sequence motif CNTCTGG is represented twice with in the 500bp sequence, each in either half of the region (Söğünmez, 2012). Interestingly, the CNTCTGG motif is candidate binding domain for zinc finger domain containing, broad tram track, brik-a-brag (zbtb) transcription factors described above, which have been found to be associated with proximal promoter sequences of OR and VR genes in mouse (Michaloski *et al.*, 2011). The zbtb transcription factors can mediate epigenetic silencing of gene expression and repression of transcription via chromatin modification (Lee and Maeda, 2012). The zbtb binding sites thus constitute attractive candidate regulatory motifs that could underlie the observed repression.

In addition, the transcriptome analysis for expression of the members of zbtb family reveals some zbtb genes are highly expressed in OE of the zebrafish and the gene with the highest expression is zbtb7b in zebrafish OE. Interestingly this zbtb7b is found also high in OE of mouse and suggested to have a repressive effect in the expression of the chemosensory receptor(Michaloski *et al.*, 2011).

2. PURPOSE

How an OSN chooses a single OR for expression and how this choice is maintained throughout life is a striking question in olfactory biology that has only partially been answered. In addition to long-range cis-acting regulators and epigenetic control mechanisms, certain aspects of OR expression strongly depend on specific transcription factors that interact with proximal promoter sequences of OR genes. Yet, only few relevant transcription factors have been functionally identified so far and a comprehensive picture of this type of regulation is still lacking.

A transgenic analysis of the zebrafish OR101-1 gene promoter previously pointed towards a relatively large genomic region located 2 kb upstream of the gene that negatively regulates expression, suggesting that it may contain binding sites for novel transcriptional repressors. This is an interesting finding because up to now only activating transcription factors have been reported and no functional evidence for inhibitory regulation has been described in the vertebrate olfactory system.

The main purposes of the research presented in this thesis are a functional and in-depth analysis of this novel inhibitory regulation and to examine its possible biological role. In particular, I wanted to understand if specific sequences within the larger genomic region contribute to the observed repression. The region contains repeats of a motifs, which are similar to repressive protein binding sites. I hypothesized that transcription factor binding to these motifs contributes to the observed repression and negatively affects transgene expression.

As a first aim, I wanted to analyze the significance of these candidate transcription factor binding sites by rendering them non-functional through site-directed mutagenesis and observe the effect of the mutation on promoter transgene expression. In an attempt to shed light onto a possible biological function of this repression, I speculated that the region

may act as an insulator or attenuator to protect the OR101-1 promoter from the influence of nearby enhancer sequences. Therefore, I wanted to analyze the degree of repression that can be observed in transgenic constructs that contain the powerful murine H-enhancer. Previously it was observed that the H-enhancer can direct transgene expression to additional chemosensory cell types. Thus, I also wanted to understand whether repression from the genomic sequence can contribute to improved cell type specificity

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Fish

The fish species used in this study was *Danio rerio*, commonly known as zebrafish. In the experiments, either the inbreed AB/AB and AB/Tü wild type strains obtained from the Zebrafish International Resource Center (ZIRC) or fish obtained from a local pet shop (PS-wt), were used. The animals were maintained in a dedicated fish room at the Boğaziçi University Life Sciences Center (Vivarium).

3.1.2. Equipment and Supplies

A detailed list of chemicals, equipments, and consumables, including manufacturers and order information can be found in Appendix A (Equipment) and Appendix B (Supplies).

3.1.3. Buffers and Solutions

Buffers and solutions that were used in molecular biology procedures were prepared according to Sambrook and Russell (2001) or obtained from the manufacturer directly. Zebrafish-specific solutions and buffers were prepared according to Westerfield (2007).

3.2. Methods

3.2.1. Maintenance and Breeding of Fish

Zebrafish strains (AB/AB, AB/Tü, and PS-WT) were kept at 28ºC under a 14 hours light / 10 hours dark light regime. Up to 5 zebrafish were kept in 1 l tanks, 6-15 zebrafish in 3 l tanks, and up to 50 zebrafish in 10 l tanks. Dedicated fish to obtain fertilized oocytes for microinjection of plasmid DNA were kept as individual couples in 1 l tanks. Artificial fresh water was prepared by supplementing 100 liters of reverse osmosis water with 2 g sea salt (Instant Ocean), 7.5 g sodium bicarbonate, and 0.84 g calcium sulfate. The flow, aeration, five-stage filtration and UV sterilization of system water was provided by professional housing systems (Stand Alone System, Aquatic Habitats). Adult zebrafish were fed three times a day with flake food (TetraMin, Sera Vipan) and supplemented with live brine shrimp larvae (*Artemia sp.*) during morning and evening feedings.

To obtain fertilized oocytes for microinjection experiments, specially designed mating tanks were used. Mating tanks are equipped with separator walls to physically isolate male and female fish during overnight premating exposure and a mesh bottom to protect freshly spawned oocytes from predation by the parents. The removable separator wall also allows for accurate timing of mating. This was necessary because fertilized oocytes can be used for microinjection only before the first cleavage, which occurs around 20 to 30 minutes after fertilization. Following evening feeding episodes, fish of both sexes were transferred to separate compartments of the mating tanks and left overnight without direct physical contact. Artificial plants were placed into the mating tanks to provide comfortable spawning areas for female fish. Separator walls were removed immediately before microinjection experiments but not earlier than the onset of the light period. Fertilized oocytes collect in a separate bottom compartment underneath a mesh bottom of the inset chamber and were collected with the help of a strainer and transferred to petri dishes containing E3 embryo medium.

3.2.3. Polymerase Chain Reaction (PCR)

Polymerase chain reactions to amplify DNA fragments for cloning purposes were performed using the GoTaq Flexi DNA Polymerase (Promega), OneTaq (NEB), or Advantage Taq (Clonetech) PCR kits according to the manufacturers recommendations. For a typical PCR reaction, 100 ng of plasmid DNA, 0,5 µM of forward and reverse primer, respectively, 1x reaction buffer, 1.5 mM $MgCl_2$ (added separately if the reaction buffer does not contain), 0.2 mM dNTP mix and 1-2 units of Taq polymerase were used. The PCR conditions were set to 3 min pre-amplification denaturation at 95˚C, 24-30 cycles of 45 sec denaturation at 95ºC, 30 sec annealing at the annealing temperature (4 degree lower than the melting temperature of the lowest melting oligonucleotide) and 1 min /1 kb target amplicon at 72°C, followed by a final elongation step for 8 min at 72˚C.

For colony PCR on templates obtained from crude lysis of bacteria to identify positive colonies following ligation, home-made Taq polymerase was used. A typical reaction mixture contained 0,5 µM of each primer, forward and reverse, 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix and 1 µL of homemade Taq polymerase. The PCR cycling conditions were set to an initial 5 min lysis and denaturation step at 95˚C, followed by 30 cycles of 45 sec denaturation at 95ºC, 30 sec at the annealing temperature (4 degree lower than the melting temperature of the lowest melting oligonucleotide) and 1 min /1 kb target amplicon at 72 °C. A final elongation step was performed for 8 min at 72˚C.

3.2.4. Restriction Enzyme Digestion of DNA

Restriction endonucleases from Promega, Invitrogen or New England Biolabs (NEB) were used. In a typical reaction mixture 3-6 units of restriction enzyme was used per microgram of DNA using appropriate buffer (1x concentration) and 1x BSA if required by the enzyme. The endonuclease digestion reactions were incubated at 37ºC for 1 – 4 hours for analytical digests or overnight for quantitative digestion reactions.

3.2.5. Agarose Gel Electrophoresis

Following amplification of DNA fragments by PCR or restriction enzyme digestion, DNA fragments were analyzed by agarose gel electrophoresis using 1% agarose gels. The gel was prepared with TAE buffer and supplemented with 0.5 µg/ml Ethidium bromide. 1x TAE was used as running buffer at 60 – 100 V. After the desired fragment separation was obtained, gels were visualized under UV light in a GelDoc XR (Bio-Rad Labs, USA) system and documented electronically as TIF images. The 1kb and 100bp DNA ladders (NEB, USA) were used as molecular weight markers.

3.2.6. PCR and Gel Purification

The High Pure PCR Purification Kit (Roche, USA) was used according to the manufacturer's instructions to purify PCR products, digested plasmids, and to extract DNA fragments from agarose gels. For gel extraction, the desired DNA fragment was cut out from the gel using a scalpel and weighed. Then 300 µl of binding buffer per 0.1 g of agarose gel was added and the mixture was incubated at 56ºC for 10 minutes to allow melting of the agarose gel. Then 150 µl isopropanol were added per 0.1g of agarose gel and the mixture was transferred to spin columns provided by the kit, washed, and eluted using wash and elution buffers provided by the kit. Eluted DNA was quantified using the NanoDrop® Spectrophotometer or visualized directly on agarose gels.

3.2.7. Site-directed Mutagenesis by overlap extension PCR

Site-directed mutagenesis by overlap extension PCR was performed to introduce targeted mutations into candidate regulatory sequences within the promoter of the OR101-1 gene. Two candidate zbtb7b binding site heptanucleotide sequences were eliminated from longer OR101-1 promoter constructs using specific mutagenesis oligonucleotides (Table 3.1).

Table 3.1. The designed primers for site-directed mutagenesis.

Primer Name	#	Sequence	Tm
500_bp_inhibitor_forward_primer	283	5’-GAACTAGTCTAATGTGCTGC-3’	56.4ºC
500_bp_inhibitor_reverse_primer	281	5’-CTGGAAGCATTGCTACCTAGG-3’	61.2 ºC
3-RP58_del_Fwd (Right mutation)	328	5’-GAGTTGGATGGAGTT TAGGAAGAGAGAATGTCATCAGC-3’	75.8 ºC
3-RP58_del_rev (Right mutation)	329	5’-CTCTCTTCCTAAACTC CATCCAACTCTGGAACAGTTC-3’	75.6 ºC
5-RP58_del_Fwd (Left mutation)	330	5’-GGAGTTCAGTAGACCT TCTAGGGATGACATATCC-3’	74.1 ºC
5-RP58_del_rev (Left mutation)	331	5’-CCCTAGAAGGTCTACTG AACTCCATTGCTGTTCAC-3’	75.6 ºC

To remove both candidate zbtb7b sites within the same DNA fragment or individually, a total of six oligonucleotide primers were designed that are complementary to the sequence around the target sites. The forward and reverse mutation primers shared overlapping sequences, excluding the target site and were paired with outside sequence-specific primers. The steps of the site-directed mutagenesis procedure for the mutation of the 5’-binding site is exemplarily summarized in Figure 3.1. Primers 330 and 331 are complementary to the sequences flanking the site to be deleted on the forward and reverse strand, respectively. Two separate reactions were performed during a first round of PCR amplification using primer pairs 283 / 330 and 331/ 281, respectively to generate 5’ and 3’ fragments that contain the mutation. Then both fragments were combined as template for overlap PCR using the 283 / 281 primer pair in a second round of amplification. This PCR reaction generated a final product of 572bp containing the desired deletion of the RP58 site. The same procedure was applied to generate a deletion of the 3’ binding site using 283 / 328 and 330 / 281 primer pairs, respectively. The composition of the reaction mixture is given in Tables 3.2 and 3.3.

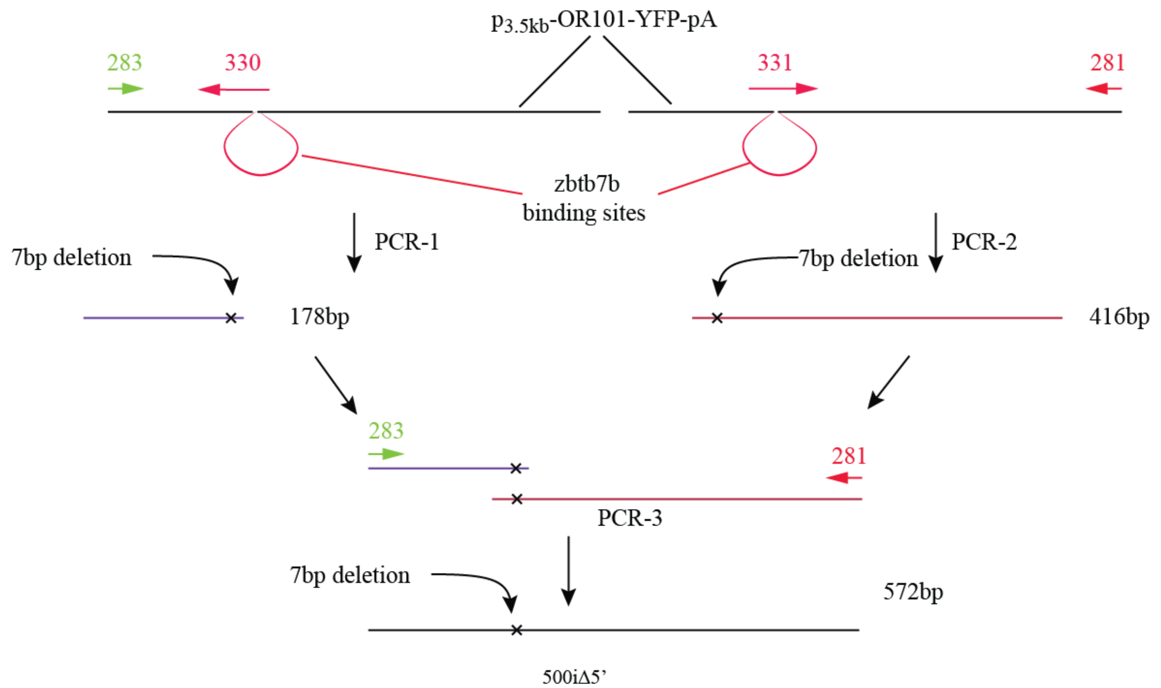

Figure 3.1. The steps of the site directed mutagenesis for 5' mutation.

Table 3.2. The composition of PCR for 5' mutation.

	PCR-1	PCR-2	PCR-3
Template	P3.5kb-OR101-YFP-pA (100ng)	P3.5kb-OR101-YFP-pA (100ng)	Amplicon 5'-1 (0,82µL) + amplicon5'2 (2µL)
Forward Primer	500_bp_inhibitor_forward_primer (2.5µL)	5-RP58_del_Fwd (2.5µL)	500_bp_inhibitor_forward_primer (2.5µL)
Reverse primer	5-RP58_del_rev (2.5µL)	500_bp_inhibitor_reverse_primer (2.5µL)	500_bp_inhibitor_reverse_primer (2.5µL)
dNTP	1 µL	1 µL	1 µL
Advantage Taq buffer	5 µL	5 µL	5 µL
Advantage Taq	1 µL	1 µL	1 µL
Obtained product	Amplicon 5'-1 (178bp, 31ng/µL)	Amplicon 5'-2 (416bp, 29,4ng/µL)	500iΔ5' (572bp)

Table 3.3. The composition of PCR reactions for 3’ mutation.

	PCR-1	PCR-2	PCR-3
Template	p3.5kb-OR101-YFP-pA (100ng)	P3.5kb-OR101-YFP-pA (100ng)	Amplicon 3’-1 (1,12μL) + amplicon3’2 (2μL)
Forward Primer	500_bp_inhibitor_forward_primer (2.5μL)	3-RP58_del_Fwd (2.5μL)	500_bp_inhibitor_forward_ primer (2.5μL)
Reverse primer	3-RP58_del_rev (2.5μL)	500_bp_inhibitor_reverse _primer (2.5μL)	500_bp_inhibitor_reverse_ primer (2.5μL)
dNTP	1 μL	1 μL	1 μL
Advantage Taq buffer	5 μL	5 μL	5 μL
Advantage Taq	1 μL	1 μL	1 μL
Obtained product	Amplicon 5’-1 (143bp, 89.3ng/μL)	Amplicon 5’-2 (448bp, 156.8ng/μL)	500iΔ3’(572 bp)

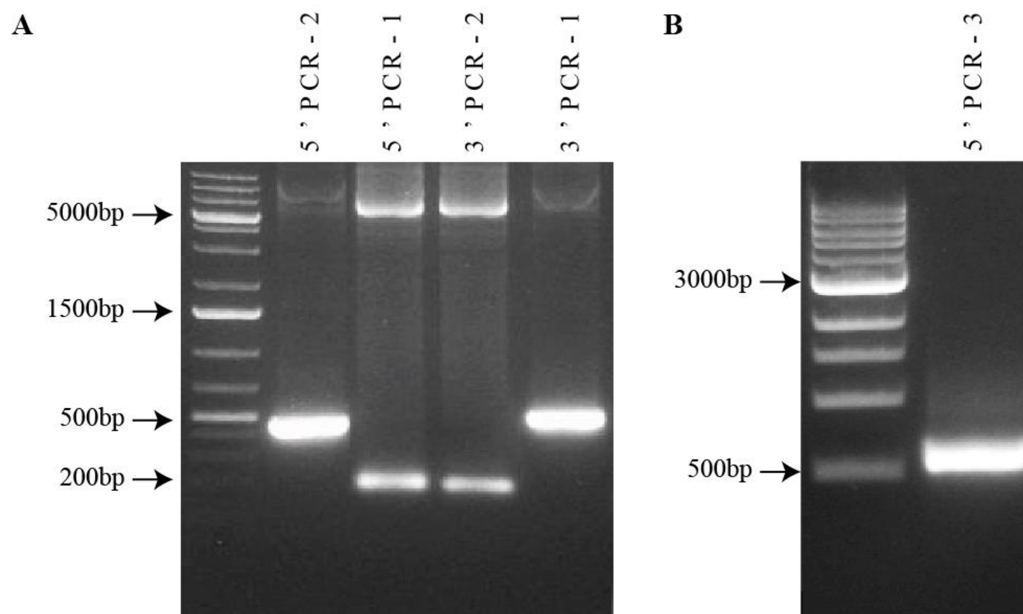

Figure 3.2. (A) Amplicons from PCR-1 and PCR-2 (lane 1-416bp, lane 2-178 bp, lane-3 143 bp, lane-4 448 bp). (B) Amplicon from PCR-3 for 5’ mutation (572 bp).

To create the combined mutation of both binding sites, first the 5’-mutation was generated and cloned into pGEM-T Easy vector. This plasmid was the used as a template for the overlap extension PCR to generate the 3’ mutation. The resulting DNA fragment harboring the combined mutation of 5’- and 3’-binding sites was cloned to the pGEM-T Easy vector. The composition for the reaction is given in Table 3.4, while Table 3.5 summarizes the reaction conditions for all PCR reactions.

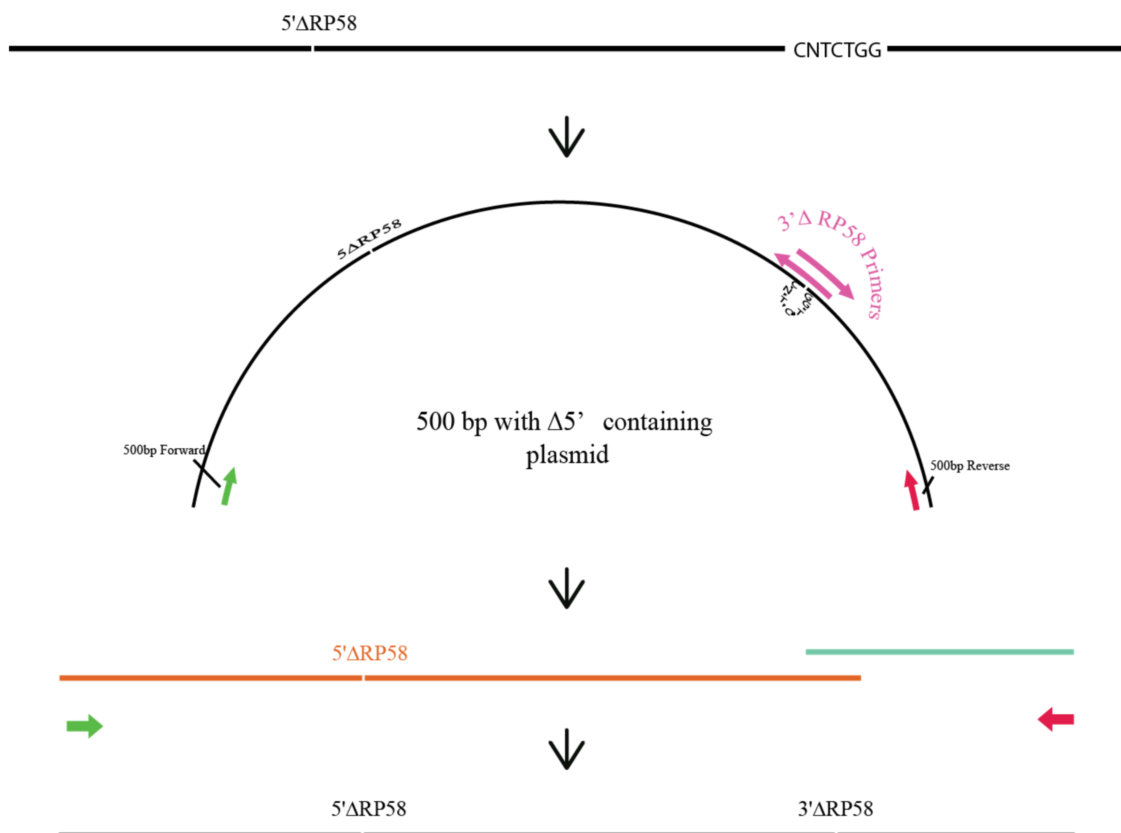

Figure 3.3. The generation of the double mutated construct.

Table 3.4. The composition of PCR reactions for 5'/3' mutation.

	PCR-1	PCR-2	PCR-3
Template	500iΔ5' in pGEM-T Easy (100ng)	500iΔ5' in pGEM-T Easy (100ng)	Amplicon 5/'3'-1 (1,6μL) + amplicon 5'/3'2 (1μL)
Forward Primer	500_bp_inhibitor_forward_primer (2.5μL)	3-RP58_del_Fwd (2.5μL)	500_bp_inhibitor_forward_primer (2.5μL)
Reverse primer	3-RP58_del_rev (2.5μL)	500_bp_inhibitor_reverse_primer (2.5μL)	500_bp_inhibitor_reverse_primer (2.5μL)
dNTP	1 μL	1 μL	1 μL
Advantage Taq buffer	5 μL	5 μL	5 μL
Advantage Taq	1 μL	1 μL	1 μL
Obtained product	Amplicon 5'/3'-1 (143bp, 15,4ng/μL)	Amplicon 5'/3'-2 (411bp, 70,9ng/μL)	500iΔ5'/Δ3' (565bp)

Table 3.5. PCR conditions for each PCR.

	PCR-1	PCR-2	PCR-3
Denaturation	3min, 95ºC	3min, 95ºC	3min, 95ºC
Denaturation	45 sec, 95ºC	45 sec, 95ºC	45 sec, 95ºC
Annealing	40 sec, 60ºC	40 sec, 60ºC	40 sec, 54ºC
Elongation	40 sec, 68ºC	40 sec, 68ºC	40 sec, 68ºC
Final elongation	7min, 68ºC	7min, 68ºC	7min, 68ºC
Cycle	28	28	24

3.2.8. Preparation of Transgenic Constructs

The DNA fragments containing the individual or combined mutations of RP58 sites within the 500 bp sequence were cloned into the pGEM-T Easy vector and screened for SP6 orientations. The orientation screening was performed by PCR using the forward primer 283 and a T7–specific primer (primer 82). 5µg of each plasmid was digested with *Sac*I and *Sac*II restriction endonucleases and purified by gel purification. 5µg of the $p_{1.2kb}$-OR101-1-YFP-pA plasmid was digested sequentially with *Sac*I and *Sac*II because in this plasmid the restriction sites are only 7 bps apart from each other. Thus, after digestion, the DNA was purified using gel purification and insert and vector were ligated in a ratio of 3:1. The ligation product was transformed into *E. coli* and positive colonies were screened by colony PCR using the 283 / 281 Primer combination. The resulting constructs were named: $p_{500i\text{-}1.2kb}$-OR101-YFP-pA, $p_{500i\Delta5'/\Delta3'\text{-}1.2kb}$-OR101-YFP-pA, $p_{500i\Delta5'\text{-}1.2kb}$-OR010-YFP-pA, and $p_{500i\Delta3'\text{-}1.2kb}$-OR101-YFP-pA.

To generate constructs that contained the mouse H-element, 5µg $p_{H-1.2kb}$-OR101-YFP-pA was digested with *Sac*I and *Sal*I and the 2.1 kb fragment of the H-element was purified from an agarose gel. Then 3 µg each of plasmid, $p_{500i-1.2kb}$-OR101-YFP-pA and $p_{500i\Delta5'/\Delta3'-1.2kb}$-OR101-YFP-pA, were digested with *Sal*I and *Sac*I sequentially because the restriction sites are close the each other and only 11bp apart. The digestion products were purified by gel purification and insert and vector were ligated in a ratio of 3:1. The ligated DNA was transformed into *E. coli* and colonies positive for the presence of the H element were screened by colony PCR using the H_CORE_SACI_F (240) and H_CORE_SACII_R (241) primer combination. Colonies that screened positive were further analyzed by colony PCR using the 500_bp_inhibitor_forward (283) and 500_bp_inhibitor_reverse (281) primer combination to confirm the presence of the H element in the correct target plasmid. The resulting constructs were named $p_{H-500i-1.2kb}$-OR101-YFP-pA and $p_{H-500i\Delta5'/\Delta3'-1.2kb}$-OR101-YFP-pA, respectively.

Table 3.6. Primers used for generation of transgenic constructs.

Primer Name	#	Sequence	Tm
500_bp_inhibitor_forward_primer	283	5'-GAACTAGTCT AATGTGCTGC-3'	56.4ºC
500_bp_inhibitor_reverse_primer	281	5'-CTGGAAGCA TTGCTACCTAGG-3'	61.2ºC
H_CORE_SACI_F	240	5'-GAGCTCAATTCA GTTCCTCTTTTCTC-3'	64.6 ºC
H_CORE_SACII_R	241	5'-CCGCGGCTGTG TTCTCTCTTAATGAT-3'	67.9 ºC
T7_highTM	82	5'-TAATACGACTCAC TATAGGGCGAATTGG-3'	67.2 ºC

3.2.9. Ligation of DNA Fragments to Vectors

To ligate DNA fragments to vector backbones typically a 3:1 or 1:1 molar ratio of insert to vector was used. The required amount of DNA was estimated by the formula:

$$\frac{\text{Amount of vector (ng) x Size of insert (kb)}}{\text{size of vector (kb)}} \text{ x } \frac{molar\ ratio}{(insert: vector)} = \text{amount of insert (ng)}$$

The concentration of DNA was measured using the NanoDrop® Spectrophotometer, and the sizes of DNA fragments were determined by agarose gel electrophoresis . For each reaction 1 µl of T4 DNA ligase (NEB), 1 µl of 10x Ligase buffer, were combined with the appropriate amount of insert and vector ion a final reaction volume of 10 µl. The reaction mixture was incubated at 25ºC for 1 hour, followed by transformation into competent *E. coli*.

Direct cloning of PCR products was performed by using the pGEM-T Easy (Promega) vector system. For each ligation reaction up to 3.5 µl of purified PCR product, 0.5 µl pGEM-T Easy vector, 5 µl of 2x ligase buffer, 1 µl of T4 DNA ligase (NEB) and dH_2O to adjust the reaction volume to 10 µl were combined and incubated at 25ºC for 1 hour and transformed into competent *E. coli*.

3.2.10. Preparation of Competent Cells (Rubidium Chloride Method)

To generate *E.coli* competent for transformation by heat shock, a single colony of strain TOP 10 MRF' was selected from a fresh streak and inoculated overnight at 37ºC in 3 ml LB medium. 100 µl of the overnight culture was used to inoculate 100 ml of fresh LB medium and incubated at 37ºC on a shaker until the OD_{550} reached a value of 0.6. The culture was chilled on ice for 15 min and centrifuged at 3.000 rpm at 4ºC for 10 minutes. The bacteria pellet was resuspended in the remaining supernatant by gently tapping the spin bottle. The resuspended bacteria were mixed with 20 lL of CT1 (30 mM potassium

acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl and 15% glycerol; pH 5.7) per 100 mL of initial culture and incubated on ice for 30 min. The bacteria solution was centrifuged again at 3.000 rpm at 4ºC for 10 min, the supernatant was discarded and bacteria were resuspended in 4 ml of CT2 solution (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl and 15% glycerol; pH 6.5). After 15 min incubation on ice, the bacteria solution was divided into 50 µl aliquots, snap frozen in liquid nitrogen and stored at -80˚C until use.

Transformation efficiency was determined by the following formula:

$$\frac{colonies\ number\ per\ plate\ \ x\ \ total\ volume(\mu l)x\ dilution\ (\ if\ any)x10^{6}\ pg/g)}{volume\ plated\ (\mu L)x\ pg\ DNA\ used}$$

3.2.11. Transformation of Plasmid DNA into Competent Cells

For each transformation reaction 50 µL competent cells were thawed on ice for 5 min, mixed with up to 10 µl of plasmid DNA or ligation product and incubated on ice for 30 min. Typically, 10-50 ng of plasmid DNA for retransformations and 10 µl of ligation reaction were used for a transformation. Following incubation on ice, the tubes were transferred to a heat block adjusted to 42ºC and incubated for 90 sec and again incubated on ice for 5 minutes to recover. The transformed bacteria were resuspended in 500 µl of LB medium and incubated for 1 hour at 37ºC on a shaking platform. 100 µl of the transformation reaction were spread on LB agar plates with appropriate antibiotic selection. The remaining reaction mixture was centrifuged for 1 minute at maximum speed, the supernatant was removed by decanting and the cells were resuspended with the remaining supernatant before being spread on appropriate selection plates. Bacteria plates were incubated at 37ºC overnight.

3.2.12. Plasmid Isolation

Depending on the desired amount of plasmid DNA, different plasmid isolation kits were used. For small scale preparations of up to 20 µg the Plasmid MiniGeneJet Isolation kit (Thermo Scientific) and for larger samples of up to 100 µg, the Qiagen midi kit were used. Plasmid DNA was isolated from bacteria according to the manufacturer's protocol.

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3.2.13. Whole Mount Antibody Staining

Wild type and transgene-injected zebrafish embryos were collected at 5 dpf, euthanized with 0.04% MS222 (tricaine methanesulfonate, Sigma), and fixed overnight in 4% PFA/1x PBS. Following overnight incubation, the samples were washed in PBS, permeabilized in 1.5% Triton-X 100 in 1x PBS at 4ºC overnight. Then, embryos were blocked with BDP (1% BSA, 0.1% DMSO, in 1X PBS) containing 5% normal goat serum for 2 h at room temperature. Primary antibodies were diluted in BDP and the samples were incubated overnight at 4ºC under gentle agitation. The incubation was followed by multiple washes in BDP at room temperature before incubation with secondary antibody diluted in BDP over night at 4˚C. The next day, embryos were washed 5 times for 30 min to 1 h and analyzed under a fluorescence microscope.

Table 3.7. The properties of antibody used in whole mount antibody staining.

Antibody type	Antibody Name	Dilutions	Details
Primary	anti-TrkA (763) antibody	1:100	rabbit IgG; 15100; sc-118, Santa Cruz Biotechnology
Secondary	Alexa Fluor® 488	1:250	Donkey anti-rabbit IgG conjugated to Alexa Fluor 488
Secondary	Alexa Fluor® 633	1:250	Goat anti-rabbit IgG conjugated to Alexa Fluor 633

3.2.14. Morpholino Design

Morpholinos are synthetic antisense oligonucleotides that bind to complementary RNA sequences and which can be used to knock-down gene expression by blocking initiation of translation or splicing of introns. Two morpholino oligonucleotides against the zbtb7b pre-messenger RNA were designed by GeneTools,LLC (USA). The zbtb7b-TB morpholino was designed to bind to the 5'-UTR of zbtb7b 31 bp before the transcription start and is supposed to block translation. A second morpholino zbtb7b-E3 was designed against the intron 2 / exon 3 boundary and is supposed to block splicing, causing the inclusion of intron 2 into the mature mRNA. The change probably affects the zing-finger, C2H2 like domain of the protein necessary for DNA binding. The sequences of the morpholino oligonucleotides are given in Table 3.8

Table 3.8. Sequences of designed morpholino oligonucleotides.

Name	Sequence	Type
Zbtb7b-TB	5'-TAGGCGAACTCCAGCAGCGCCGCCA-3'	Translation blocking
Zbtb7b-E3	5'- TCGTTCCTGAAAACAGACGTGAGGT- 3'	Splice blocking (inclusion of intron 2)

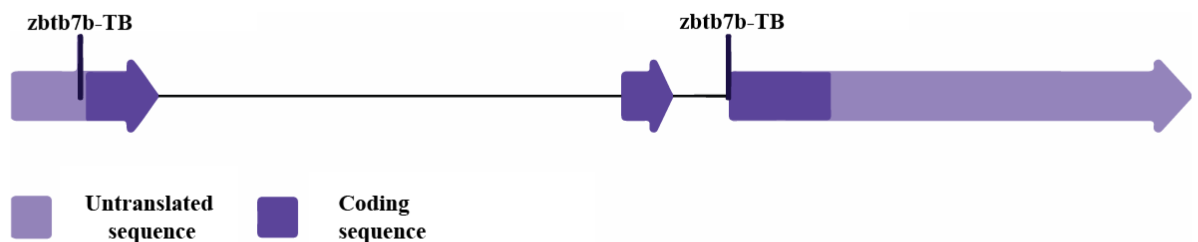

Figure 3.4. Binding sites of zbtb7b-TB (translation blocking morpholino for zbtb7b) and zbtb7b-E3 (splice blocking morpholino for zbtb7b, target site:intron2-exon3).

3.2.15. Microinjection into Zebrafish Oocytes

The day before injection of plasmid DNA or morpholino oligonucleotides, fish were set up in mating tanks with a separator wall to prevent premature mating. The separator was removed after the onset of the light cycle and fertilized oocytes were collected into specially prepared agarose injection molds.

Glass capillary needles were prepared for injection by backfilling with the plasmid DNA dissolve in injection solution (10mM KCL an 0.01% Phenol red). The concentration of plasmid DNA was adjusted to be 50 ng/µl for each construct (100 ng/µl total in co-injection experiments). For morpholino oligonucleotide injection, the stock solution (65mg/mlin water) was diluted to several concentrations ranging from 2 to 10 ng/l with 1x Danieu solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES; pH 7.6) and 0.5% Phenol red solution. With the help of Pasteur pipettes, fertilized eggs were lined up into the agarose mold under a stereo micorscope. A total amount of 2 to 4 nl of plasmid or morpholino oligonucleotide solution was injected into the yolk directly underneath the blastomere at the one cell stage using a FemtoJet® Express pressure injector (Eppendorf). The injection volume was calibrated by injection into an oil droplet on top of a micrometer slide and estimated from the droplet size (Figure 3.5). Following injection, embryos were removed from the mold and transferred to petri dishes containing E3 embryo medium supplemented with 20 µl of 0.03 M methylene blue per liter of E3 medium to get prevent the growth of mold. Embryos were kept at 28ºC in E3 medium until 3 dpf, the medium was renewed each day and dead embryos were removed.

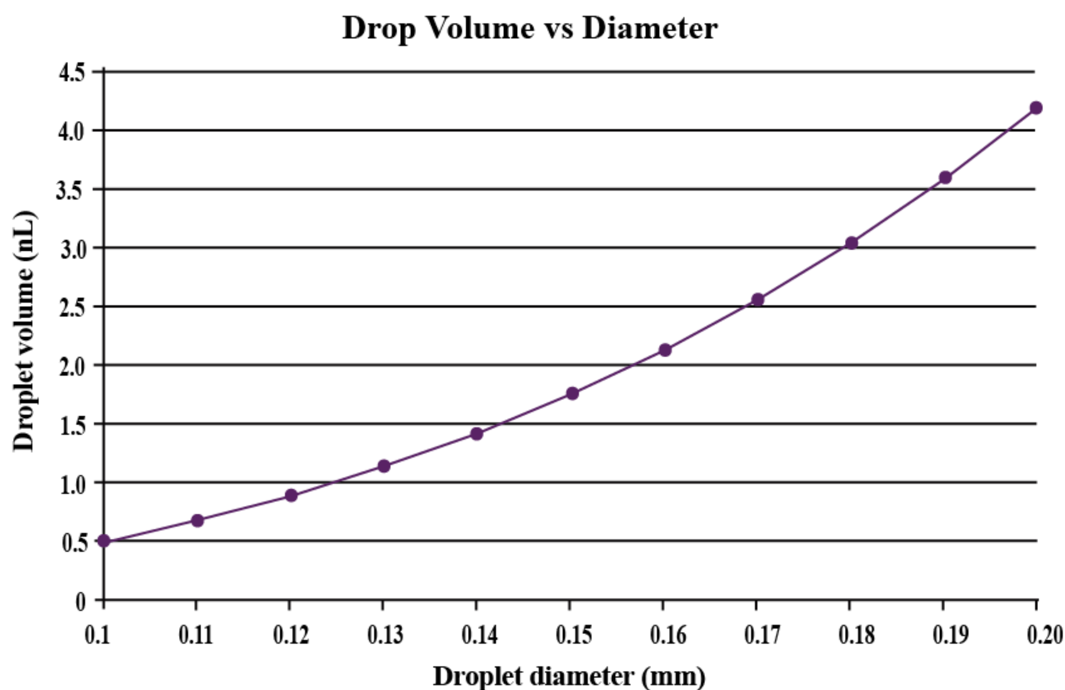

Figure 3.5. Calibration table for estimation of droplet diameter corresponds to volume (Derived from Mullins, 2013).

3.2.16. Imaging of Zebrafish Embryos

For live imaging and expression analysis, plasmid DNA-injected embryos were anesthetized with 0.04% MS222 (Sigma, USA) and mounted in specially designed agarose molds. (Figure 3.6). The embryos were then immobilized with low melting agarose and covered with a coverslip. Prepared agarose molds were submersed into MS222-containing petri dishes to guarantee that the embryo remain motionless. Mounted embryos were then imaged using a SP5-AOBS laser scanning confocal microscope with the 20X objective (Leica, Germany).

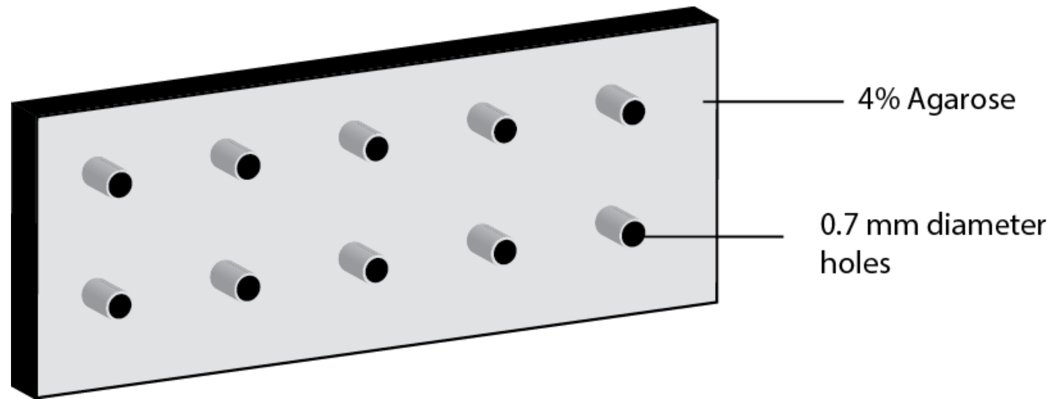

Figure 3.6. The custom designed agarose mold to hold embryos during screening and imaging process. (15mm X 40mm X 3mm thick approximately.) Each mold has at least 8 holes with diameter 0.7mm.

4. RESULTS

The selection of an OR gene is probably the most critical event during the final steps of OSN differentiation (Mori and Yoshihara, 1995). Analogous to lymphocytes in the immune system, OSNs are monospecific and exclusively select one allele of a single OR gene from a large genomic repertoire for expression (L. Buck and Axel, 1991). Although conclusive experimental proof is lacking, this mechanism may hold true for zebrafish OSNs as well. Using multi-color in situ hybridization for a limited number of 16 out of 171 zebrafish OR genes, Sato *et al.* (2007) showed that most of these OR genes from are not coexpressed by the same OSN. An exception, however, was found for the OR103-1 / OR103-5 pair, which appears to be coexpressed systematically by the same OSN. Even though, the full mechanism of OR gene choice is not completely understood, selection of OR genes for expression appears to be largely stochastic (Chess *et al.*, 1994; Qasba and Reed, 1998; Serizawa *et al.*, 2000; Vassalli *et al.*, 2002b). The OR gene choice mechanism may be to some extent controlled by long-range activating elements, such as the H- and P-elements in mouse or the E15-1 and E15-2 enhancers in zebrafish (Fuss *et al.*, 2007; Khan *et al.*, 2011; Nishizumi *et al.*, 2007; S Serizawa *et al.*, 2000). These long-range cis-activating elements are supposed to interact with more proximal regulatory sequences located within OR gene promoters. In contrast to this type of regulation, however, rather short proximal DNA sequence surrounding the TSS of OR genes by less than 500 bps have been shown to drive transgene expression independent of long-range enhancers for some OR genes (Qasba and Reed, 1998; Vassalli *et al.*, 2002a; Vasalli et al 2011). Recently, it has also been shown that the subnuclear organization of OR gene loci is not random; silent OR alleles that are not expressed appear to cluster in specific nuclear regions, whereas the expressed allele locates in a region of dynamic heterochromatin (Armelin-Correa *et al.*, 2014; Clowney *et al.*, 2012). Accordingly, several epigenetic modifiers and transcription factors that could recruit chromatin remodeling complexes to OR gene loci have been described (Lyons *et al.*, 2013; Markenscoff-Papadimitriou *et al.*, 2014). Yet, regardless of downstream mechanisms that enable stable expression of a single OR gene per OSN, additional factors acting on promoter / enhancer sequences most likely contribute to other aspects of OR expression, such as zonal and temporal expression profiles.

In this study, the promoter sequence of a model zebrafish OR gene, OR101-1, was used to understand aspects of OR gene expression control by proximal cis-regulatory sequences upstream of the OR101-1 TSS. In a previous study, sequences upstream of the OR101-1 gene were analyzed in a promoter bashing screen to pinpoint the position of relevant regulatory sites that positively or negatively affect OR101-1 expression (Söğünmez, 2012). In this study, a rather broad region of 500 bp (termed 500i) located 2 kb upstream of the OR101-1 coding sequence was identified, which had a repressive effect on transgene expression. This was a significant finding, because up to now no repressive regulatory factors controlling OR expression have been identified functionally. The focus of the experiments described below is an in-depth analysis of this rather large negative-regulatory sequence aiming at the identification of specific transcription factor (TF) binding sites that mediate the observed reduction in OR101-1 transgene expression. In addition, experiments are described that aim to elucidate the functional role of the observed repression of OR101-1 promoter activity.

4.1. Effect of the 500i region on promoter activity

The sequence of the proximal OR101-1 gene promoter has been dissected previously and properties of expression have been described for various segments of the first 3.5 kb upstream of OR101-1 (Söğünmez, 2012). It could be shown that 1.2 kb of sequence upstream of the OR101-1 gene coding sequence, which corresponds to approximately 600 bp of sequence upstream of the TSS, promote robust reporter gene expression in 61.3% of zebrafish embryos that have been injected with a transgenic construct driving fluorescent reporter genes under control of this sequence. Interestingly, longer construct that comprised additional sequences further upstream resulted in a dramatic loss of transgene expression. Detailed analysis pinpointed a region located between 2.0 and 2.5 kb upstream of OR101-1 that mediates the observed repression. It has been demonstrated that exclusion of this 500i sequence from transgenic constructs rescued high levels of transgene efficiency.

Bioinformatics analysis of the 500i sequence revealed the presence of two sequence motifs with the consensus sequence CNTCTGG (Söğünmez, 2012). This motif bears strong similarity to a binding site for repressor protein 58 (RP58). RP58 (aka ZBTB18, C2H2-171, MRD22, TAZ-1, or ZNF238) is a member of the BTB-ZF (broad tramtrak bric-a-brac zinc-finger domain) or POK (POZ and Krüppel) families of transcription factors and contains both Krüppel-like zinc finger DNA binding and BTB domains. RP58 was shown to recruit DNMT3 as a transcriptional co-repressor that targets HDAC1 to transcriptionally silent heterochromatin through its ATRX domain (Fuks *et al.*, 2001). Thus, binding of RP58 or closely related factors to 500i might be responsible for the observed repressive effect on transgene expression from the OR101-1 promoter. A strong candidate binding factor would be zbtb7b, which has been identified to be expressed in the mouse olfactory tissue and occurrence of related binding sites has been found to be overrepresented in OR and VR gene proximal sequences (Michaloski *et al.*, 2011).

4.1.1. Confirmation of high transgene expression using a1.2 kb promoter construct

A transgenic construct containing 1.2 kb of sequence upstream of the OR101-1 gene coding sequence exhibits strong penetrance of expression when microinjected into fertilized zebrafish oocytes (Söğünmez, 2012). Up to 65% of injected zebrafish embryos were found to express a fluorescent reporter gene from this transgenic construct. In a first experiments I wanted to replicate this finding by using the existing $p_{1.2kb}$-OR101-YFP-pA construct (Figure 4.1A) to assure that comparable levels of transgene efficiency can be obtained. Therefore, the $p_{1.2kb}$-OR101-YFP-pA construct was simultaneously injected with a $p_{1.3kb}$OMP-mCherry-pA construct that was cloned into the pT2AL200R150G vector. The $p_{1.3kb}$OMP-mCherry-pA construct could, in principle, be expressed by all ciliated OSNs and typically 80 – 90% of embryos injected with this construct are positive for the transgene. Thus, the OMP promoter construct can serve as a general control for injection efficiency and can be employed to normalize results across different injection experiments. For all experiments described below, the embryos surviving the injection procedure were analyzed at 3 dpf by confocal microscopy and scored as the percentage of $p_{1.3kb}$OMP-

mCherry-pA-expressing embryos, using expression of the mCherry reporter by at least one OSN as cut-off .

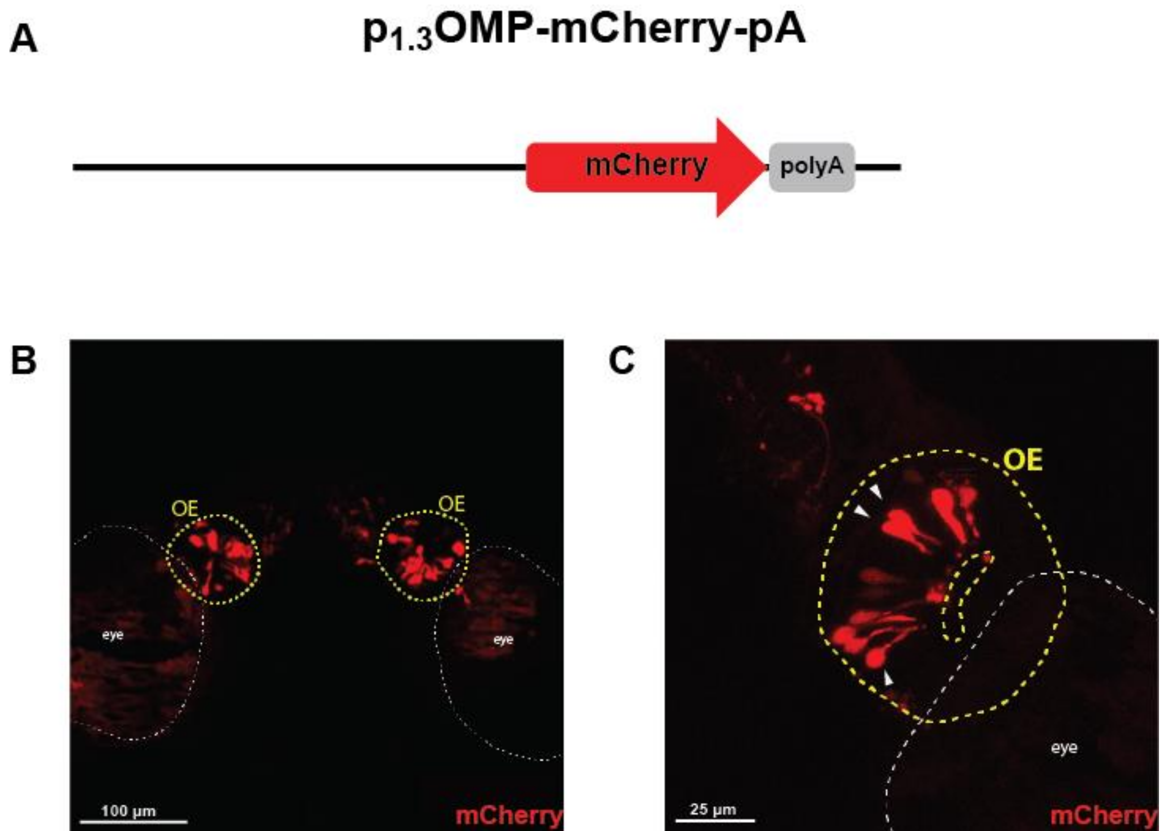

Figure 4.1. Transgene expression of a $p_{1.3kb}$-OMP-mCherry-pA. (A) Graphical representation of the $p_{1.3kb}$-OMP-mCherry-pA. (B) Confocal z-projections of both OE at 3 dpf (10x, red: mCherry fluorescence) (C) Confocal z-projections of a representative OE at 3 dpf (20x, red: mCherry fluorescence). Arrowheads point to mCherry-expressing OSN, the yellow line indicates the circumference of the OE.

A total of 657 embryos were injected with the $p_{1.2kb}$-OR101-YFP-pA construct in 7 independent injection experiments and 293 embryos survived until analysis at 3 dpf. Of those, 189 fish (64.5%) scored positive for mCherry expression and 154 fish for YFP expression from the $p_{1.2kb}$-OR101-YFP-pA construct (Figure 4.1B; individual numerical results are detailed in appendix C). Thus, in my hands the transgene efficiency of p1.2kb-OR101-YFP-pA normalized to $p_{1.3kb}$OMP-mCherry-pA was 82.6 ± 5.6%. Surprisingly, this

efficiency was slightly higher than previously reported by Söğünmez (2012) and might be attributed to personal differences in the injection procedure, purity of DNA prepared for injection, or differences in the effective amount of plasmid DNA that is deposited into the oocyte.

As an alternative measure of transgene efficiency, the number of YFP-positive OSNs per embryo was determined and expressed relative to the number of mCherry-positive cells. A total of 87 mCherry-positive OEs were analyzed and the number of YFP- and mCherry-expressing OSNs was counted under the fluorescence microscope. The number of YFP-expressing cells was divided by the number of mCherry-expressing OSNs for each OE separately and averaged over all analyzed samples. The average ratio of YFP-/mCherry-positive cells for p1.2kb-OR101-YFP-pA was found to be 0.59 ± 0.04. In two samples, the number of YFP-expressing cells was higher than the number of mCherry-expressing OSNs.

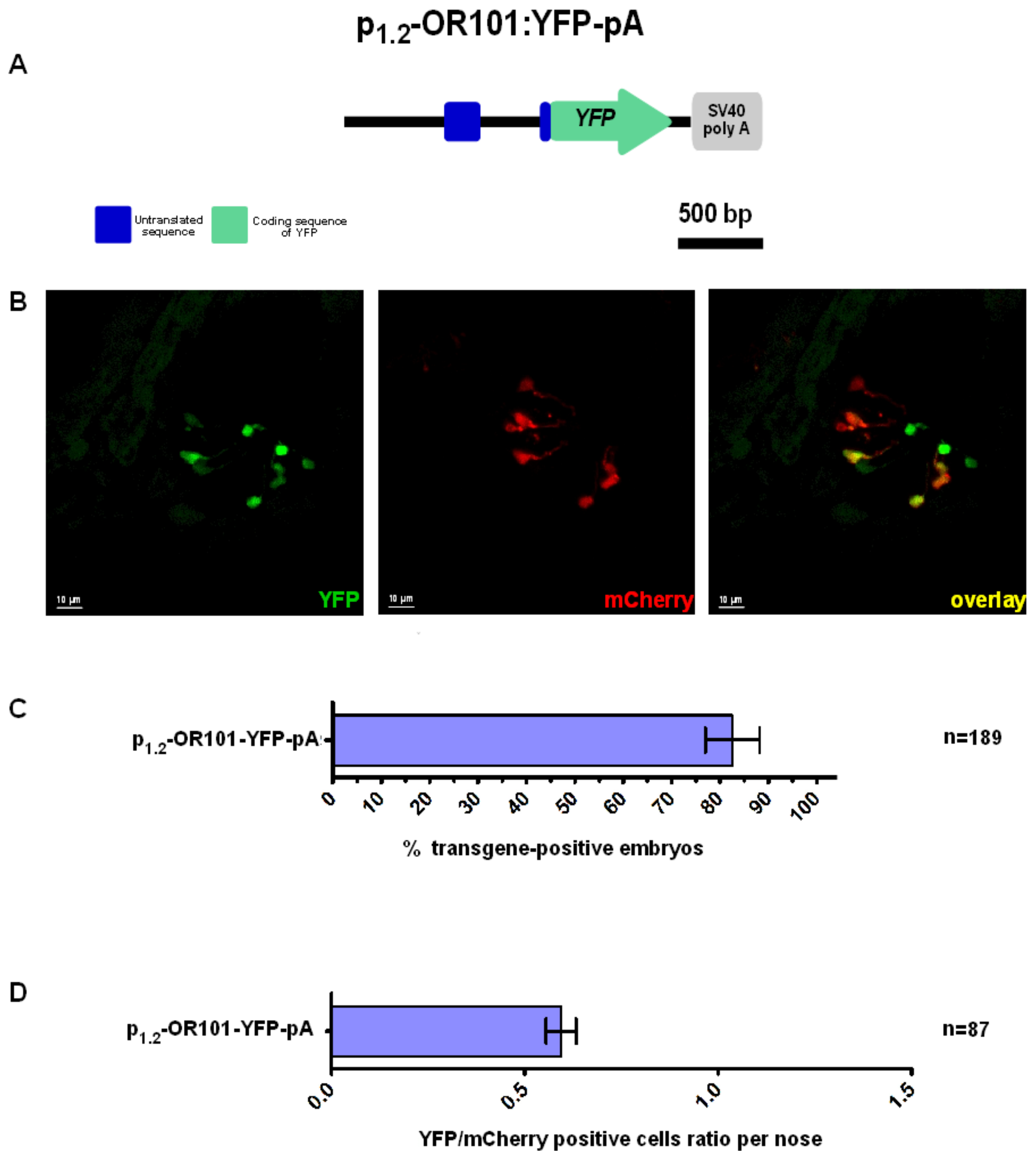

Figure 4.2. Transgene expression analysis of a $p_{1.2kb}$-OR101-YFP-pA construct. (A) Graphical representation of the $p_{1.2kb}$-OR101-YFP-pA construct. (B) Confocal z-projections of a representative OE at 3dpf (green: YFP fluorescence, red: mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of $p_{1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of YFP-positive / mCherry-positive OSNs per OE.

4.1.2. The effect of 500i on $p_{1.2kb}$-OR101-YFP-pA expression

A previous analysis of the genomic region upstream of OR101-1 indicated that the sequence located between 2.0 and 2.5 kb upstream of the OR101-1 coding sequence strongly represses transgene expression. Transgenic promoter constructs containing this 500i sequence showed poor penetrance when injected into zebrafish oocytes. The specific exclusion of 500i from larger transgenic construct efficiently rescued transgene expression. Similarly, cloning of 500i to the 5'-end of the highly efficient $p_{1.2kb}$-OR101-YFP-pA construct resulted in a significant decrease in expression efficiency (Söğünmez, 2012). Since in my hands injection of $p_{1.2kb}$-OR101-YFP-pA resulted in transgene efficiencies that were higher than previously reported, I wanted to understand which efficiency I would obtain with a similar $p_{500i\text{-}1.2kb}$-OR101-YFP-pA construct (Figure 4.2A). The $p_{500i\text{-}1.2kb}$-OR101-YFP-pA construct was coinjected with $p_{1.3kb}$OMP-mCherry-pA into fertilized zebrafish oocytes. A total of 788 embryos were injected in 9 independent experiments and 418 embryos survived until 3 dpf for analysis. Of those, 245 embryos (58.6 %) were positive for mCherry expression in at least one OE and 113 embryos were positive for YFP expression. Thus, the normalized transgene effciency of the $p_{500i\text{-}1.2kb}$-OR101-YFP-pA construct was 48.0 ± 3.5% (Figure 4.2C). Thus, the presence of 500i resulted in a 41 % decrease of transgene efficiency when compared to the $p_{1.2kb}$-OR101-YFP-pA control construct, which promoted transgene expression in 82.6 % of embryos.

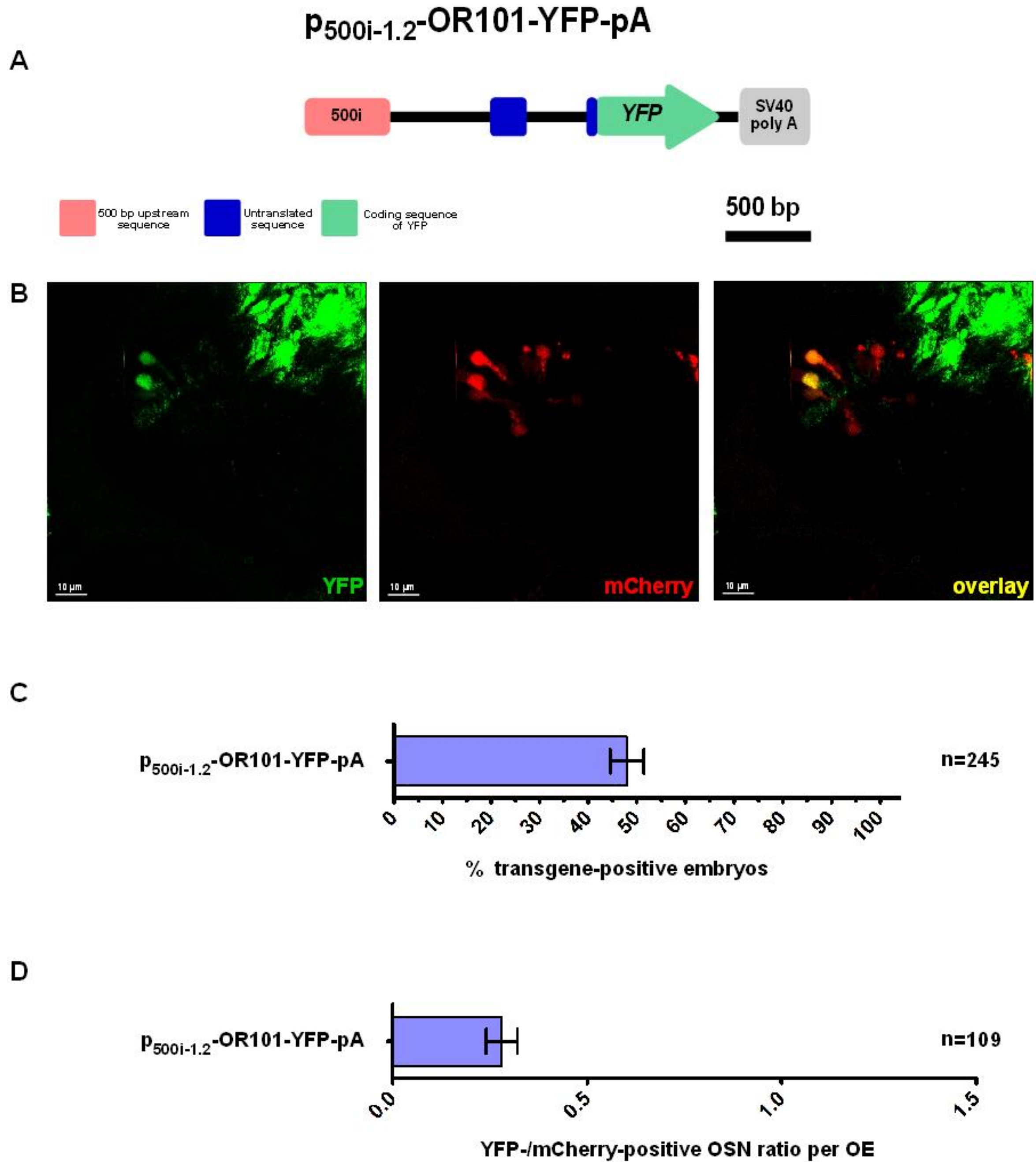

Figure 4.3. Transgene expression analysis of a $p_{500i-1.2kb}$-OR101-YFP-pA control construct. (A) Graphical representation of $p_{500i-1.2kb}$-OR101-YFP-pA construct. (B) Confocal z-projections of a representative OE at 3dpf (green: YFP fluorescence, red: mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of $p_{500i-1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of YFP-positive / mCherry-positive OSNs per OE.

A similar reduction in efficiency was evident at the level of transgene-expressing OSNs. A total of 109 OEs, which were positive for mCherry, were analyzed and in none of the OEs was the number of YFP-expressing cells higher than the number of mCherry-positive cells. The ratio of YFP-/mCherry-positive cells for $p_{500i\text{-}1.2kb}$-OR101-YFP-pA was 0.27 ± 0.04 cells, again a 54% decrease when compared to the $p_{1.2kb}$-OR101-YFP-pA construct (Figure 4.2D). Thus, the presence of the 500i sequence in transgenic promoter constructs affects cell number as well as overall efficieny of the trangenic construct. However, at this point it is not clear how tightly the number of transgene-positive cells and penetrance of expression are functionally correlated. Yet, it is conceivable that penetrance of transgene expression at the level of trangenic embryos is a consequence of the frequency of choice of the transgenic constructs at the level of individual OSNs. Yet, from this analysis and others (Kazcı, 2015) it appears that differences in the relative number of transgene-positive cells are a more sensitive measure of differences in promoter strength than the overall number of transgenic embryos. The results of this experiment also indicate that the repressive effect of 500i on the $p_{1.2kb}$-OR101-YFP-pA construct is reproducible, even though the overall efficiency of all constructs was higher in my hands than previously reported.

4.1.3. The effect of mutations within two candidate RP58 binding sites on the repressive function of 500i

As demonstrated above, 500i has a suppressive effect on transgene expression, both on the percentage of transgene-positive fish and and the number of transgene-positive OSNs. The sequence contains two candidate binding sites for the inhibitory transcription factor RP58 with the consensus sequence CNTCTGG. Transcription factors of this class recruit histone modifying enzymes that turn flanking genomic loci into transcriptionally silent heterochromatin. Thus, the two presumptive RP58 binding sites represented good candidates for regulatory sequences that may underlie the repressive effect of 500i. Interestingly removal of either one site in the context of deleting either the entire 5’- or 3’-half of 500i only partially disinhibits transgenic promoter constructs.

To test the hypothesis that the two CNTCTGG motifs mediate binding of repressive factors, the sites were deleted from transgenic constructs by site-directed mutagenesis. In total three different constructs were generated in which either the 5'-site , the 3'-site, or both were deleted from 500i and cloned in front of $p_{1.2kb}$-OR101-YFP-pA.

First, a construct in which both RP58 sites have been deleted, $p_{500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA, was injected into fertilized zebrafish oocytes along with the $p_{1.3kb}$OMP-mCherry-pA control plasmid. From a total of 679 embryos that were injected in 7 independent experiments, 399 embryos survived until analysis at 3 dpf. Of those 249 embryos (62.4 %) showed mCherry expression in the OE and 179 embryos simultaneously expressed the YFP reporter. Thus, the overall penetrance of transgene expression for this construct was as high as 72.3 ± 4.1%. The efficiency of the mutated construct is 1.5-times higher than the 48.0% obtained for the $p_{500i\text{-}1.2kb}$-OR101-YFP-pA construct, which contains the intact 500i sequence, and almost as high as the $p_{-1.2kb}$-OR101-YFP-pA construct, which does not contain 500i and for which a penetrance of 82.6 % was observed.

Analysis of the relative cell number for 161 OEs that were positive for mCherry revealed a ratio of YFP- / mCherry-positive cells of 0.51 ± 0.03 cells. Again, this ratio is similar to the results obtained for $p_{1.2kb}$-OR101-YFP-pA injections (0.59) and significantly different from the $p_{500i\text{-}1.2kb}$-OR101-YFP-pA construct (0.27), which contains an intact 500i sequence. Thus, removal of two heptanucleotide sequences located at different positions within 500i appears to restore nearly full transgene efficiency of the 1.2 kb promoter constructs, both at the level of transgene-expressing embryos and the number of transgene-positive cells.

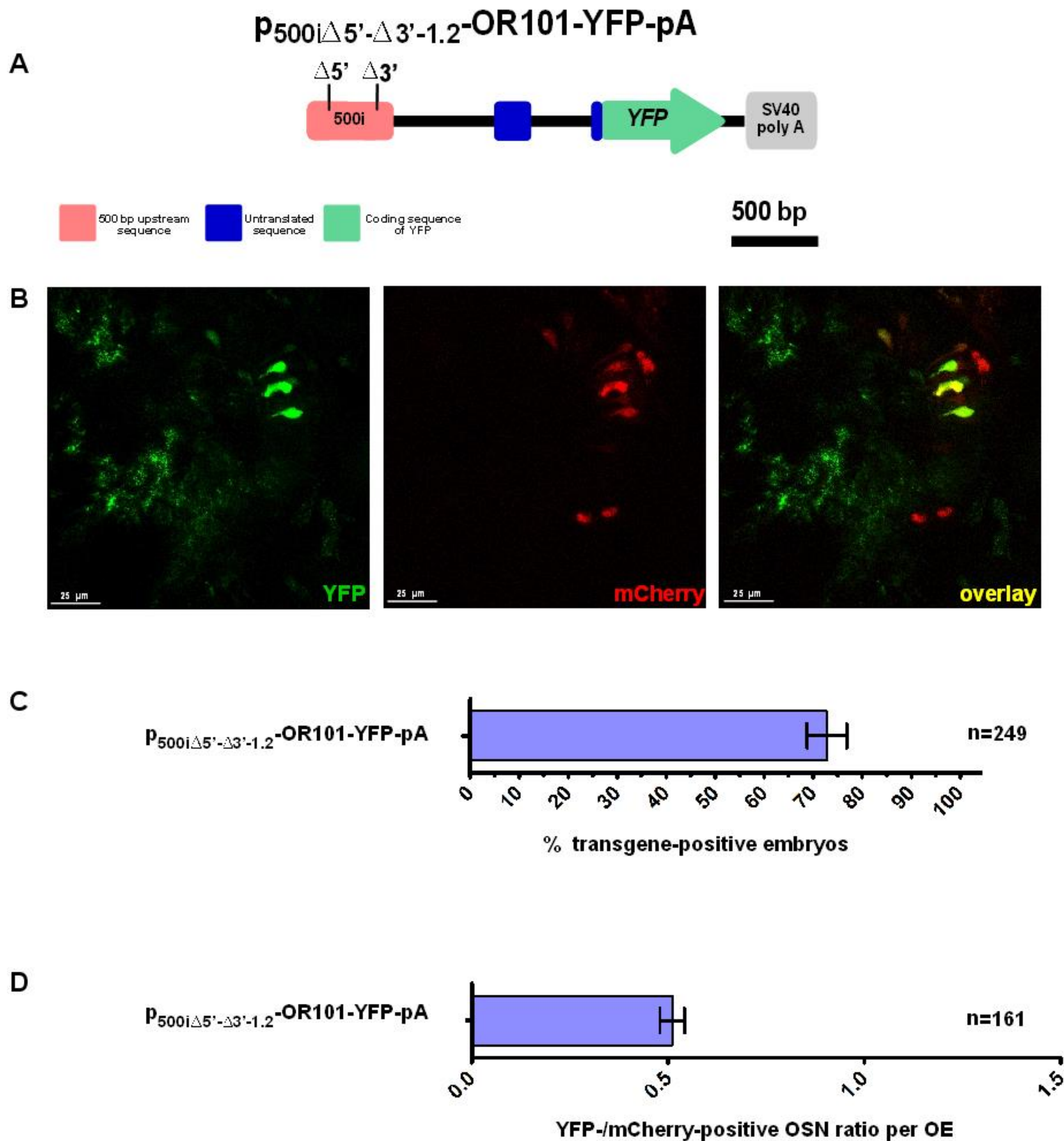

Figure 4.4. Transgene expression analysis of a $p_{500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA construct. (A) Graphical representation of $p_{500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA construct. (B) Confocal z-projections of a representative OE at 3dpf (green: YFP fluorescence, red: mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of $p_{500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of YFP-positive/mCherry-positive OSNs per OE.

4.1.4. The effect of a mutation within the 5'-RP58 binding site on the repressive function of 500i

Removal of both candidate RP58 motifs from 500i restores high levels of transgene efficiency and raises the question what the individual contribution of either representation of the motif may be. Previously it was shown that the deletion of a larger 5'- or 3'-segments of 500i that each contained one representation of the motif resulted in a partial rescue from repression (Söğünmez, 2012), suggesting that both motifs function independently and synergistically. To better understand the differential contribution of 5'- and 3'-RP58 sites, each motif was mutated independently.

When the $p_{500i\text{-}\Delta5'\text{-}1.2kb}$-OR101-YFP-pA (Figure 4.5A) construct was injected into 265 fertilized oocytes, 93 embryos from 5 independent injection experiments could be analyzed at 3 dpf. Of those, 64 expressed mCherry (68.8 %) and 50 were positive for EYFP expression. The normalized transgene efficiency was 76.90 ± 4.85%, which was slightly higher than the efficiency observed for the construct in which both RP58 motifs had been mutated.

An analysis of the number of transgene-positive OSNs from 90 OEs revealed a ratio of YFP- / mCherry-positive OSNs of 0.64 ± 0.10 cells. This ratio is slightly higher than the ratio observed for $p_{1.2}$-OR101-YFP-pA (0.59) and significantly different from $p_{500i\text{-}1.2kb}$-OR101-YFP-pA (0.27). Thus, a single mutation in the 5'-located RP58 motif is capable of restoring high transgene efficiency and to render the repressive effect of 500i inactive.

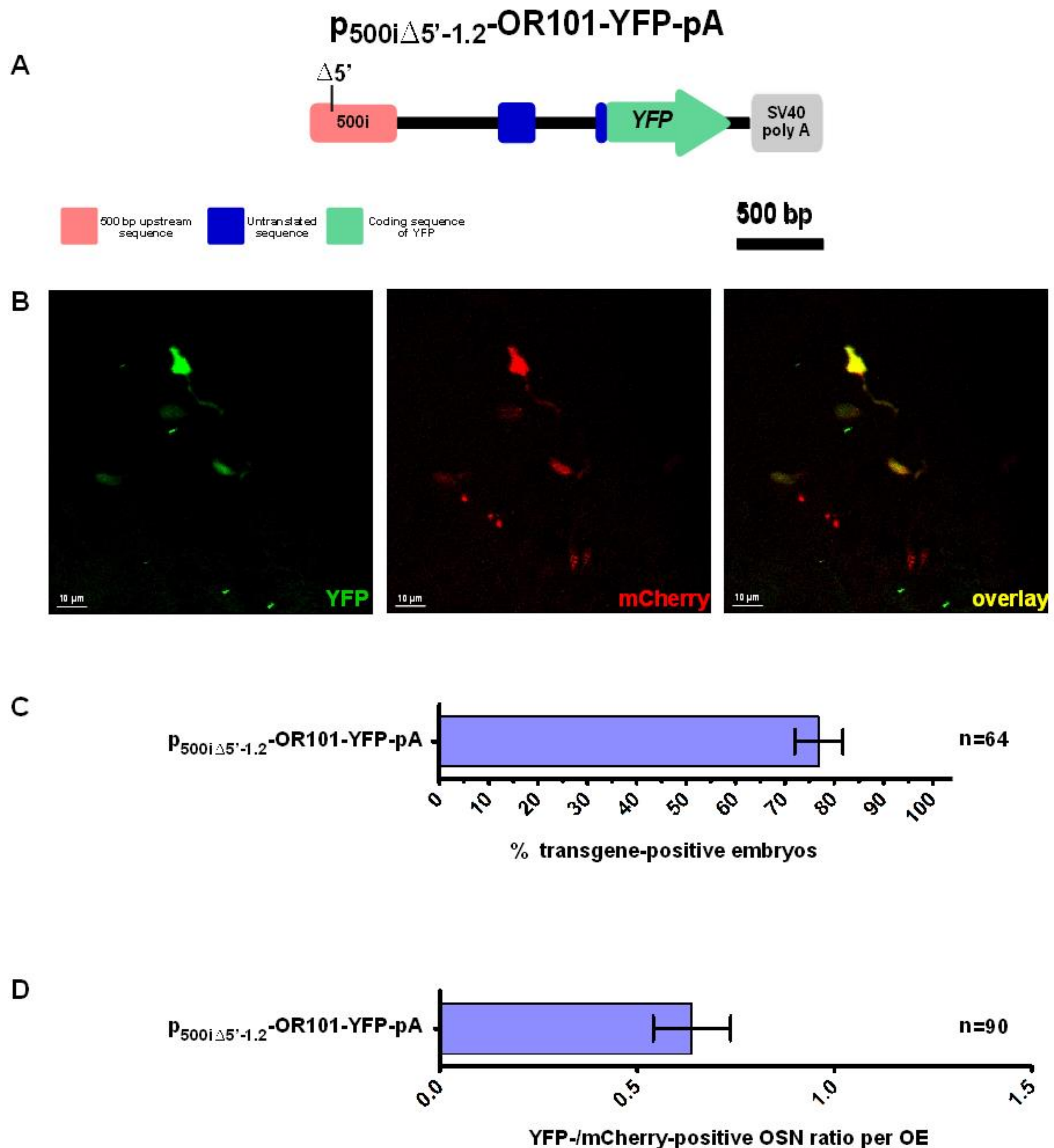

Figure 4.5. Transgene expression analysis of the $p_{500i\text{-}\Delta 5'\text{-}1.2kb}$-OR101-YFP-pA. (A) Graphical representation of $p_{500i\text{-}\Delta 5'\text{-}1.2kb}$-OR101-YFP-pA. (B) Confocal z-projections of a representative OE at 3dpf (green: EYFP fluorescence, red: mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of $p_{500i\text{-}\Delta 5'\text{-}1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of EYFP-positive / mCherry-positive OSNs per OE.

4.1.5. The effect of a mutation within the 3'-RP58 binding site on the repressive function of 500i

Removal of both or the 5'-motif fully restored transgene efficiency from OR101-1 promoter constructs. Thus, a question that remained was whether the 3'-located RP58 motif is redundant or non-functional. Deletion of the entire 3'-half of 500i from transgenic constructs resulted in a partial rescue of transgene efficiency that was similar in size to the effect of the deletion of the 5'-half.

From a total of 4 independent injection experiments in which $p_{500i\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA construct was injected, 131 embryos survived until analysis at 3d pf and 76 of those embryos expressed mCherry (58.0 %) while 56 were positive for YFP expression. Thus deletion of the 3'-motif restored expression efficiency to 73.17 ± 2.77 %, a level that is similar to the effect observed for the mutation of both or the mutation of the 5'-motif alone.

For the $p_{500i\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA construct, a total of 101 OEs were analyzed at the single cell level and the ration of YFP- / mCherry-positive cells was determined to be 0.51 ± 0.04 cells, which is equal to the cell ratio observed when both instances of the motif or only the 5'-motif had been mutated. Different from the 5'-mutation, in 3 noses the EYFP-positive cell number was higher than the number of mCherry-positive cells.

Thus, in summary, the mutation of either the 5'- or the 3'-motif is sufficient to fully restore transgene efficiency and to render 500i non-functional. The effect of deletion of both RP58 motifs was not synergistic and suggests that the repressor may need to bind twice to 500i in order to exert its repressive effect.

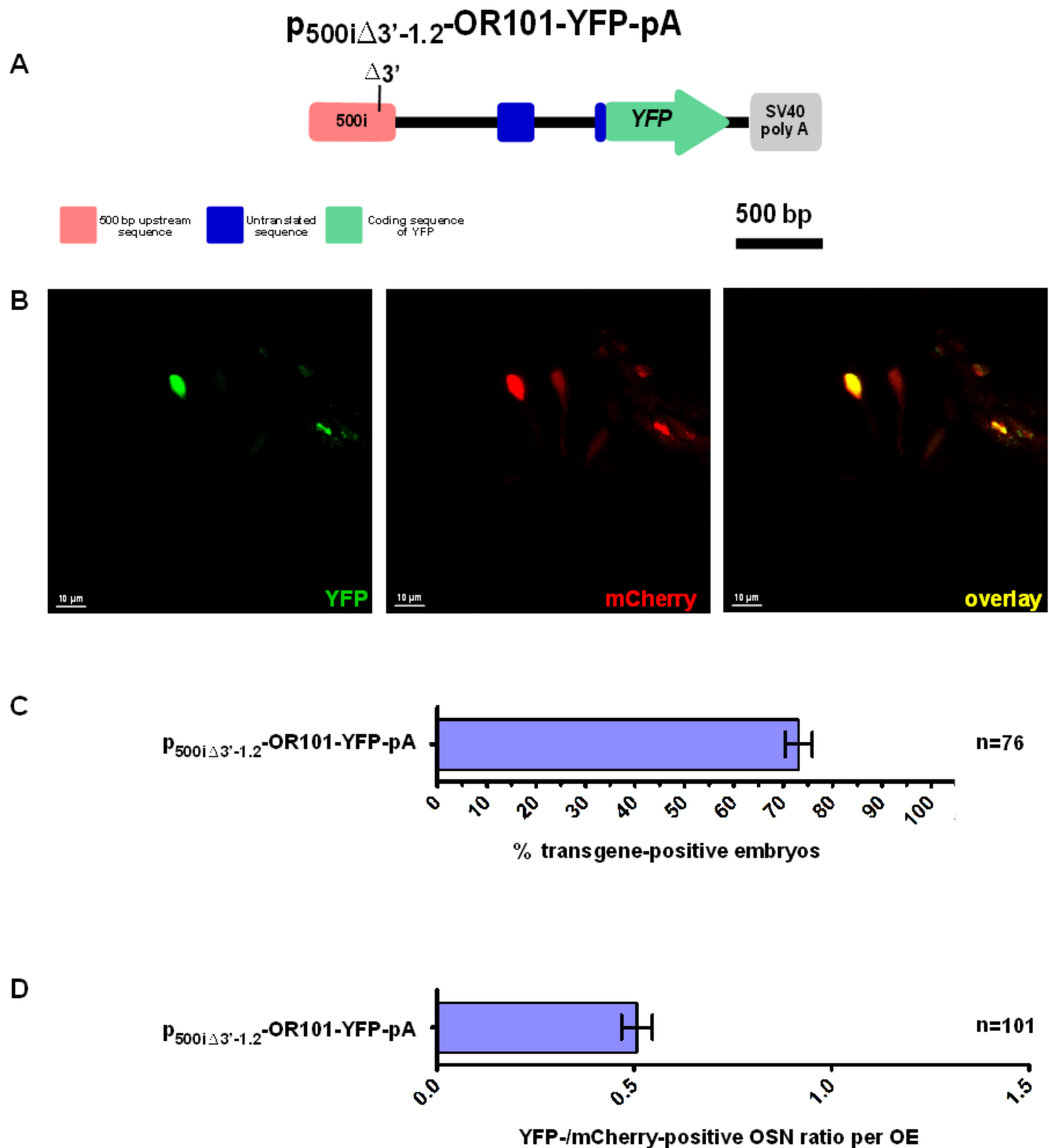

Figure 4.6. Transgene expression analysis of the $p_{500i\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA. (A) Graphical representation of $p_{500i\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA. (B) Confocal z-projections of a representative OE at 3dpf (green: EYFP fluorescence, red: mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of $p_{500i\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of EYFP-positive / mCherry-positive OSNs per nose.

4.1.6. Statistical and Comparative analysis

The analysis of different transgenic constructs that did or did not contain functional RP58 binding sites revealed that the combination of two binding sites has a negative effect on promoter transgene expression, while loss of binding mutations in any of the two sites restores full functionality of the OR101-1 promoter.

Table 4.1. Summary of comparison of constructs with *p* values.

Constructs		***p* values**	
Control	**Tested**	**Transgene expression**	**Cell ratio**
$p_{1.2kb}$-OR101-YFP-pA	$p_{500i-1.2kb}$-OR101-YFP-pA	< 0,0001	<0,0001
	$p_{500i\Delta5'/\Delta3'-1.2kb}$-OR101-YFP-pA	0,1848	0,1000
	$p_{500i\Delta5'-1.2kb}$-OR101-YFP-pA	0,5145	0,6769
	$p_{500i\Delta3'-1.2kb}$-OR101-YFP-pA	0,2153	0,1157
$p_{500i-1.2kb}$-OR101-YFP-pA	$p_{500i\Delta5'/\Delta3'-1.2kb}$-OR101-YFP-pA	0,0004	<0,0001
	$p_{500i\Delta5'-1.2kb}$-OR101-YFP-pA	0,0006	0,0002
	$p_{500i\Delta3'-1.2kb}$-OR101-YFP-pA	0,0004	<0,0001

To understand whether the differences in expression that were observed for the different constructs represent meaningful results the analyzed data were subjected to a statistical analysis. A two-tailed, unpaired t-test assuming unequal variance was used to compare the data obtained from different constructs against the $p_{1.2kb}$-OR101-YFP-pA control construct. According to this analysis the $p_{500i-1.2kb}$-OR101-YFP-pA varies significantly ($p<0.0001$) from $p_{1.2kb}$-OR101-YFP-pA. The other constructs that contain either a single or the combined mutation of the CNTCTGG motif were not significantly different from $p_{1.2kb}$-OR101-YFP-pA. The differences between p500i-1.2kb-OR101-YFP-

pA with the mutated construct were also analyzed. The analysis shows that the expression obtained with $p_{500i\text{-}1.2kb}$-OR101-YFP-pA was also significantly different from the expression obtained from mutated versions of the construct. The result was the same, regardless whether penetrance of expression in % transgene-positive embryos or in the relative number of transgene-positive OSNs was analyzed.

Thus, the observed negative regulatory function of 500i may be conferred to the constructs through the presence of RP58 binding sites, as any mutation in those sites resulted in expression that was indistinguishable from a transgenic construct that does not contain 500i (Figure 4.7). It is surprising that the mutation of just a single RP58 site, either the 5'- or 3'-site, was sufficient to rescue the construct from repression and to restore full activity. This is in contrast to serial deletions of larger regions flanking these motif that only partially restored transgene efficiency.

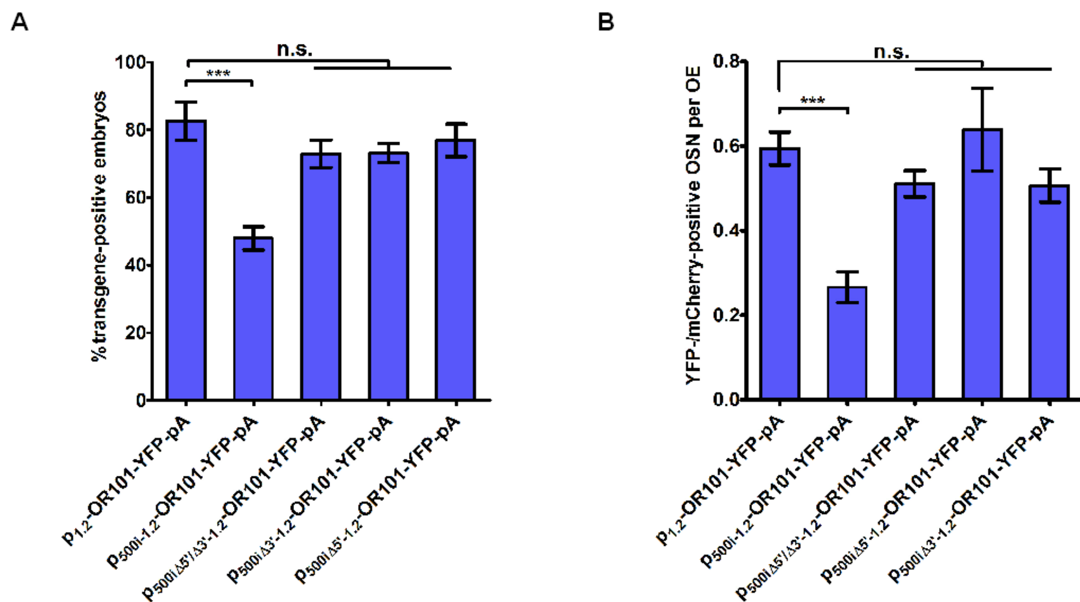

Figure 4.7. Summary of the transgene efficiency for constructs that do or do not contain RP58- binding sites. (A) Penetrance expressed as percent transgene expressing embryos relative to $p_{1.3kb}$OMP-mCherry-pA (B) Ratio of YFP- / mCherry-positive OSNs per OE.

4.2. Effect of 500i on enhancer activity

4.2.1. Effect of the mouse H-element on the OR101-1 promoter

Finding that 500i, which naturally resides within 2 kb upstream of the OR101-1 promoter, has a strong impact on promoter activity raises the question as to what the biological significance of this repression might be. It is the current view in the field that OR gene expression depends on both, proximal and long-range regulators (Fuss and Ray, 2009). It has been demonstrated that OR promoters are sensitive to flanking enhancer sequences in a distance-dependent manner; the closer the enhancer to the TSS of an OR gene the stronger the impact of the enhancer (Fuss *et al.*, 2007; Nishizumi *et al.*, 2007). Thus, OR genes located close to an enhancer would be overrepresented by an unproportionally high number of OSNs that express this OR. Thus, it is possible that 500i acts as insulator between an OR cluster and neighboring genes or as an attenuator for OR genes that are closely situated near olfactory-specific enhancer sequences.

To understand, whether 500i has the capacity to shield the OR101-1 promoter from the effect of a nearby enhancer, the mouse H element was inserted into transgenic constructs that do or do not contain 500i. Even though the H-enhancer element is a mammalian-specific sequence, it drastically increases transgene efficiency and cell number in the zebrafish OE when fused to moderate zebrafish OR gene promoters (Nishizumi *et al.*, 2007; Söğünmez, 2012; Taştekin, 2012; Uzel, 2014)

To initially test the basic effect of the H enhancer on the 1.2 kb OR101-1 promoter a construct containing the H-region upstream of the 1.2 kb OR101-1 promoter sequence ($p_{H-1.2kb}$-OR101-YFP-pA Figure 4.8A) was microinjected along with $p_{1.3kb}$OMP-mCherry-pA. From 3 independent injection experiments, 107 fish survived until the analysis at 3 dpf. Of those, 69 expressed mCherry (64.5 %) and all 69 embryos were positive for YFP. Thus, the transgene efficiency at the level of positive embryos was 100%, probably attributable to the strong positive regulatory effect of H. This is further illustrated by the observation that

different from previous experiments, embryos that only expressed $p_{H\text{-}1.2kb}$-OR101-YFP-pA but not $p_{1.3kb}$OMP-mCherry-pA could be found in the sample.

Moreover, addition of the H-enhancer also dramatically increased the number of transgene-positive cells (Figure 4.8B). For the 1.2 kb promoter construct that does not contain the H-element an average number of $6.17 \pm 0{,}60$ cells was observed per OE, while number of transgene-positive cells increased 3.4-fold to an average of $20.66 \pm 1{,}85$ cells per OE in the related construct that contained the H-element. When the ratio of cells that expresses YFP and mCherry per OE was analyzed to get an alternative measure of transgene efficiency, from 119 OEs that were analyzed an average ratio of 1.04 ± 0.03 cells could be observed. Thus, the presence of the H-element increased the ratio of transgene positive cells 2-fold. Additionally, in 52 OEs the number of YFP-expressing cells was higher than the number of mCherry expressing cells an observation that was rarely made for other constructs that did not contain the H-enhancer. It should be noted, however, that in the co-injection experiments described here, an increase in the average number of mCherry-positive cells per embryo was observed as well. Thus, the H-element appears to increase the number of cells that express the OMP promoter construct in trans or by cointegration of the coinjected constructs into the genome in a way that the OMP promoter became under the influence of H. For this reason, the actual effect of the H-element on the OR101-1 promoter is underestimated when the number of YFP-positive cells is expressed as the ratio of mCherry-positive OSNs.

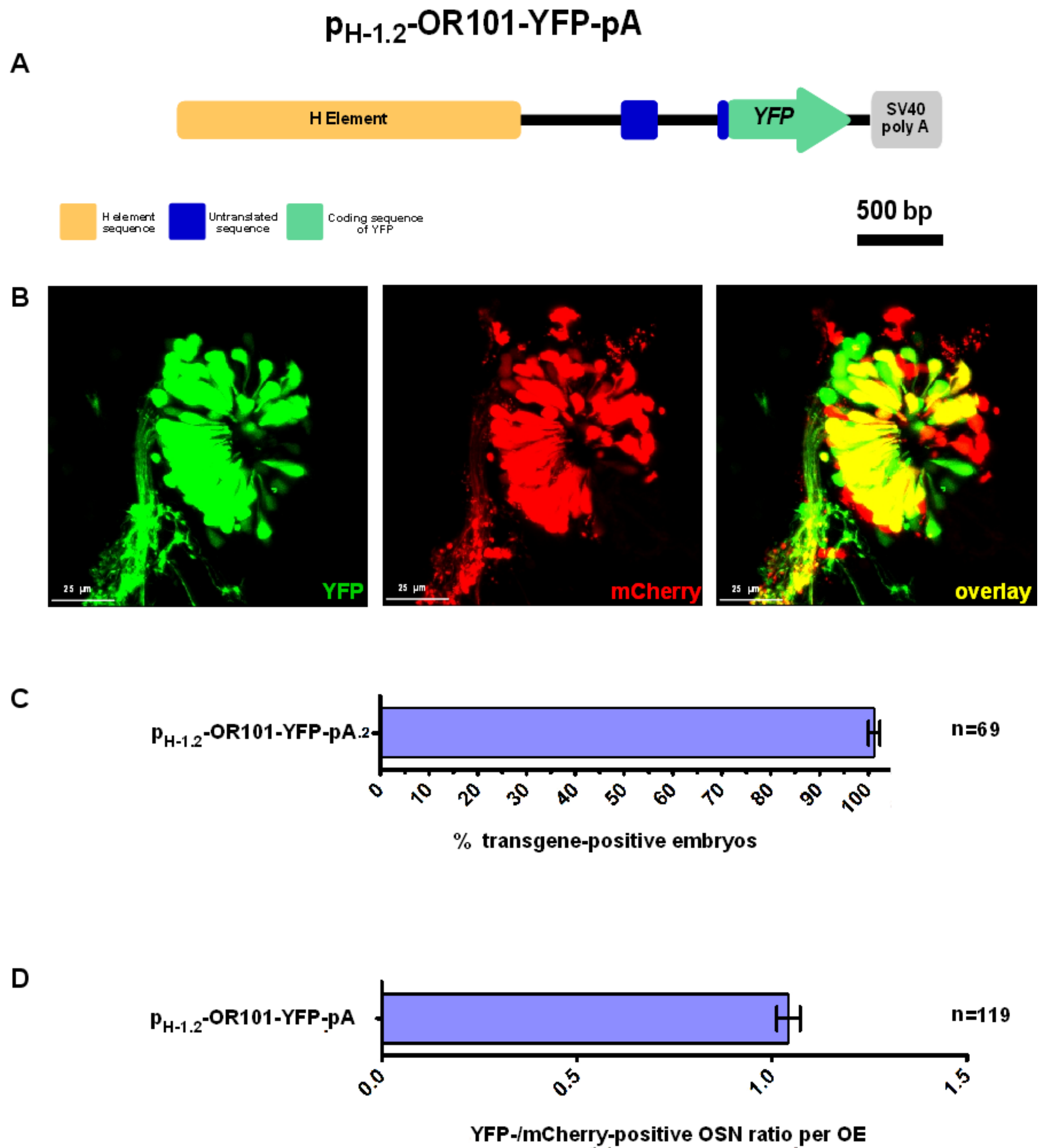

Figure 4.8. Transgene expression analysis of a $p_{H\text{-}1.2kb}$-OR101-YFP-pA construct. (A) Graphical representation of $p_{H\text{-}1.2kb}$-OR101-YFP-pA. (B) Confocal z-projections of a representative OE at 3dpf (green: YFP fluorescence, red: mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of $p_{H\text{-}1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of YFP-positive / mCherry-positive OSNs per OE.

4.2.2. Effect of 500i on H-enhancer activity

Next, the effect of 500i on the constructs containing the H-enhancer was examined. In previous experiments, it has been shown that a construct containing 2.5 kb of sequence upstream of OR101-1 and therefore the full 500i sequence reduces the efficiency of the H enhancer. However, in this construct, the H-enhancer is located 1.3 kb away from the OR101-1 promoter and distance-related effects that might account for the difference in expression cannot be excluded. Therefore, a new $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA construct was generated in which the H-enhancer is separated from the 1.2 kb OR101-1 promoter only by 500i.

From 6 independent injection experiments, 201 fish survived until analysis at 3dpf. Among those, 164 embryos expressed mCherry (81.6%) and 155 were positive for YFP. Thus, the normalized transgene efficiency was 86.75 ± 7.39% for this construct, which is only sightly less than the 82.6% penetrance observed for $p_{H\text{-}1.2kb}$-OR101-YFP-pA.

When compared to $p_{H\text{-}1.2kb}$-OR101-YFP-pA the number of transgene-positive cells appears to be lower for this construct, but higher than for $p_{1.2kb}$-OR101-YFP-pA. (Compare Figure 4.1B with 4.9B). The ratio of YFP- / mCherry-positive cells per OE was reduced by one-fourth to 0.75 ± 0.03 cells when compared to $p_{H\text{-}1.2kb}$-OR101-YFP-pA (1.04), but 50 % higher than for $p_{1.2kb}$-OR101-YFP-pA. In 15 OEs a higher number of YFP- than mCherry-expressing cells could be observed. The average number of YFP-expressing OSNs for this construct was 10.51 ± 0,97,almost half the number of YFP-expressing cells observed for $p_{H\text{-}1.2kb}$-OR101-YFP-pA (20.66). Thus, the presence 500i could not abolish, but significantly diminish the effect of the H enhancer on transgene expression when the sequence was intercalated between the enhancer and the core promoter.

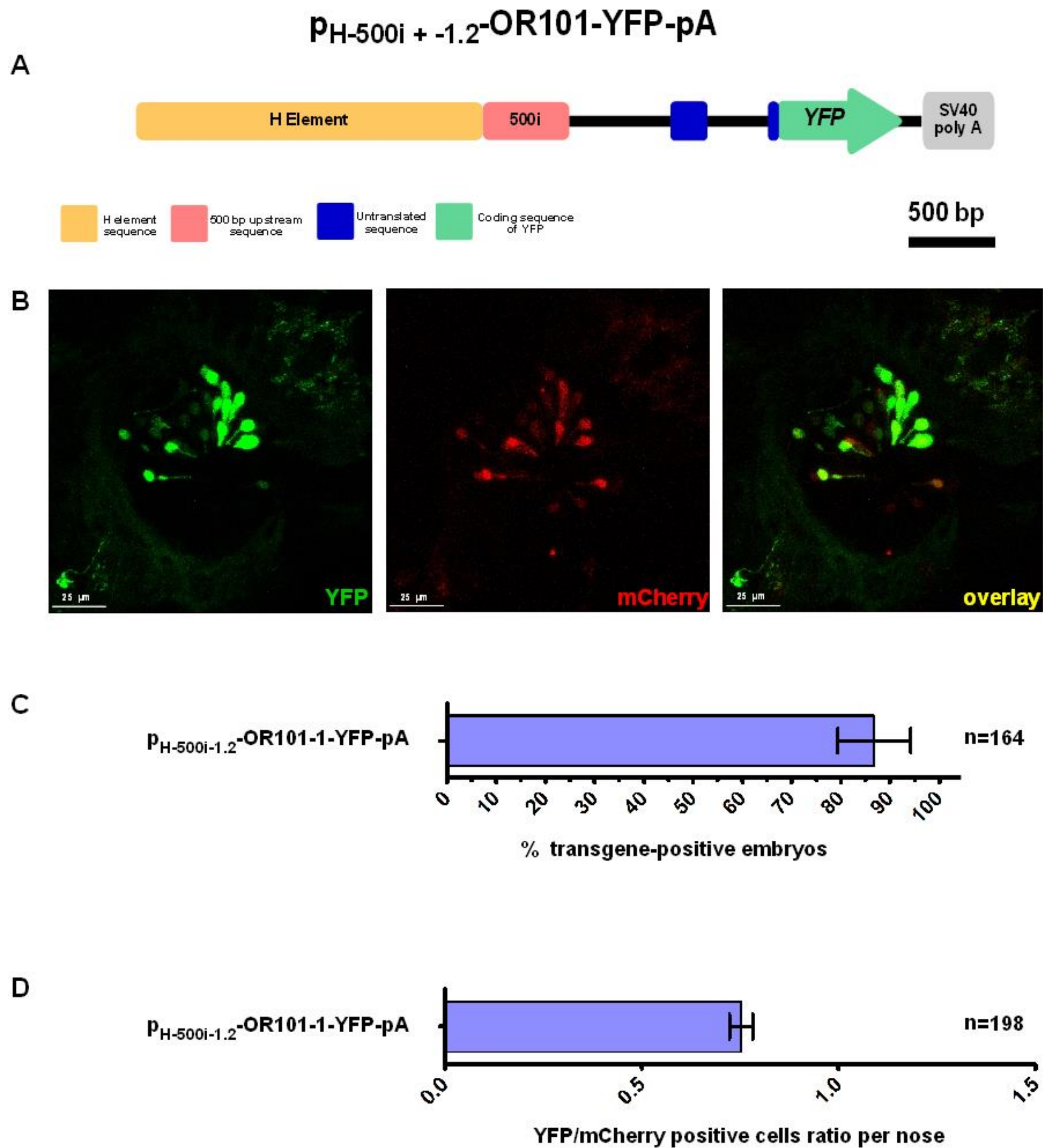

Figure 4.9. Transgene expression analysis of a $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA construct (A) Graphical representation of $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA. (B) Confocal z-projections of a representative OE at 3dpf (green:YFP fluorescence, red:mCherry fluorescence, right:overlay. (C) Transgene efficiency as percentage of $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of YFP-positive /mCherry-positive OSNs per OE.

4.2.3. Effect of a double mutation in 500i on H-enhancer activity.

As shown above, deletion of the RP58 motifs from 500i reduce the negative regulatory influence of this sequence. Thus, I was curious to understand whether the deletion of RP58 sites has a similar effects in the context of strong enhancers. Therefore, a $p_{H\text{-}500\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA construct was created.

Of 284 fish that were injected with this construct and that survived until analysis at 3 dpf, 182 embryos expressed mCherry (64.1 %) and all 182 embryos were positive for YFP. An additional embryo that only expressed YFP but not mCherry could also be identified. Thus, the normalized transgene efficiency was 100 ± 0, similar to the parent $p_{H\text{-}1.2kb}$-OR101-YFP-pA construct but different from the results obtained with $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA, which contains the intact 500i sequence.

Similarly, high numbers of transgene-positive cells could be detected in embryos that expressed this construct. The detailed analysis of 362 OEs showed a ratio of 0.90 ± 0.02 cells for YFP-/ mCherry-positive OSNs. Comparing to the ratio of 0.75 for $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA construct to the ratio of 1.04 for $p_{H\text{-}1.2kb}$-OR101-YFP-pA, it appears that deletion of the RP58 sites in 500i at least partially disinhibits the construct from expression. The average number of YFP-expressing cells was 15.94± 0,86. Thus, the effect of the mutation on the absolute cell number is similar to the effect on the ratio of YFP- / mCherry-positive OSNs. The 15.9 YFP-expressing cells observed for $p_{H\text{-}500\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA are right between the numbers of 20.66 and 10.51, which were observed for $p_{H\text{-}1.2kb}$-OR101-YFP-pA and $p_{H\text{-}1.2kb}$-OR101-YFP-pA respectively. In a total of 85 noses, the number of YFP-expressing cells was higher than the number of mCherry-positive cells similar to $p_{H\text{-}1.2kb}$-OR101-YFP-pA, and which is probably due to the strong regulatory effect of H on the OR101-1 promoter in those two constructs.

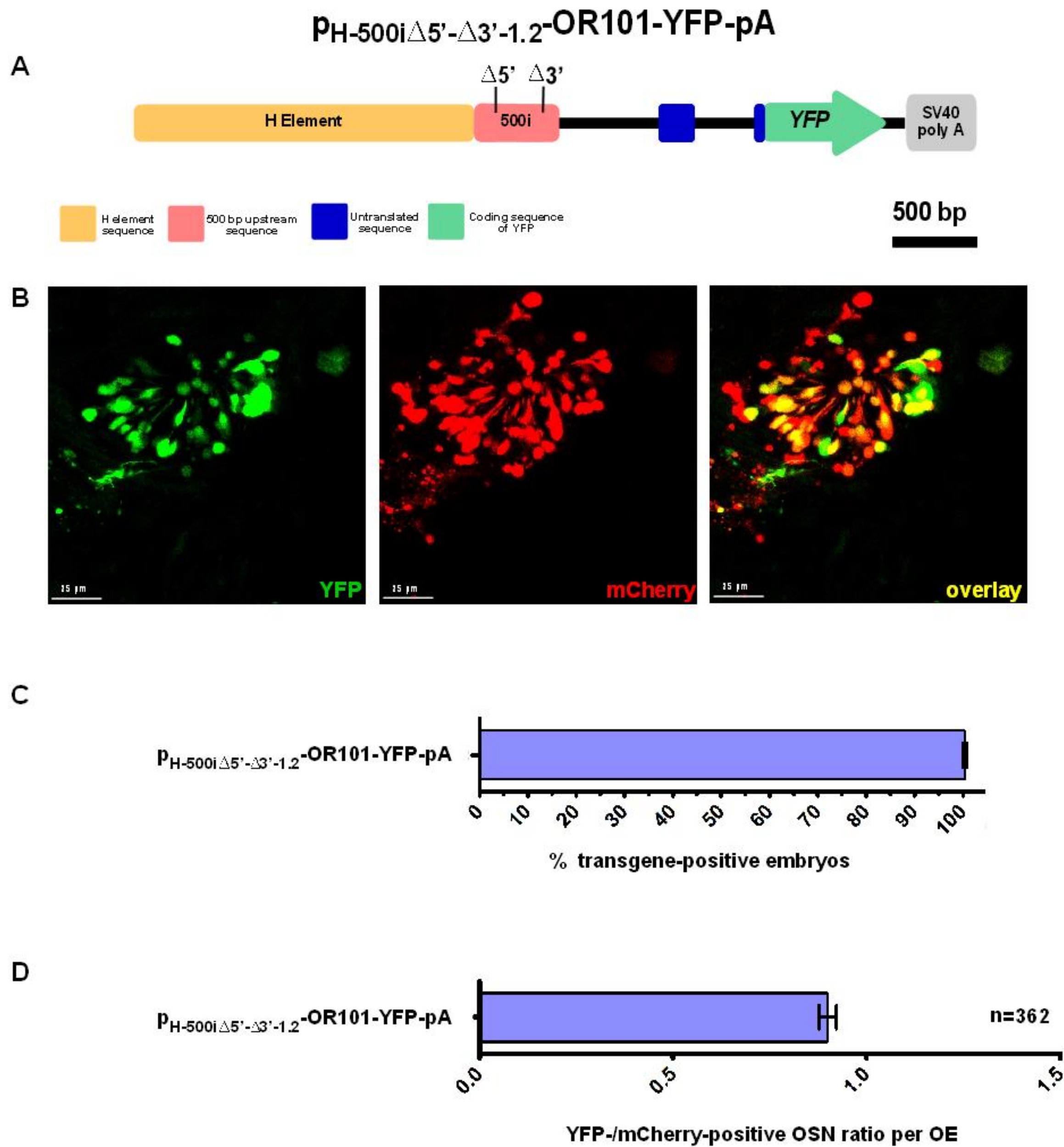

Figure 4.10. Transgene expression analysis of a $p_{H\text{-}500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb1.2kb}$-OR101-YFP-pA. (A) Graphical representation of $p_{H\text{-}500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb1.2kb}$-OR101-YFP-pA. (B) Confocal z-projections of a representative OE at 3dpf (green:YFP fluorescence, red:mCherry fluorescence, right: overlay. (C) Transgene efficiency as percentage of p1.2kb-OR101-YFP-pA over $p_{1.3kb}$OMP-mCherry-pA positive embryos (D) Average ratio of YFP-positive/mCherry-positive OSNs per OE.

4.2.4. Comparison and statistical analysis

Similar to the analysis above the data obtained within this set of experiments were subjected to a statistical analysis. First, the difference between $p_{1.2kb}$-OR101-YFP-pA and $p_{H-1.2kb}$-OR101-YFP-pA was examined and both, the differences in cell number and in penetrance between $p_{1.2kb}$-OR101-YFP-pA and $p_{H-1.2kb}$-OR101-YFP-pA were found to be statistically significant (Table 4.2; Figure 4.11).

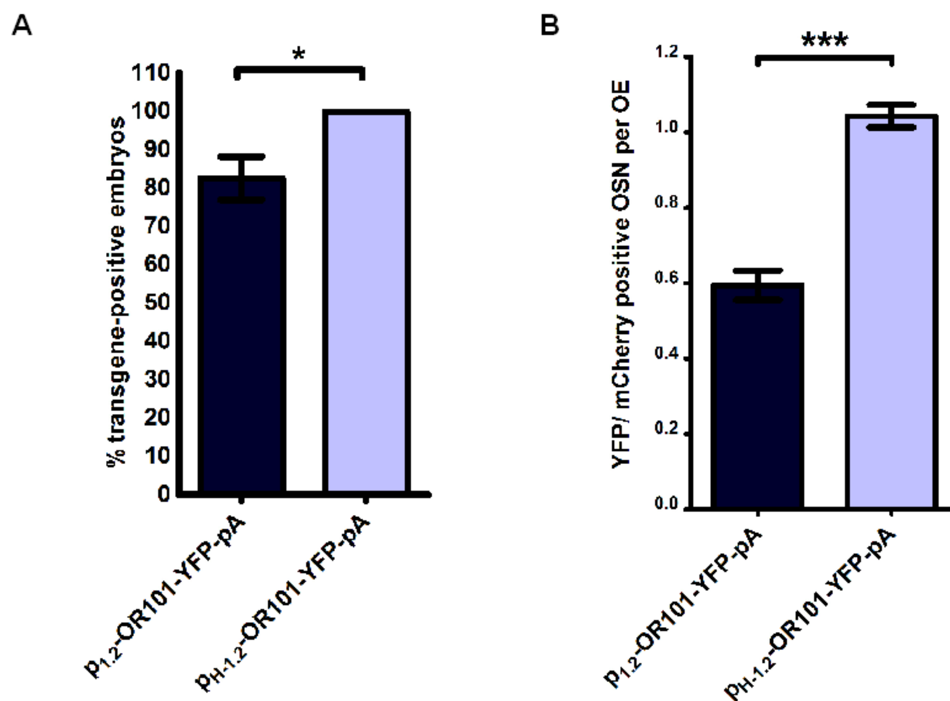

Figure 4.11. Comparison of the $p_{1.2kb}$-OR101-YFP-pA with $p_{H-1.2kb}$-OR101-YFP-pA (A) Comparison of $p_{H-1.2kb}$-OR101-YFP-pA transgene efficiency with $p_{1.2kb}$-OR101-YFP. (B) Comparison of $p_{H-1.2kb}$-OR101-YFP-pA YFP-positive/mCherry-positive cell ratio with $p_{1.2kb}$-OR101-YFP.

However, no statistically significant difference could be found at the level of transgene-expressing embryos for the constructs that contained 500i, regardless whether the sequence was mutated at RP58 sites or not (Table 4.2.). However, a statistically significant effect can be found for the relative number of YFP-positive cells. Yet, although

the mutated $p_{H\text{-}500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA had a much higher ratio than $p_{H\text{-}500i\text{-}1.2kb1.2kb}$-OR101-YFP-pA, it did not reach the level of $p_{H\text{-}1.2kb}$-OR101-YFP-pA and the mutations in RP58 sites only partially rescued expression.

Table 4.2. Summary of comparison of H containing constructs with *p* values.

Constructs		***p* values**	
Construct 1	**Construct 2**	**Transgene expression**	**Cell ratio**
$p_{1.2kb}$-OR101-YFP-pA	$p_{H1.2kb}$-OR101-YFP-pA	0,0210	<0,0001
$p_{H1.2kb}$-OR101-YFP-pA	$p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA	0,133	<0,0001
$p_{H1.2kb}$-OR101-YFP-pA	$p_{H\text{-}500i\Delta5'/\Delta3'\text{-}1.2kb}$-OR101-YFP-pA	n/a	0,0002
$p_{H\text{-}500i\Delta5'/\Delta3'\text{-}1.2kb}$-OR101-YFP-pA	$p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA	0,133	0,0001

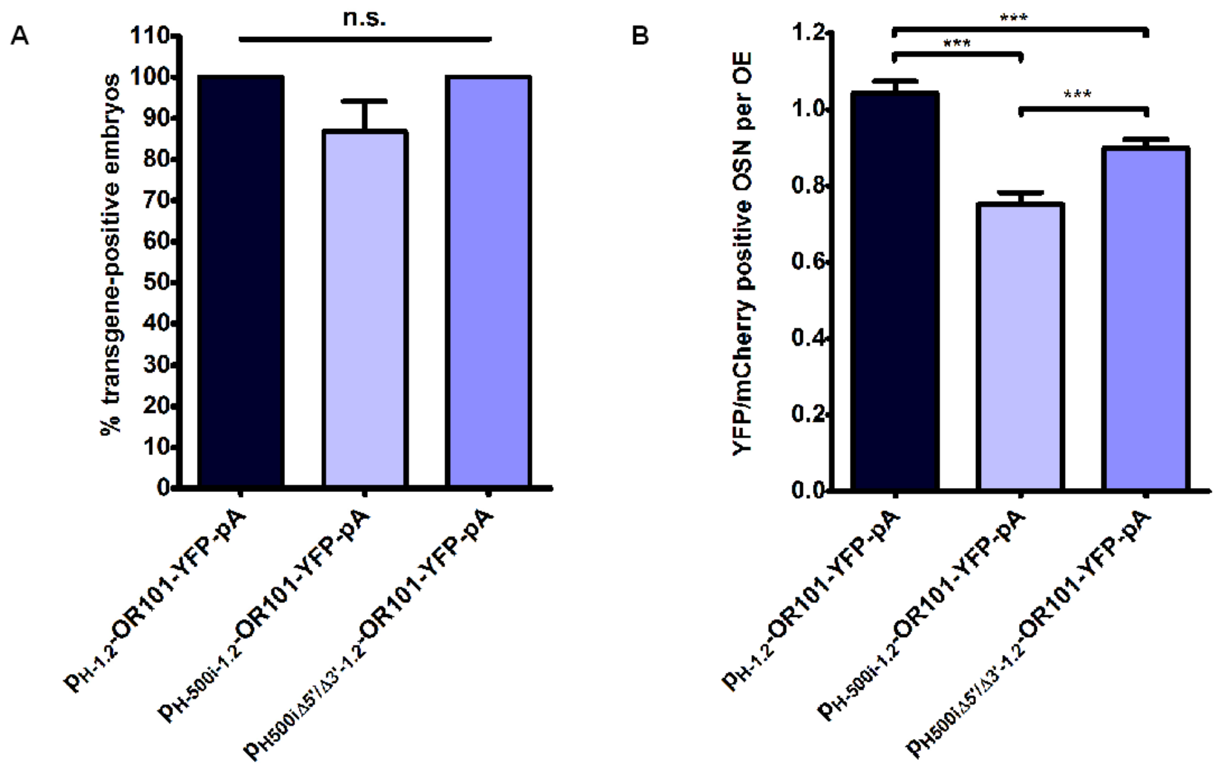

Figure 4.12. Comparison of the H containing constructs (A) Transgene expression efficiency with $p_{H\text{-}1.2kb}$-OR101-YFP-pA (B) Comparison of YFP-positive/mCherry-positive cell ratio ($p_{H\text{-}1.2kb}$-OR101-YFP-pA vs $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA; $p_{H\text{-}1.2kb}$-OR101-YFP-pA vs $p_{H\text{-}500i\Delta5'/\Delta3'\text{-}1.2kb}$-OR101-YFP-pA; $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA vs $p_{H\text{-}500i\Delta5'/\Delta3'\text{-}1.2kb}$-OR101-YFP-pA).

Surprisingly, for all constructs that contained the H-element, a certain percentage of embryos could be observed in which the number of YFP-expressing cells exceeded the number of mCherry-expressing OSNs. In $p_{H\text{-}1.2kb}$-OR101-YFP-pA, 43.7 % of OEs had more YFP- than mCherry-positive cells. However, this percentage was different for the different constructs. For the 500i-containing construct $p_{H\text{-}500i\text{-}1.2kb}$-OR101-YFP-pA the number of YFP-positive cells was higher than the number of mCherry-positive cells in only 7.9 %. Interestingly, when the two RP58 sites were rendered nonfunctional, an intermediate percentage of 23.6 % fish showed higher numbers of YFP- than mCherry expressing cells for $p_{H\text{-}500i\text{-}\Delta5'\text{-}\Delta3'\text{-}1.2kb}$-OR101-YFP-pA.

In summary, it can be concluded that 500i does not block the activity of the H element completely but significantly weakens its effect at least at the level of transgene-positive OSNs. In line with observations on the basic OR101-1 promoter, mutation of both RP58 motifs within 500i restores H-element activity, albeit only partially. It cannot be ruled out, however, that the effect of 500i on a more distantly located enhancer would be more significant. Here only one condition was tested in which the H enhancer was separated from the core promoter only by 500i. Since the influence of the enhancer on expression could decrease successively if it were located further upstream, 500i might become strong enough a modulator to efficiently protect the OR101-1 promoter from excessive expression.

4.3. Morpholino

To analyze the effect of the zbtb7b protein, two morpholino were designed as translation blocking morpholino (zbtb7b-TB) and splice blocking morpholino (zbtb7b-E2). From those, zbtb7b-TB was injeted. For the first trial 4ng of the zbtb7b-TB was injected as suggested concentration however neitheir malformation of the embryo nor the delay of the development was observed. Then to determine minumum amount that required to obtain a phenotypical effect, morpholino was titrated. From 2 injection sets, at 6ng -4nl injection cause delay in the development and cause some morphological disruptance.

4.4. Effect Of Enhancer on Cell Type

Enhancer-containing construct showed that there is a significant increase in the number of cells expressing the reporter gene. Nevertheless, it is not known which OSN subtype is sensitive to enhancer function in these experiments. In the zebrafish OE four types of OSN have been reported: ciliated and microvillous OSNs, as well as crypt and kappe cells. All these cell types differ from each other in their morphological and molecular features (Oka and Korsching, 2011; Oka *et al.*, 2012; Yoshihara, 2009). In previous experiments, it was shown that constructs that contain 500i have a higher

tendency to be expressed in ciliated OSNs, whereas $p_{1.2kb}$-OR101-YFP-pA were also expressed in microvilluos cells and in indistinct round cells. The latter could either be crypt/ kappe cells or immature ciliated are microvillous OSNs that have not yet extended a dendrite. Thus, I wanted to analyze the transgene-expressing cell types molecularly by using an anti TrkA antibody that can be used to selectively label crypt cells. (Ahuja *et al.*, 2013).

In initial experiments wild-type embryos were subjected to immunohistochemistry with the anti-TrkA antibody to understand the morphology and distribution of crypt cells in the embryonic OE. As described, crypt cells have large spherical profiles and are few in number in the 5 dpf embryo. They can easily be identified by their characteristic indentation of their apical cell body when stained against TrkA (Figure 4.13.).

Following these initial characterizations of crypt cell morphology in the embryo, the next step was to perform TrkA-immunohistochemistry on $p_{1.2kb}$-OR101-YFP-pA-injected embryos and analyze both markers using confocal microscopy. All cells detected with the TrkA antibody had crypt cell morphology and an average number of 3.6±0.68 crypt cells were found per OE. A total of 2.5% of the YFP-positive cells were found to be positive for TrkA (Figure 4.14. A,C). Thus, the majority of $p_{1.2kb}$-OR101-YFP-pA-expressing cells did n not belong to the crypt cell population, regardless of their cell morphology.

Next, I was curious about the effect of enhancer on the cell type density. Therefore, oocytes were injected with $p_{H\text{-}1.2kb}$-OR101-YFP-pA and stained with TrkA antibody. The results of the staining was very surprising, as an increase in TrkA-labeled cells was observed. However, not all cells showed the typical crypt cell morphology but had an appearance similar to ciliated neurons with cilia at the apical site. In addition, the center of the OE was heavily stained (Figure 14.14B). Since crypt cells are small and short, they mostly located in the central OE. Therefore, it is hard to determine if the labeled cells are true crypt cell or not. The average number of the TrkA positive cells was 7.7%. However, in this experiment 50% of the YFP-positive cells were stained with TrkA (Figure 14.14C).

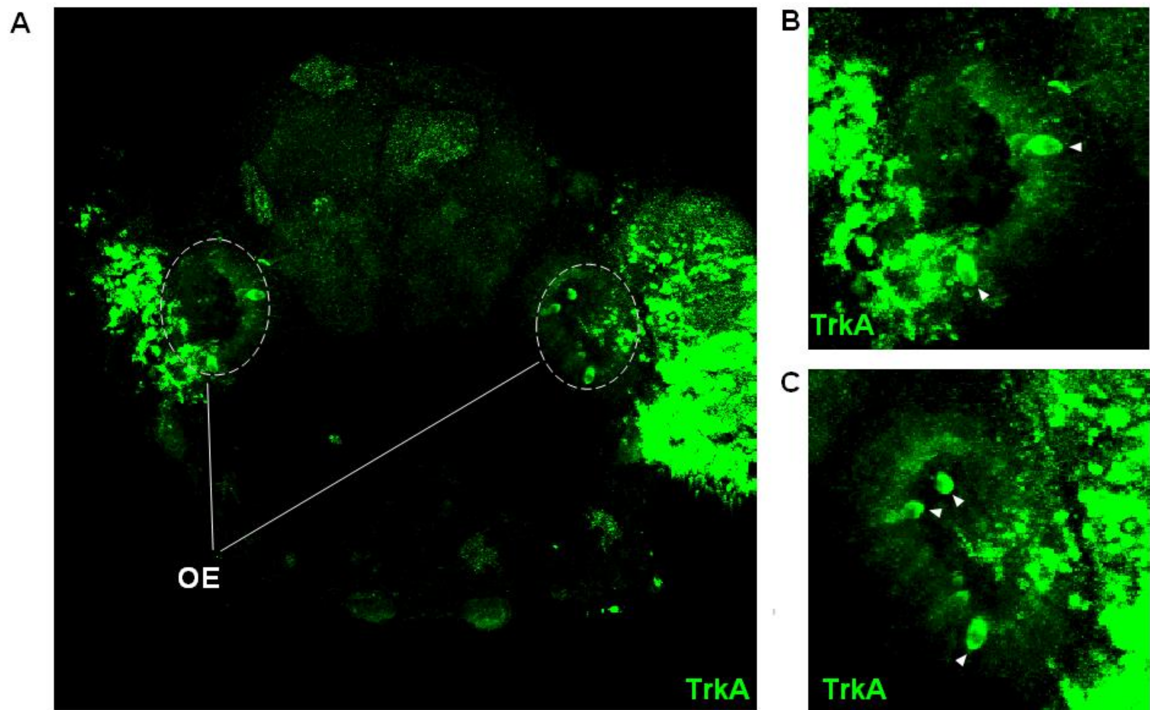

Figure 4.13. 5dpf wild-type embryo stained against TrkA. (A) Front view of the 5dpf embryo (B) Left OE. White arrowheads show the stained crypt cells. (C) Right OE. White arrowheads show the stained crypt cells.

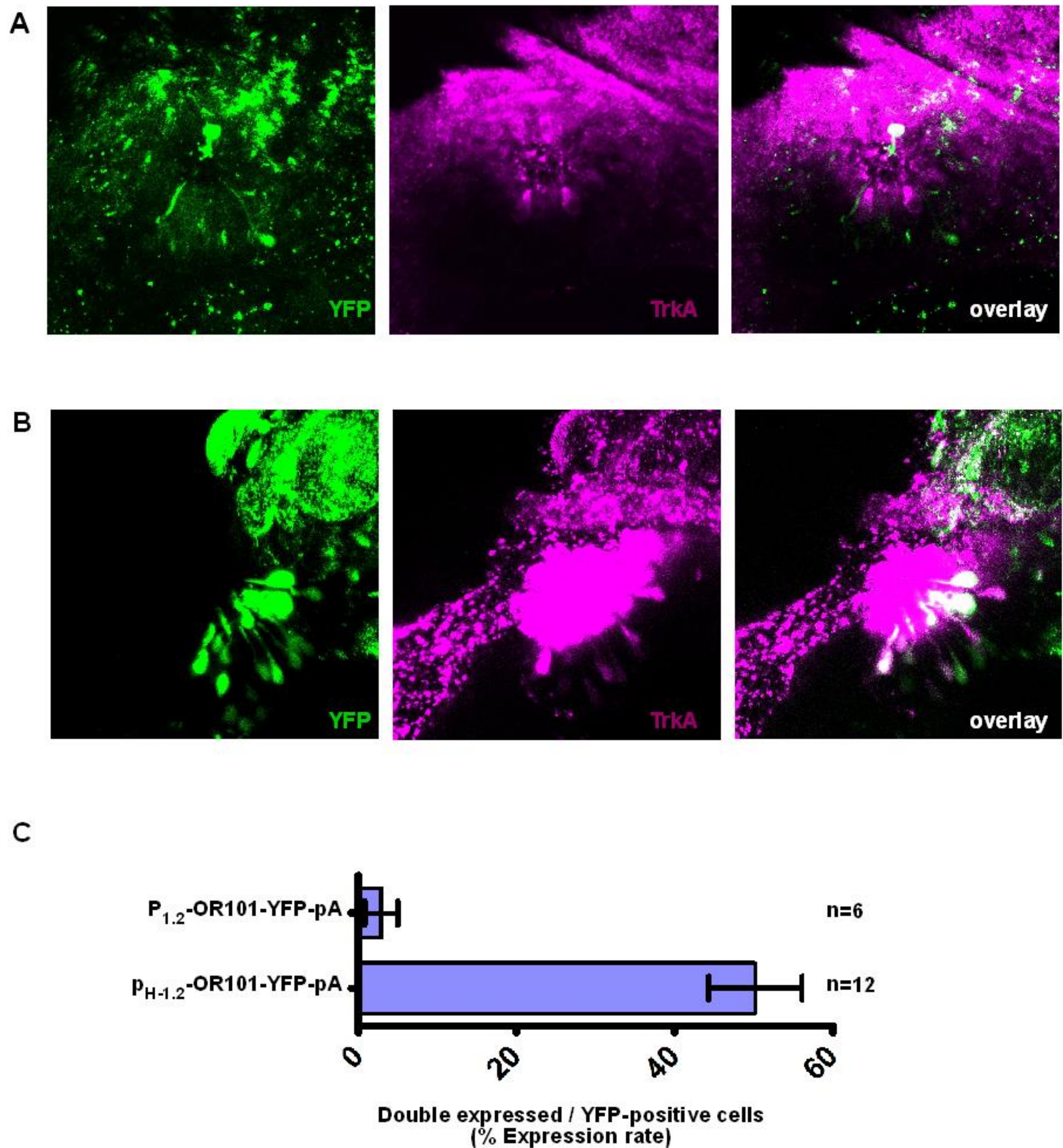

Figure 4.14. TrkA antibody staining of 5dp injected larvae. (A) Confocal z-projections of a representative OE at 5dpf, $p_{1.2kb}$-OR101-YFP-pA embryo (Green:YFP fluorescence, pink:Alexa 633, right: overlay). (B) Confocal z-projections of a representative OE at 5dpf, $p_{H1.2kb}$-OR101-YFP-pA embryo (Green:YFP fluorescence, pink:Alexa 633, right: overlay). (C) Double expressed / YFP-positive cell rates for each construct.

5. DISCUSSION

The decisive step during OSN maturation is the selection of a single OR gene for expression. The monospecificity of OSNs is necessary to avoid sensory confusion at the systems level. In analogy to the visual system, where expression of more than one of the three different opsin genes by the same cone photoreceptor would result in color blindness, random coexpression of OR genes in OSNs would compromise the function of the olfactory system and render it 'odor blind'. Thus, OSNs have to ensure that they obey the 'one neuron – one receptor' rule (Peter Mombaerts, 2004b). Those OSNs that express more than one OR per neuron cannot reach a mature state and are eliminated by apoptosis (Tian and Ma, 2008).

The problem of establishing singular OR expression is trivial due to the very large number of virtually identical receptor genes that a given OSN could theoretically choose from:1.400 OR genes in the mouse, 800 in humans, 1.000 in dogs, and 170 in fish (Malnic *et al.*, 2004; Olender *et al.*, 2004; Zhang *et al.*, 2004; Niimura and Nei, 2005; Alioto and Ngai, 2005). The OR gene choice mechanism also has to ensure that the initial choice of an OR gene remains stable throughout the lifetime of the neuron, because the expressed OR instructs how the OSN established synaptic connections in the olfactory bulb (Wang *et al.*, 1996).

Over the last decade considerable progress has been made in understanding various aspects of the OR gene choice mechanism. The picture of the mechanism that emerges is rather complex and involves classical regulation by transcription factors that act on proximal regulatory sites in OR promoters, long-range cis- and eventually trans-regulatory enhancer sequences, epigenetic modifications, and intracellular signaling pathways.

The current model of OR gene choice suggests that the entire range of OR gene loci becomes epigenetically silenced in immature OSN precursors (Lyons *et al.*, 2013). In those

cells, all OR loci are found in constitutive heterochromatin as indicated by repressive H3K9me3 and H4K20me3 histone marks. A specific lysin demethylase LSD1 then removes methyl groups from lysine residues and thereby randomly releases one or more OR gene loci from repression. Because persisting LSD1 activity would successively demethylase and thereby activate an increasing number of OR gene loci, LSD1 activity is silenced upon translation of the first OR that escaped epigenetic repression. This negative feedback regulation depends on the forming OR protein which initiates the unfolded protein response pathway and the Perk signaling cascade (Dalton *et al.*, 2013; Shou Serizawa *et al.*, 2003). Thus, activation of additional OR gene loci in the same cell is prevented because they remain silenced by repressive epigenetic marks, which cannot be reversed due to LSD1 inactivity.

Attractive as this model is, it proposes that OR gene choice would be equal for all 1.400 OR genes in the genome and that all ORs would be expressed by an equal number of OSNs across the entire olfactory epithelium. Yet, OSNs expressing a given OR are confined to narrow expression domains called ‘zones’ (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Miyamichi *et al.*, 2006) and even within a zone, the frequencies of OSNs expressing different ORs differ significantly (Shykind *et al.*, 2004; Miyamichi *et al.*, 2006). Thus OSNs situated in different spatial positions of the OE have a different potential to express different subsets of ORs. An attractive hypothesis is that specific DNA-interacting factors guide LSD1 activity to distinct subsets of OR gene loci in a position-specific manner. Thus, OR gene choice would be non-random but determined by the combination of factors that would be able to interact with a given number of OR gene loci and the effectiveness by which they could recruit LSD1 to a given locus.

Powerful activators of OR gene choice have been identified with the H and P enhancers in the mouse (Serizawa *et al.*, 2003; Fuss *et al.*, 2007; Nishizumi *et al.*, 2007; Khan *et al.*, 2012). Those enhancers are located within or next to OR gene clusters and in mice that are deficient of these sequences, nearby OR genes are not chosen for expression. Recently, an archipelago of similar elements has been uncovered that are associated with virtually all OR gene clusters (Markenscoff-Papadimitriou *et al.*, 2014). These enhancers

appear to cluster together in the nucleus and may act as anchorpoints to recruit LSD1 to a nuclear hub with the function of a ‘choiceosome’.

The model outlined above might account for uneven choice probability of different OR genes as different enhancers might be recruited with different efficiency to the choice some and genes located further away from the enhancer might be chosen less frequently than more proximal genes (Fuss *et al.*, 2007). It cannot, however, account for zonal expression patterns, as genes from the MOR28 cluster that are controlled by the same H element are expressed in spatially non-overlapping domains in the dorsal and ventral OE (Fuss *et al.*, 2007). Thus, additional regulators have to be involved that direct OR gene choice to individual OR genes in a temporal and spatial fashion and with distinct probabilities.

In addition to these long-range interacting elements a limited number of short-range cis-activating elements have been identified (Plessy *et al.*, 2012; Qasba and Reed, 1998; Rothman *et al.*, 2005; Vassalli *et al.*, 2011b, 2002). The most intensively studied regulatory sites in proximal OR promoters are the O/E and homoeodomain-like sites (Vassalli *et al.*, 2001,2011; Rothman *et al.*, 2005; Hirota and Mombaerts, 2004; Hirota *et al.*, 2007; McIntyre *et al.*, 2008). The O/E and homeodomain-like sites are recognition sites for the Olf1 and Lhx2/Emx2 transcription factors, respectively, can be found in the promoter of most OR and VR genes (Michaloski *et al.*, 2006). Importantly, they have been shown to direct OR expression to particular spatial domains (Rothman *et al.*, 2005) or are required for specific subsets of ORs to be expressed at all. When O/E and homeodomain-like sites of the M71 OR gene were mutated by targeted genome engineering, a ventral shift of the M71 expression domain was observed (Rothman *et al.*, 2005). Mice lacking expression of the interacting transcription factors Lhx2 or Emx2 are devoid of expression of specific OR subsets (Hirota *et al.*, 2007; McIntyre *et al.*, 2008). Thus, specific instructions as to how, when, and where a particular OR should be expressed are contained within short proximal promoter sequences of OR genes.

All of the hierarchically organized regulatory mechanisms described so far have in common that they act positively on an OR gene locus. Interaction of OR promoters with enhancers or binding of transcription factors to OR promoters appear to increase their probability of choice and expression by the OSN. It is conceivable, albeit not a necessary condition, that OR gene choice is also under negative regulation to ensure proper spatial expression profiles or to counteract the unproportional influence of enhancers on proximal OR genes.

Using a bioinformatic approach, Michaloski *et al.*, (2011) found that candidate binding sites for the Repressor Protein 58 (RP58) are overrepresented in OR and VR gene promoters. One variant of the sequence motif, CNTCTGG, was present in 49of the analyzed V1R and 40% of OR promoters. This motif resembles binding sites for RP58 (aka ZBTB18, C2H2-171, MRD22, TAZ-1, or ZNF238), which is a member of the BTB-ZF (broad tramtrak bric-a-brac zinc-finger domain) or POK (POZ and Krüppel) families of transcription factors and contains both Krüppel-like zinc finger DNA binding and BTB domains. RP58 was shown to recruit DNMT3 as a transcriptional co-repressor that targets HDAC1 to transcriptionally silent heterochromatin through its ATRX domain (Fuks *et al.*, 2001).

However, bioinformatic analysis can only guide the identification of factors and binding sites but does not provide functional prove that the identified sequences and factors are indeed involved in OR gene regulation. Even though the bioinformatic findings are striking, and the suspected interacting factor zbtb7b is highly expressed in olfactory tissue, no functional data are available on any negative regulatory effect on vertebrate OR promoters. The situation is different in Drosophila, where combinatorial codes of negative and positive transcription factors have been shown to direct expression of OR genes to individual OSNs or to distinct sense organs, the antenna, and the maxillary palp (Fuss and Ray, 2009).

5.1. RP58-like binding motifs mediates repression

In this study, I worked with the zebrafish OR101-1 gene promoter to identify and characterize possible negative regulatory sites and transcription factors that may bind to these sites. Promoter analysis by Nuray Söğünmez (2012) revealed that 500i reduces the number of transgene-positive embryos at 3 dpf, regardless whether the sequence was in its natural position or moved closer to the transcription start. Interestingly, partial deletion of the sequence from transgenic constructs only partially rescued transgene efficiency. A striking feature of the sequence is the presence of two DNA motifs with the sequence CNTCTGG, one in each half of the sequences that Söğünmez deleted in her studies.

Here I tested, whether these CNTCTGG sequences could be responsible for the observed repression. By generating transgenic constructs that neatly remove either one or both of the two sequences, I could show that high transgene efficiency of promoter constructs can be rescued by these relatively small mutations. The 500i sequence is 565 bp in size and each of the two sequence motifs contributes only 7 bp to the overall sequence. Interestingly, mutation of only one site was sufficient to restore full promoter activity and the combined mutation was not synergistic. This is in contrast to the observation of Söğünmez, where only the removal of the full 500i sequence, but not smaller fragments, was sufficient to restore high promoter activity. These finding suggest two conclusions. First, the two motifs most likely act cooperatively in the way, that the relevant transcription factor has to bind to both sequences simultaneously and binding to just one site is not sufficient, otherwise the mutation of a single site should have had either no or only a mild effect. It would be interesting to test, whether the exact spacing between the motifs is important for the interaction of binding factors. Such observations have been made for instance for two DNA –binding E-box and GATA motifs and the Ets and IRF motif-pair which occurs in gene regulatory sequences associated with haematopoiesis and lymphoid development (Li *et al.*, 2012). Second, additional binding sites or binding sites for complementing factors might be located either downstream or upstream of 500i because the partial deletions by Söğünmez were performed on longer transgenic constructs that contained an additional kilobase of sequence upstream of 500i. In the olfactory system, such observations have been made by Rothman *et al.*, (2005) and O/E and homeodomain-like sites are context dependent. In isolated sequences of transgenic constructs, the mutation of O/E sites had a

strong effect on OR expression, whereas the same targeted mutation in the genome had no or mild effects.

Regardless of the mechanism and interaction, the fact that only a seven bp deletion is sufficient to abolish the repressive activity of 500i is a good indicator that the RP58-like sites are indeed the sequences that mediate repression. Theoretically, the sequence could span other transcriptional regulators, such as miRNAs or lncRNAs that have been disrupted by the mutations. Transcriptome analysis from olfactory tissue, however, does not reveal any transcription around the 500i region, which makes this possibility unlikely.

5.2. How good is the assay?

The study presented here builds on previous data obtained by other investigators (Söğünmez). Thus, it was essential to validate the assay and to check reproducibility of the results. Validation of the assay was necessary to understand whether any effect of a mutation could be detected and could reach (statistical) significance. Thus, in a first set of experiments I determined the baseline of my assay. The basic $p_{1.2kb}$-OR101-YFP-pA construct was injected to see whether the expression levels obtained by me are reliable and high. The construct drives expression of the EYFP reporter from a 1.2kb promoter that does not contain the 500i region. The average percentage of embryos expressing this construct was 82.57%, which was higher than previous findings of 61.3%. Similarly, for the construct $p_{500bp\text{-}1.2kb}$-OR101-YFP-pA, which in addition to the 1.2 kb promoter contains the 500i sequence, a reduction in efficiency, but overall higher percentages of transgene-positive embryos were found. In my hands, 47.97% of embryos expressed the construct as compared to the previously observed 12%.

What could account for this difference in expression? Transient transgenic assays are powerful because they allow to check promoter expression in a fast and relatively simple manner. Within only three days, the time point that was used for expression analysis in this and the previous study, conclusive results can be obtained without the need to rear the

animal and wait for germline transmission. Nevertheless, the assay is sensitive to a variety of technical parameters. First, the genetic background of the fish used may matter. In both studies, fish of the AB/AB outbread strain were used, thus in this case the strain may not be of a big concern. The assay is also sensitive to the amount of DNA injected and the location where the DNA is deposited in the oocyte. I used similar DNA concentrations in my experiments as Söğünmez (50 ng/µl for each construct), yet the amount of DNA that is ejected by the micromanipulator is hard to control and differences in the total amount of deposited DNA might exist. Similarly, the my DNA and the DNA used in previous experiments may be of different purity after linearization. Ultimately it matters how much DNA can enter the blastocyst, thus injections directly into the blastocyst are preferable but harder to achieve than injection into the underlying yolk. Thus differences in the target location of DNA cannot be ruled out. Eventually it would be helpful to titrate the injection to reach similarly low expression efficiency and to check whether a stronger difference between $p_{500bp-1.2kb}$-OR101-YFP-pA and $p_{1.2kb}$-OR101-YFP-pA could be observed.

With respect to the bandwidth of the assay and to ensure that the difference between repressed and basic constructs is high enough I did a statistical analysis and confirmed that the difference in transgene expression between $p_{1.2kb}$-OR101-YFP-pA, p500bp-1.2 kb-OR101-YFP-pA is significant. The observed difference was about half, which seemed sensitive enough to detect the effect of the mutations that I investigated later in this study.

5.3. In as far does the assay reflect OR gene choice?

The studies presented her aimed to shed light onto a critical aspect of OR gene choice. However, the transgenic constructs used throughout the study do not contain sequences coding for the OR gene but are replaced with a sequence coding for a reporter gene instead. Thus, technically the constructs resemble OR deletions (Serizawa *et al.*, 2003; Shikind *et al.*, 2005; Fuss *et al.*, 2012). Loci in which the OR coding sequence has been replaced with reporter genes are chosen and expressed but because they do not produce a functional OR protein, or in this case no OR protein, do not activate the UPR

pathway that locks in gene choice (Dalton *et al.*, 2013). It has been shown many times over that OSNs expressing these unfunctional loci make a second choice and switch OR expression (Serizawa *et al.*, 2003; Shikind *et al.*, 2005; Fuss *et al.*, 2012). Expression from the non-functional allele is successively extinguished. By first approximation, the OR gene choice machinery cannot know a priory whether a functional OR gene will be produced from a chosen locus and the deletion construct assay may indeed resemble regular OR gene choice, at least during initial steps.

Another critical point is that the 'OR locus' offered for choice in my assay was a transgene, rather than a full genomic locus. However, it has been demonstrated in the mouse that transgenes are treated by the choice mechanism as additional alleles (A Tsuboi *et al.*, 1999) that are subject to the same criteria. This is remarkable, but makes sense in the light of the evolutionary history of OR gene loci and clusters that arose by multiple rounds of gene duplications and each duplication added novel alleles to the pool from which an OSN can choose. Yet, in the zebrafish assay used here, multiple (up to several thousand) copies of the transgene might be injected or integrated into the genome and be available for choice (Stuart *et al.*, 1988; Vassalli *et al.*, 2002). Thus, the transgene should be favored over endogenous OR loci. However, this does not change the conclusion of the assay for OR gene choice but shifts the numbers into a favorable range. Eventually BAC transgenesis guided by Tol2-mediated genome integration (Suster *et al.*, 2009; Yang *et al.*, 2006) might give results that are more closely related to naturally occurring OR choice, but would lower the number of cells expressing the transgene and therefore the number of positive embryos that can be obtained.

Here, I primarily scored the percentage of transgene-expressing embryos compared to a control construct. In as much does the number of embryos that express the transgene reflect OR gene choice. The outcome of OR gene choice is the number of OSNs that express a given OR and normally all animals express the OR. The argument goes as follows. An increased number of OSNs expressing the transgene increases the likelihood of embryos being positive as well. In my hands the number of transgene-positive OSNs per embryo were low (6.17), thus, in some fish OSNs chose the transgene in others they did

not. If the likelihood of OSNs to express the transgene increases, so would the number of embryos. Nevertheless, it is a rather indirect readout of OR gene choice.

Therefore, I also examined and compared the number of transgene-positive cells for each construct. Results were comparable and the reduction of the cell number was significant for the comparison of $p_{1.2kb}$-OR101-YFP-pA and $p_{500i\text{-}p1.2kb}$-OR101-YFP-pA. Deletion of the RP58-like motifs also increased the number of transgene-expressing cells. However, I no correlation analysis between penetrance and cell number was performed.

5.4. Relationship to other transcription factors

So far only transcription factor binding to OR promoters were known that positively affect OR expression. Removal of the homeodomain or O/E binding sites from the M71 promoter resulted in reduced expression of the transgene (Rothmann *et al.*, 2005). Similarly, removal of the factors that are supposed to bind to these sites, such as Lhx2 (Hirota *et al.*, 2007), Emx2 (McIntyre *et al.*, 2008), or Olf-2/3 (Wang *et al.*, 2004) result in disturbances of OR gene expression. Differently, however, removal of RP58-like motifs from the OR101-1 gene promoter increases expression as compared to constructs that contain the full sequence, which is a natural part of the OR101-1 promoter. Both transgene efficiency and transgene expressing cell number increased. Thus, the results for the first time functionally demonstrate negative modulation of OR promoter activity. This is a highly significant finding because it conceptually expands how OR gene choice might be regulated.

The known transcription factors that act on OR promoters such as Emx2 and Lhx2 have different effect on different OR genes and classes. Lhx2 deficiency affects Class II OR genes whereas Emx2 affects a subset of Class II ORs (Hirota and Mombaerts, 2007; McIntyre at al., 2008). Therefore, OR genes from different classes are regulated with different elements. A similar target distinction might hold true for zbtb7b. OR101-1 gene is the only member of Class II OR genes in zebrafish and therefore OR101-1 and its

regulation might differ from other zebrafish ORs. Also, the RP58 site has so far only been identified for the OR101-1 promoter and might be unique among the zebrafish OR repertoire. Therefore, it is possible that a loss-of-function deletion of the true binding factor might only affect this gene but not others. Yet, these studies have not been performed yet. I began TALEN-mediated inactivation of zbtb7b but i could not generate desired mutation. . Even though TALEN system is reported for efficient mutagenesis in zebrafish (Sander *et al.*, 2011), in some genes the efficiency of the mutation is very low and sometimes it could not even generate the mutation (Moore *et al.*, 2012). Yet, we do not now the efficiency for zbtb7b.

5.5. Which factors bind to the RP58-like motifs?

For simplicity and by analogy to the study by Michaloski *et al.* (2011) the motifs tested in this study were called RP58 binding sites or RP58-like sites. Yet, the factor binding to these sites is not known. The zbtb family of transcription factors comprises 41 genes in zebrafish, but only 3 of them are expressed at high levels in the olfactory epithelium (Zbtb7a, Zbtb7b and Zbtb10). In the mouse OE, Zbtb7b is the factor that is expressed at high levels (Michaloski *et al.*, 2011). Zbtb7b is a transcription factor that causes repression of gene expression. Generally, in the promoter region, several binding sites are presents, and also it has the ability to generate homodimer and multimeric aggregates due to btb domain interactions (Bardwell and Treisman, 1994; Stogios *et al.*, 2007). Zbtb7b has been identified as a transcriptional repressor of Col1A1 and Col1A2 gene expression and necessary element for the CD4 cell fate decision during T lymphocyte development (He *et al.*, 2010; Kappes *et al.*, 2006; Park, 1996). The cells that express zbtb7b proteins (also known as Th-pok) in immature T- cell precursor turns to CD4 cell lineage since the zbtb7b protein cause the repression of the CD8 by changing heterochromatin structure and making the related gene unavailable during CD4 cell development (He *et al.*, 2010; Kappes *et al.*, 2006; L. Wang *et al.*, 2008). Consequently, in the immune system zbtb7b provides mutually exclusive expression of the CD8 and CD4 surface markers, MHC class I or MHC class II respectively.

It is likely that similar mechanism occur in the olfactory system. Because of the high level of zbtb7b expression in the OE, the sites that were referred to as RP58-like sites might in fact be a binding site for zbtb7b. The deletion of the sites might cause loss of docking sites for the protein, and consequently it cannot repress the target gene. As discussed above, both sites might be necessary for zbtb7b to be effective and homodimerization of zbtb7b might be the underlying cause for this observation.

Yet, that zbtb7b indeed is the factor that binds to the two sites investigated here awaits further experimental confirmation. The sites can be used in yeast-one-hybrid studies to identify the true binding factor. Indeed, I have begun to clone constructs for use in yeast-one-hybrid experiments but in the time of this study the work could not be completed.

Assuming that zbtb7b is the repressive binding factor that act on of the OR101-1 gene promoter, we designed morpholino oligonucleotides to knock down the protein in zebrafish embryo. Two types of morpholinos were designed that either block translation or splicing of zbtb7b. The splice-blocking morpholino was designed against second intron-third exon boundary. Probably it will cause disruption of the binding ability of the protein since the exon codes zing-finger, C2H2 like domain of the protein. However, of those, only the translation blocking morpholino was injected. Unfortunately, due to time limitation, this experiment could not be completed. Therefore, the effect of the zbtb7b knockdown on OR101-1 gene is still not known.

5.6. Biological role of the repression

What might be a biological role for the observed repression by RP58-like sites? OR genes are arranged in clusters and cluster-specific enhancer sequences have been identified (e.g. Fuss *et al.*, 2007). Recent data suggests that enhancers of different OR clusters come together in the nucleus to form a nuclear hub or choiceosome (Markenscoff-Papadimitriou *et al.*, 2015). It has also been shown that genes located closer to the enhancer have a higher frequency of choice (Khan *et al.*, 2011). The RP58-like site-mediated repression may

shield the OR101-1 promoter from the effect of a nearby enhancer. Thus, the sites would function as insulators or attenuators of enhancer activity similar to transcriptional repressor CTCF (Ling *et al.*, 2006). So far, an enhancer at the edge of the OR115-family cluster is not known. It could be an OR cluster specific enhancer, an OR101-1-specific enhancer or an enhancer of a nearby gene. OR101-1 is at the edge of the cluster and the next gene is the nicotinic acetylcholine receptor subunit alpha 10. It is possible that the repressor also works in the other direction or in both (Geyer, 1997; Kellum and Schedl, 1991; Sun *et al.*, 1999). and protects the acetylcholine receptor gene from the activity of an enhancer located in the OR115 cluster and from inappropriate expression in OSNs.

The insulator hypothesis by examining the effect of 500i on the H enhancer. H dramatically increases transgene expression and cell number. (Markenscoff-Papadimitriou *et al.*, 2014; Nishizumi *et al.*, 2007). As a consequence, all embryos expressed the transgene and the reporter protein expressing cell number increase 3.4-fold when the H element was present in the construct. The inclusion of 500i between H and the 1.2kb upstream region caused a decrease in the transgene expression and the cell number, in accordance with its proposed insulator function. However, the effect was not 100% and a remaining 1.25 fold increase remained, even though it is not as high as H containing one. A statistical significant difference could be found for the number of transgene-expressing OSNs, but not the fish. It should be noted, however, that expression levels were close to each other, ranging between 80%-100%. Thus, it could be that the assay reached saturation due to the presence of H. The H element is a strong enhancer and it was cloned in close proximity to 500i, therefore, its effect its effect might even be stronger. Another reason, why no complete cancellation of the enhancer effect might have been observed, could be that transgene expression is, at least in part, from integrated plasmid DNA and the sequence would integrate may times over in tandem. Thus, there would always be instance in which the H enhancer would have direct access to the OR101-1 promoter without the 500i sequence between the H element and the promoter. This problem could be circumvented by flanking the entire promoter-reporter section by 500i, an experiment that has not been done yet.

Interestingly, mutations of the RP58-like motif also abolished, at least partially, the effect of 500i on the H-enhancer. A double mutation of the motif restored full transgenic efficiency, but surprisingly, only recovered the number of transgene-expressing cells halfway. Thus, additional sequences other than the RP58-like motifs might have a role in the blocking of enhancer activity. Mechanistically, zbtb7b belongs to a family of proteins that generally function as transcriptional repressors that recruit histone modification complexes and thereby cause chromatin remodeling. Certain insulators are also generated by histone modifications that physically block the interaction of promoters with the enhancer (Bilic and Ellmeier, 2007; Dhordain *et al.*, 1997; Fujita *et al.*, 2004). Therefore, it is possible that zbtb7b causes histone modification to prevent enhancer-promoter interaction.

5.7. Enhancer element and cell type specifity

Another intriguing possibility is that negative regulation restricts the cell type in which a gene is expressed. Cell type is used loosely here, as it could refer to different classes of chemosensory neurons or of different functional subtypes of the same class. The zebrafish OE, different from the mouse, comprises at least four distinct sensory neurons, ciliated, microvillous, crypt and kappe neurons (Ahuja *et al.*, 2014; Hamdani and Døving, 2007; Hansen and Zeiske, 1998). A functional subdivision by cell lineage has been shown in the dorsal OE of the mouse (Thomas Bozza *et al.*, 2009). Enhancers are not necessarily cell type-specific and could direct OR gene choice to an inappropriate cell type if not restrained by negative regulation. We have observed that the H-element increases expression of reporter genes in microvillous cells from OR promoters, although ORs are only expressed in ciliated cells (Sato *et al.*, 2005). I wanted to test the hypothesis that 500i might increase cell type specificity when a OR promoter is combined with the H enhancer.

The presence of 500bp region may restrict the OR101 gene to specific cell type. Previous analyses show a higher tendency in expression in ciliated neurons with the presence with the inhibitory region. However, the analysis is done by comparing the morphology of the neurons and not with the use of molecular markers. A large number of cells had round profiles, which could be either crypt / kappe neurons or immature ciliated

or microvillous OSNs. Using immunostaining for TrkA, which stains crypt neurons, I as able to analyze this possibility with better accuracy. An initial analysis was done on $p_{1.2kb\text{-}OR101}$-YFP-pA injected embryos. Immunopositive cells shows crypt morphology with round cell body and were few in number similar to wild type embryo. On $p_{H\text{-}p1.2kb}$-OR101-YFP-pA injected embryos, an increase in TrkA and double-positive cells was observed. However; many cells did not have crypt cell morphology. With their long axons and apparent cilia, they resembled ciliated cells. It could be that in the construct TrkA or some other antigen is upregulated that reacts with the antibody and that the antibody looses its specificity for crypt cells. TrkA normally is not a true marker protein for crypt cells but it is found that crypt cells have TrkA like immunoreactivity (Catania *et al.*, 2003; Germanà *et al.*, 2004). Also TrkA protein only detected in the crypt cells and it is shown the antibody stains only crypt cell on the wild type embryos. However how enhancer effect the TrkA protein synthesis is unknown. For that reason, possible unspecific staining might be due to the enhancer activity. Unfortunately, the difficulties in the staining procedure make the determination of the effect of the enhancer element on the OR101-1 gene expression problematic.

5.8. Conclusion

Overall, in this study I could show that two short sequence motifs located 2 kb upstream of the OR101-1 gene are the basis of inhibition of gene expression from OR promoter constructs. Therefore, these results, for the first time, demonstrate negative control of an OR gene. Even though the true interacting factor is not known, it is likely that a member of the zbtb family binds to the repressor motifs. More direct analysis can be done by determining protein–DNA interaction for the sequence motif with yeast-one hybrid. The sites also appear to restrain enhancer activity, which allows for interesting speculations on the function of this type of regulation. The sequence analyzed here might act as an insulator between an OR cluster and a neighboring locus, but the direction of the possible enhancer cross-talk is not yet known.

APPENDIX A: EQUIPMENT

Table 6.1. Equipment.

4 °C Room	Birikim Elektrik, Turkey
Autoclaves	Astell Scientific, UK
Centrifuge	Eppendorf, Germany (5417R)
Confocal Microscope	Leica SP5-AOBS, USA
Electronic Balance	Sartorius, Germany (TE412)
Electrophoresis Supplies	Bio-Rad Labs, USA (ReadySub-Cell GT Cells)
Fluorescence Microscope	Leica Microsystems, USA (MZ16FA)
Freezer 1 -20 °C	Arçelik, Turkey
Freezer 2 -80 °C	Thermo Electron Corp., USA (Farma 723)
Gel Documentation	Bio-Rad Labs, USA (GelDoc XR)
Glass Bottles	Isolab, Germany
Incubating Shaker	Thermo Electron Corp., USA
Incubator 1	Weiss Gallenkamp, UK
Incubator 2	Nuve, Turkey
Microinjector	Eppendorf, Germany (FemtoJet)
Micropipetters	Eppendorf, Germany (Research)

Table 6.1. Equipment (cont’).

Microwave Oven	Vestel, Turkey
Refrigerator	Arçelik, Turkey
Softwares	Vector NTI (Invitrogen, USA)
Thermal Cyclers	Bio-Rad Labs, USA (C1000)
Vortex	Scientific Industries, USA
Microwave Oven	Vestel, Turkey

APPENDIX B: SUPPLIES

Table 6.1. List of Supplies.

1 kb DNA Ladder	New England Biolabs, U.S.A. (N3232)
100 bp DNA Ladder	New England Biolabs, U.S.A. (N3231)
Advantage 2 Polymerase Mix	Promega, U.S.A. (M890A)
Bovine Serum Albumin	New England Biolabs, U.S.A. (B9001)
EDTA Disodium Salt	Sigma-Aldrich., U.S.A. (E5134 - 1 kg).
Ethanol Absolute	Sigma-Aldrich, U.S.A. (34870)
Ethidium Bromide	Sigma Life Sciences, U.S.A. (E1510-1 ml)
GeneRuler 1 kb Plus DNA Ladder	Thermo Scientific (SM1331)
Glycerol	Sigma-Aldrich, U.S.A. (G5516-500 ml)
KpnI	New England Biolabs, U.S.A. (R0142L)
LB Agar	Sigma Life Sciences, U.S.A. (SL08394)
LB Broth	Sigma-Aldrich, U.S.A. (L7658- 1 kg)
Magnesium Chloride, 25 mM	Promega, U.S.A. (A3511)

Table 6.2. List of Supplies (cont').

Magnesium Sulfate	Sigma-Aldrich, U.S.A. (M7506)
NcoI	New England Biolabs, U.S.A. (R0193 L)
NotI	New England Biolabs, U.S.A. (R0189 L)
OneTaq® Standard Reaction Buffer	New England Biolabs, U.S.A. (B9022S)
pGEM®-T Easy Vector System	Promega, U.S.A. (A1360)
SacII	New England Biolabs, U.S.A. (R0157S)
Potassium Chloride	Sigma-Aldrich, U.S.A. (P9541)
SacI	Invitrogen (15240-201)
SeaKem® Agarose	Cambrex, U.S.A. (50004)
Sodium Acetate	Sigma-Aldrich, U.S.A. (S8625)
Sodium Chloride	Sigma-Aldrich, U.S.A. (S7653 - 1 kg)
Sodium Hydroxide	Sigma-Aldrich, U.S.A. (S8045 - 1 kg)
SpeI	New England Biolabs, U.S.A (R0133L)
SalI-HF	New England Biolabs, U.S.A (R3138S)

Table 6.2. List of Supplies (cont'.).

T4 DNA Ligase	New England Biolabs, U.S.A (M0202L)
Trizma® Base	Sigma-Aldrich, U.S.A. (T6066)
OneTaq® DNA Polymerase	New England Biolabs, U.S.A (M0480L)

APPENDIX C: RESULTS FOR INDIVIDUAL EXPERIMENTS

Construct: p1.2-OR101-YFP-pA

Experiment 1

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
80	24	18	15	83,33333333

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
7	6	2	4
6	0	4	0
8	8	8	6
3	0	0	0
3	0	1	0
4	3	1	0
1	0	1	0
2	0	0	0
6	9	5	4
1	1	1	0
4	0	3	0
3	3	3	3
2	0	1	0
2	1	1	0
2	1	1	0
9	1	9	0

Experiment 2

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
89	3	3	3	100

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
7	7	4	6
5	4	3	2
0	4	0	3

Experiment 3

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
109	70	40	37	92,5

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
3	8	2	8
17	11	8	11
11	15	11	15
13	12	12	7
14	13	10	10
2	13	1	4
13	13	12	11
17	10	12	10
15	14	10	14
17	8	12	3
20	18	3	10
16	8	12	4
2	0	1	0
7	0	6	0
11	19	7	19
7	10	3	6
22	28	21	24
1	0	0	0
21	20	18	18
17	14	10	6
9	1	5	0
16	14	14	10
11	14	13	1
8	7	14	3
13	9	8	0
7	4	2	1
9	9	9	0
21	17	17	4
15	10	3	0
5	4	5	1

Experiment 4

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
79	56	20	12	60

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 5

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
152	77	61	45	73,7704918

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 6

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
87	26	14	10	71,42857143

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 7

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
61	37	33	32	96,96969697

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Construct: p500i-1.2-OR101-YFP-pA

Experiment	Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
Experiment 1	86	61	35	10	28,57142857
Experiment 2	102	48	28	11	39,28571429
Experiment 3	71	45	27	11	40,74074074
Experiment 4	89	34	25	12	48
Experiment 5	92	29	11	6	54,54545455
Experiment 6	100	57	42	22	52,38095238
Experiment 7	40	19	11	7	63,63636364
Experiment 8	114	84	44	22	50
Experiment 9	94	41	22	12	54,54545455

Positive cell number — Experiments 1–6: **NO DATA**

Experiment 7 — Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
6	0	0	0
7	0	0	0
4	0	2	0
4	2	0	0
11	2	1	0
5	10	2	6
10	5	5	3
10	4	0	4

Experiment 8 — Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
1	0	0	0
3	0	0	0
1	0	0	0
2	6	2	1
1	3	0	2
0	9	0	7
0	1	0	0
2	1	0	1
0	1	0	1
2	3	2	3
0	3	0	0
1	1	0	1
1	2	1	0
3	5	0	2
2	2	2	0
4	3	2	0
8	3	4	1
1	1	0	1
1	1	0	0
2	1	0	0
2	3	1	0
1	0	0	0
11	3	11	0
0	1	0	0
5	1	1	0
0	2	0	0
1	1	0	0
5	0	0	0
3	0	1	0
8	1	0	0
7	5	2	0
1	0	0	0
1	0	0	0
2	0	0	0
4	0	1	0
2	0	0	0
1	0	0	0
1	3	0	0
4	0	0	0
1	0	0	0
2	0	0	0
1	2	1	1
1	1	0	0

Experiment 9 — Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
1	1	0	0
1	1	1	1
2	0	1	0
7	1	2	0
2	5	0	3
1	5	0	0
0	1	0	0
0	1	0	1
1	3	1	0
4	1	0	0
3	0	0	0
1	2	1	2
4	0	0	0
1	1	0	1
10	5	0	0
4	0	3	0
8	3	0	0
1	1	0	0
1	0	0	0
4	0	1	0

Construct: p500iΔ5'/Δ3'-1.2-OR101-YFP-pA

Experiment 1

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
72	49	26	15	57,69230769

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 2

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
100	37	23	17	73,91304348

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 3

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
116	79	56	35	62,5

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 4

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
82	56	35	25	71,42857143

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 5

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
120	76	52	45	86,53846154

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
6	0	2	0
6	0	1	0
3	0	0	0
3	0	2	0
16	18	6	15
4	0	0	0
18	0	14	0
7	0	7	0
3	0	2	0
3	5	2	3
6	5	4	3
3	0	1	0
11	3	9	3
1	0	1	0
5	0	2	0
10	10	9	4
13	6	11	5
14	0	14	0
8	3	7	1
10	3	7	0
6	0	5	0
5	0	3	0
4	0	3	0
5	6	1	0
10	11	5	10
1	0	0	0
4	6	4	5
1	0	0	0
7	4	5	1
1	1	0	0
10	4	8	0
2	0	1	0
7	7	5	5
8	0	7	0
3	3	2	3
8	0	6	0
7	11	3	12
3	2	1	2
8	0	2	0
1	0	0	0
8	7	7	2
1	1	1	0
3	0	2	0
5	6	4	4
9	0	0	0
8	0	7	0
4	7	3	5
0	1	0	0
1	0	1	0
6	0	3	0

Experiment 6

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
157	95	50	36	72

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
8	0	5	0
3	1	2	1
1	0	1	0
4	0	0	0
5	1	2	0
2	1	2	1
1	0	0	0
13	6	11	5
1	0	0	0
1	0	1	0
2	0	0	0
2	4	0	0
6	5	6	3
3	1	2	1
5	1	4	0
6	10	5	7
1	2	0	0
6	0	0	0
3	3	2	0
1	0	0	0
1	0	0	0
16	4	5	4
6	1	3	1
2	3	2	3
4	3	4	0
3	1	0	1
3	2	2	0
2	4	0	4
5	0	3	0
1	2	0	1
13	6	9	6
2	0	0	0
1	7	0	0
4	0	2	0
4	1	4	0
4	0	1	0
3	3	2	1
1	0	0	0
2	1	0	1
1	1	0	0
17	11	17	3
2	3	1	0
3	2	3	1
1	0	1	0
3	1	3	0
3	2	2	0
2	0	1	0

Experiment 7

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
32	7	7	6	85,71428571

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
8	20	8	20
12	14	11	9
6	8	2	3
13	9	11	9
6	5	1	0
8	7	0	0

Construct: p500iΔ5'-1.2-OR101-YFP-pA

Experiment	Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
Experiment 1	80	19	17	15	88,23529412
Experiment 2	85	18	3	2	66,66666667
Experiment 3	61	38	28	20	71,42857143
Experiment 4	39	18	16	13	81,25

Experiment 1 – Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
18	5	12	3
4	4	2	1
9	8	0	2
5	0	0	0
5	5	4	2
10	3	8	1
4	0	1	0
6	1	6	0
1	1	0	0
4	3	2	1
11	0	10	0
4	2	1	1
1	3	1	1
1	4	1	2
7	5	6	1
4	4	1	1
4	0	3	0

Experiment 2 – Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
6	1	0	1
8	1	0	1
13	3	0	0

Experiment 3 – Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
1	0	0	0
2	1	0	0
5	4	1	0
1	0	0	0
4	1	3	0
5	6	4	4
4	0	2	0
5	1	5	1
1	0	1	0
6	7	4	5
1	0	0	0
5	0	3	0
4	2	2	0
2	0	0	0
10	0	10	0
2	0	2	0
6	0	3	0
3	0	0	0
6	6	4	2
1	0	0	0
2	0	1	0
1	1	0	0
11	5	1	1
4	3	4	1

Experiment 4 – Positive cell number

mcherry Nose 1	mcherry Nose 2	YFP Nose 1	YFP Nose 2
9	0	0	0
14	5	10	5
4	3	2	2
4	19	4	17
4	13	3	13
14	0	7	0
10	10	9	10
15	15	6	14
5	4	0	0
9	5	6	2
12	0	8	0

Construct: p500iΔ3'-1.2-OR101-YFP-pA

Experiment 1

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
80	31	21	17	80,95238095

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
16	6	14	0
18	16	23	7
12	17	8	17
2	0	0	0
4	8	2	1
3	0	3	0
1	0	1	0
7	5	3	5
15	5	8	4
7	0	4	0
5	0	0	0
4	0	0	0
2	0	2	0
4	3	0	0
9	5	9	4
14	13	13	8

Experiment 2

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
85	31	17	12	70,58823529

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
1	0	1	0
3	0	1	0
3	0	0	0
1	0	1	0
4	0	4	0
4	4	2	3
12	10	12	13
8	4	5	3
4	8	2	6
9	9	4	3
15	5	14	2
4	2	4	0
3	3	3	1
13	9	9	5
4	8	2	6

Experiment 3

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
59	23	12	9	75

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
8	1	2	1
11	3	9	1
2	0	1	0
6	12	5	10
3	0	3	0
7	1	7	0
1	0	0	0
10	0	0	0
6	0	3	0
2	0	0	0
3	0	3	0

Experiment 4

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
55	19	14	9	64,28571429

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
1	5	8	1
2	2	0	1
4	2	0	1
7	3	2	0
8	3	5	2
11	6	0	0
12	11	0	5
14	9	10	5
15	7	0	6
16	1	0	0
17	2	1	0
18	3	0	0
19	3	1	1

Experiment 5

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
53	27	12	9	75

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
5	8	1	5
2	0	1	0
2	0	1	0
3	2	0	0
3	5	2	0
6	0	0	0
11	0	5	0
9	10	5	10
7	0	6	0
2	1	0	0
3	0	0	0
3	1	1	0

Construct: **pH-500iΔ5'/Δ3'-1.2-OR101-YFP-pA**

Experiment 1

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
172	120	73	74	101,369863

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
8	5	7	4
7	1	7	0
3	2	1	0
1	4	0	3
14	13	14	12
12	39	12	45
11	19	11	13
7	2	5	0
6	9	6	5
3	7	4	7
31	11	15	8
21	22	14	9
2	13	1	12
22	1	1	1
43	42	41	40
10	2	9	2
8	14	6	4
6	4	3	2
29	33	25	24
2	1	2	1
1	4	1	3
4	17	22	14
3	9	3	8
11	23	11	18
4	2	3	1
5	0	4	0
25	9	25	8
2	14	2	13
2	10	1	11
1	4	1	4
30	30	27	33
8	13	6	14
1	8	0	7
0	7	0	5
8	14	1	11
7	22	7	22
7	50	5	41
8	2	8	2
6	15	4	14
39	9	30	5
4	1	2	1
14	11	12	8
17	26	16	21
0	5	0	4
12	16	9	17
13	18	8	18
0	2	0	1
37	15	27	17
34	15	32	17
12	11	10	10
10	12	10	13
0	4	0	4
8	1	4	0

Experiment 2

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
110	62	53	53	100

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
79	15	77	14
39	26	31	37
27	14	43	17
40	37	45	40
43	32	38	34
12	26	11	32
7	17	6	17
91	24	93	25
12	0	9	0
8	12	5	8
19	17	15	28
9	7	8	5
11	47	20	43
29	31	27	37
7	0	7	0
0	12	0	4
6	0	5	0
8	5	7	2
18	14	20	14
19	7	12	5
4	0	1	0
39	43	48	52
5	10	3	8
15	9	13	9
32	18	35	25
19	20	21	24
0	6	0	5
51	88	60	84
6	5	4	2
10	10	4	5
20	9	18	9
3	5	2	2
5	0	3	0
8	3	2	0
10	9	15	10
4	0	3	0
67	56	70	65
28	21	25	21
19	0	18	0
27	13	23	9
8	22	8	17
32	41	27	37
62	23	62	25
39	17	8	22
6	6	4	2
47	25	38	18
34	52	34	52
5	0	4	0
11	7	9	3
3	0	3	0
35	15	38	15
7	32	13	35
50	49	43	41

Experiment 3

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
118	74	36	36	100

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
24	25	30	25
12	10	12	8
16	13	18	9
11	3	11	3
35	0	38	0
58	46	58	30
6	9	4	11
6	19	15	15
10	17	6	11
6	14	6	11
5	28	4	25
2	0	1	0
9	6	12	6
53	46	57	44
6	30	4	30
50	13	55	13
43	23	44	26
1	6	1	8
63	11	63	11
3	2	1	1
16	0	14	0
11	9	10	12
13	10	13	10
12	13	13	6
0	5	0	5
3	4	3	4
19	13	19	11
31	12	30	6
4	30	5	32
63	2	65	2
33	39	43	30
4	6	3	6
2	20	0	20
22	5	23	4
7	20	8	20
9	9	9	7
8	8	8	6
10	33	10	26
6	2	5	2
16	38	16	40
13	7	13	7
43	32	43	29
26	15	23	25
11	4	16	3
16	5	12	4
12	2	8	2
32	38	31	45
30	70	36	72
10	14	13	14
4	16	3	16
3	5	3	5
0	26	0	22

Experiment 4

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
125	28	20	20	100

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
8	11	8	10
67	18	65	19
21	12	21	11
7	4	6	5
90	1	91	1
6	21	4	19
2	35	1	41
5	7	4	3
35	15	47	17
27	8	27	5
17	11	12	17
13	19	11	20
8	0	7	0
9	0	6	0
11	18	14	19
23	9	20	8
22	22	25	25
10	0	12	0
3	21	13	20

Construct: **pH-500i-1.2-OR101-YFP-pA**

Experiment 1

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
80	4	2	1	50

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 2

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
94	44	34	32	94,11764706

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
NO DATA			

Experiment 3

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
46	23	15	14	93,33333333

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
2	7	0	0
4	2	4	0
10	2	9	2
11	8	10	5
4	7	0	6
4	2	4	0
10	9	9	8
1	0	1	0
2	1	1	0
4	0	2	0
1	3	1	2
7	0	5	0
1	0	1	0
10	0	9	0
8	0	1	0

Experiment 4

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
145	56	49	47	95,91836735

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
13	0	12	0
9	7	7	8
12	0	1	0
5	0	4	0
3	0	3	0
7	8	0	5
11	5	8	3
4	0	3	0
3	0	5	0
5	16	5	12
3	16	2	13
11	19	11	28
12	5	8	4
12	11	11	8
5	0	5	0
7	6	5	6
15	0	9	0
8	11	7	10
16	6	11	1
0	4	0	3
3	5	2	3
8	0	2	0
19	8	20	6
2	0	0	0
46	59	57	70
16	8	16	6
2	12	4	8
8	15	1	11
15	13	22	11
33	13	30	11
5	9	4	7
16	14	22	17
26	27	31	26
21	0	20	0
15	39	13	44
5	0	5	0
8	13	14	17
21	5	4	18

Experiment 5

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
109	12	11	10	90,90909091

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
5	4	0	0
1	0	1	0
9	6	7	2
21	11	10	8
3	0	2	0
34	1	29	1
23	48	16	39
4	11	3	5
48	48	39	45
2	7	0	6
13	14	8	8

Experiment 6

Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
116	62	53	51	96,22641509

Positive cell number			
mcherry		YFP	
Nose 1	Nose 2	Nose 1	Nose 2
6	6	5	2
25	19	24	21
3	0	2	0
37	23	39	23
5	2	3	1
18	13	5	6
11	30	8	32
3	0	2	0
7	2	5	1
2	0	1	0
3	0	1	0
6	0	3	0
4	0	2	0
4	0	3	0
3	0	1	0
2	1	1	1
43	15	42	8
10	1	5	1
67	72	81	70
13	5	9	5
2	0	2	0
1	0	0	0
1	17	1	17
6	4	3	1
6	3	3	2
8	10	6	7
2	0	2	0
3	0	0	0
12	4	12	1
16	7	11	3
5	1	2	1
5	17	2	25
21	36	17	21
10	5	9	2
2	0	1	0
3	0	3	0
28	30	43	36
4	9	3	7
9	16	4	8
9	13	4	13
20	6	20	6
46	4	50	2
23	21	27	14
9	4	9	3
38	22	34	22
12	12	3	7
0	4	0	4
45	5	28	5
2	6	0	3
15	6	23	2
10	9	6	2
24	32	29	21
4	34	2	25

Construct:	**pH-1.2-OR101-YFP-pA**													
Experiment 1					Experiment 2					Experiment 3				
Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression	Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression	Injected Fish #	Survived Fish #	mCherry Positive embryos	YFP positive embryos	Transgene expression
120	38	14	14	100	102	37	27	27	100	90	38	28	29	103,5714286
Positive cell number					Positive cell number					Positive cell number				
mcherry		YFP			mcherry		YFP			mcherry		YFP		
Nose 1	Nose 2	Nose 1	Nose 2		Nose 1	Nose 2	Nose 1	Nose 2		Nose 1	Nose 2	Nose 1	Nose 2	
23	20	23	20		23	24	35	26		27	23	29	27	
14	4	18	3		19	14	27	16		17	0	12	0	
25	17	25	13		9	0	9	0		25	0	25	0	
11	8	3	8		76	98	72	106		28	8	28	9	
4	0	4	0		3	0	7	0		29	0	30	0	
8	26	6	35		11	8	17	18		5	3	5	3	
10	16	17	20		0	10	2	10		46	34	45	29	
18	40	22	53		32	44	24	44		6	12	4	14	
36	0	40	0		90	101	94	96		14	0	14	0	
2	3	1	4		4	13	3	18		6	9	4	16	
30	0	37	0		27	0	39	0		5	0	5	0	
42	43	43	40		12	5	9	0		35	58	37	62	
4	3	2	3		22	15	24	21		10	23	12	25	
47	25	38	18		6	0	9	0		5	2	4	1	
67	50	70	65		25	23	21	21		13	12	14	10	
9	16	12	6		33	12	29	13		6	4	5	4	
					3	17	3	11		53	0	53	0	
					2	0	12	0		11	0	10	0	
					30	35	31	30		6	3	5	3	
					3	6	2	11		10	0	12	0	
					15	12	14	17		7	2	7	2	
					11	0	9	0		3	0	3	0	
					7	0	8	0		11	22	10	25	
					3	0	3	0		20	0	22	0	
					10	0	11	0		3	0	5	0	
					21	31	20	31		33	46	42	57	
					0	0	0	1		0	13	0	13	
					14	2	11	3		1	2	1	13	
					39	30	39	30						

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