Genetic network underlying the induction and formation of cranial placodes in the Preplacodal Region

Safia B. Khatri
Department of Neuroscience, Baylor College of Medicine, BCM295, 1 Baylor Plaza, Houston, TX 77030, USA
Email: safiakhatri@yahoo.com

Abstract
Vertebrate cranial sensory organs and their ganglia originate from thickened ectoderm called cranial placodes. Despite the cellular and functional diversity of cranial sense organs, their precursors derive from the ectoderm adjacent to the anterior neural plate region called preplacodal region (PPR). The PPR is characterized by the expression of a unique set of transcription factors referred as PPR genes, which include *Foxi, ERNI, Datch, GATA, Dlx, Six*, and *Eya*. The expression analysis of these genes does not correlate precisely with phenotypes in the PPR. Knockouts of all PPR genes do not show complete loss of any cranial placodes. However, *Foxi3* and *Dlx5* genes are expressed early in the PPR in complementary fashion, with *Dlx5* localized anteriorly and *Foxi3* localized posteriorly. In addition, mutants of *Dlx* gene family members show defective anterior cranial members do not show defects in the induction of cranial placodes. All these genes are considered as PPR genes because of their expression in the PPR, but not all of them contribute to PPR induction. Placodes, and *Foxi3* knockout mice show defects in posterior placodes. On the other hand, *Six* and *Eya* gene family members, mentioned as definitive PPR genes in previous studies, are expressed after *Foxi3* and *Dlx5.* Moreover, mutants of *Six* and *Eya* gene family Vertebrate cranial sense organs largely arise from ectodermal thickening, the placodes. Albeit diverse in function, precursors of cranial sensory placodes are intermingled initially and derive from a common region called preplacodal region (PPR) (Baker and Bronner-Fraser, 2001; Couly and Le Douarin, 1985; Couly and Le Douarin, 1988; D'Amico-Martel and Noden, 1983; Litsiou *et al.,* 2005; Streit, 2004). Several transcription factors have been identified as fate determining genes for the PPR and/ or cranial placodes, but the gene regulatory network that establishes PPR is not well understood. Based on knockout studies of different PPR genes, I have selected to discuss *Foxi, Dlx, Six,* and *Eya* gene families in this review. I will refer to *Foxi* and *Dlx* as competence transcription factors (CTFs) and the *Six* and *Eya* as definitive placodal genes (DPGs). The purpose of this review is to understand the relationship of CTFs and DPGs during the induction of PPR and cranial placodes**.**

Keywords: cranial placodes, preplacodal region, preplacodal region genes

Preplacodal region: a platform for cranial placode

Vertebrate cranial paired sensory organs and their ganglia (the lens of eye, nasal epithelium, and lateral line) originate from specialized tissue called placode. Cranial placodes are evolutionary conserved vertebrate novelties. Each placode has an individual identity with the capability of generating different cell types (Litsiou *et al.,* 2005). Nonetheless, their precursors are initially mixed and are derived from a common region called PPR (Baker and Bronner-Fraser, 2001; Couly and Le Douarin, 1985; Couly and Le Douarin, 1988; D'Amico-Martel and Noden, 1983; Litsiou *et al.,* 2005; Streit, 2004). In some species, the PPR is identified as continuous primitive placodal thickening (Knouff, 1935; Streit, 2004), whereas in others it can be identified only by the expression of molecular markers (Bhattacharyya *et al.,* 2004; Streit, 2002; Streit, 2004 and Streit, 2008).There is a common consensus about the genes that are expressed within the PPR. Some of these molecular markers, described as PPR marker genes in previous studies, are members of *Foxi*, *ERNI*, *Dlx*,

GATA, *Six,* and *Eya* gene families (McLarren *et al.,* 2003; Litsiou *et al.,* 2005; Khatri and Groves, 2013; Schlosser, 2006). These molecular markers are entitled as PPR genes because of their expression in the PPR (Baker and Bronner-Fraser, 2001; Streit, 2004).

Detailed expression analysis of PPR genes revealed that, though these genes are expressed within the PPR, none of them can precisely define this region because their expression extends to either neural, neural crest, and/or epidermal regions. For example, *Erni* is initially expressed broadly in neural domains and later confined to the PPR (Litsiou *et al.,* 2005). Similarly, *Foxi3* expression is seen in the neural domain at the tip of the primitive streak both in mice and chicks (Ohyama and Groves, 2004; Khatri and Groves, 2013). *Eya 1/2* and *Six1/4* expression is observed in the PPR as well as in the anterior neural plate (Litsiou *et al.,* 2005). Moreover, the time of the onset of PPR genes is also different. For instance, *ERNI*, *Dlx*, *Foxi*, and *GATA* genes are expressed at the early gastrula stage in different vertebrates (Khatri *et al*, submitted, Litsiou *et al.,* 2005), whereas *Six* and *Eya* genes are first observed at late gastrula and early neurula stages in the PPR (Khatri *et al*, submitted)**.**

Knockout studies of different PPR genes have been carefully carried out to understand their role in the induction of the PPR and cranial placodes. Based on the knockoutdata I have selected *Foxi, Dlx, Six* and *Eya* gene families to discuss in detail their role in the induction of PPR and cranial placodes.

***Dlx* gene family**

Dlx homeobox genes are mammalian homologs of the Drosophila *Distal-less* (*Dll*) gene. *Dll* is expressed in the distal portion of the developing appendages in *Drosophila* and has been shown to be critical for the appendages. The *Dlx/Dll* gene family is one of the earliest gene families that play a role in the development of appendages in most of the species where it has been identified. Members of the *Dlx* gene family are expressed in both craniofacial and cranial neural crest ectoderm in vertebrates (Akimenko et al., 1994; Bulfone et al., 1993; Ellies et al., 1997; Dollé et al., 1992; Qiu et al., 1997; Robinson and Mahon, 1994; Simeone et al., 1994; Yang et al., 1998). Different members of the *Dlx* family show different spatial and temporal expression patterns during vertebrate development. However, all *Dlx* family members are expressed in branchial arches suggesting an important functional role in the development of organs derived from branchial arches. *Dlx5/6* are initially expressed in the non-neural ectoderm and later limited to the PPR ectoderm. *Dlx5* is the first gene to be expressed in non-neural ectoderm and later localizes in the PPR in chick, mouse and *Xenopus* models (Acampora, 1999; Khatri et al, submitted; Litsiou et al., 2005; Luo et al., 2001; Pieper et al., 2012). *Dlx3* is expressed after *Dlx5* in the PPR. Conversely, *Dlx1* and *Dlx2* are never expressed within the PPR; *Dlx3* and *Dlx5/6* are later expressed in olfactory, otic and mandibular arch placodes (Qiu et al.,1997; Simeone et al., 1994; Yang et al., 1998).

Targeted disruption of *Dlx1, Dlx2, Dlx1/2*, or *Dlx5* genes in mice results in craniofacial, bone, and vestibular defects (Acampora *et al.,* 1999; Depew *et al.,* 1999; Qiu *et al.,* 1997) without any noticeable limb abnormalities. The lack of limb defects could be due to the compensatory effect of other members of the *Dlx* gene family. Homozygous *Dlx5* mutant mice (Acampora *et al.,* 1999; Depew *et al.,* 1999) die shortly after birth. Some *Dlx5*$^{-/-}$ mice show exencephaly whereas some don't, but have hypomineralized calvaria. Generally *Dlx5*$^{-/-}$ have common defects in nasal capsule, otic capsule, and proximal mandibles, which correlate with the early expression pattern of *Dlx5*. Double knockout of *Dlx5* and *Dlx6* genes in mice results in severe axioskeletal, craniofacial, and inner ear defects, which lead to perinatal lethality. *Dlx5/6* $^{-/-}$ mice also phenocopy human split hand/split foot malformation (SHFM1). The craniofacial and ear defects in *Dlx5/6* $^{-/-}$ mice are more severe than the ones previously reported in *Dlx5*-deficient

mice (Acampora *et al.,* 1999; Depew *et al.,* 1999) suggesting that both genes have unique and redundant functions. It has been shown that numerous genes are required for proper ear and craniofacial development. Since the deletion of genes important for ear and craniofacial development show similar defects to the *Dlx5/6* $^{-/-}$ mice (Clouthier *et al.,* 2000; ten Berge *et al.,* 1998; Thomas *et al.,*1998), *Dlx5/6* may function as global coordinators of a number of signaling pathways critical for craniofacial development (Robledo *et al.,* 2002) but not for the induction.

The earliest expression of *Dlx5* in the non-neural domain in chicks has been shown to be important for ectodermal patterning (Pera *et al.,* 1999). Recently, *Dlx5* has been identified in chicks as a mediator of cell fate decision at the border of the neural plate that promotes the expression of neural crest markers, *MSX1* and *BMP4,* and the PPR marker, Six4. The over-expression of *Six1* and *Eya2* together resulted in the induction of *Dlx5* suggesting the presence of a positive regulatory loop (Christophorou *et al.,* 2009).

Dlx knockout mice show defective cranial sensory organ formation without affecting the induction of cranial placodes, whereas ectopic placodes could not be generated when *Dlx* was over-expressed in chicks. These data further support the role of *Dlx5* as a mediator of neural plate border rather than in the specification of the PPR.

Foxi gene family

Soon after the expression of *Dlx5*, another important transcription factor, *Foxi3,* is expressed in the non-neural domain; its expression domain extends inward the neural domain at the tip of the primitive streak in chicks and mice. *Foxi1* in fish and amphibians is the closest functional homolog of mouse and chick *Foxi3* based on sequence homology (Solomon *et al.,* 2003). *Foxi1* in fish and *Foxi3* in mouse and chick are expressed in the PPR and later in pharyngeal arches. The over-expression of *Foxi1* extends the expression domain of *Six4.1*, *Eya1,* and *Dlx3b* in zebrafish. Nonetheless, Foxi1-Morpholino (MO) alone was not sufficient to inhibit the expression of *Six4.1*, *Eya1,* and *Dlx3b* (Solomon *et al.,* 2003). Similar induction of *Dlx5*, *Six1,* and *Eya2* was observed in chicks when [illegible] was over-expressed ectopically (Khatri *et al.,* submitted). These results suggest a positive regulatory network among different PPR genes.

Mutation in zebrafish *foxi1* results in interrupted otic placode induction, defects in branchial arches, and in jaw formation (Solomon *et al.,* 2003). Similarly, defective ear and jaw are seen in foo/foo mutants (Nissen *et al.,* 2003). Foxi3-MO in chicks resulted in the down-regulation of otic specific marker like *Foxg1* and *Pax2*. However, *Foxi3* alone or in combination with other PPR marker genes was not able to induce otic marker *Pax2* when cultured in the presence of the fibroblast growth factor (FGF) (Khatri *et al.,* submitted). These results suggest that Foxi3 is necessary but not sufficient for otic placode induction.

Eya and *Six* gene family members

Eya and *Six* genes are currently the most popular candidates for the specification of PPR and the induction of generic placodes in vertebrates because they are expressed in the PPR and in some cranial placodes (Schlosser, 2006). Moreover, a wide gamut of deficiencies in various placodes is seen in mutants of *Six/Eya* gene families from humans to zebrafish.

Eya genes encode proteins that affect transcription indirectly by binding to other transcription factors with direct DNA-binding capacity encoded by *Six* genes (Ikeda *et al*. 2002; Li *et al*. 2003; Ohto et al. 1999; Pignoni et al. 1997). There are typically four members of *Eya* (*Eya1–4*) in vertebrates. Mutations in *EYA1* gene in humans is associated with branchiootorenal (BOR) dysplasia syndrome, branchiootic syndrome, sporadic cases of congenital cataracts, and ocular anterior segment anomalies. *Eya1* heterozygotes result in renal

abnormalities and a conductive hearing loss similar to BOR syndrome, whereas *Eya1* homozygotes lack ears and kidneys. Also, the development of the inner ear is arrested at the otic vesicle stage and specific cranial ganglia formation is affected as well (Xu *et al.,* 1999). *EYA2* is also expressed in the eye, ear, and craniofacial mesenchyme very early during development (ninth week after conception) in humans (Abdelhak *et al.,* 1997), but no mutation in *EYA2* has been identified affecting humans (Vieira *et al.,* 2002). The expression of *Eya3* revealed by in-situ hybridization and β-Gal-staining is observed in the primordium of multiple organs like brain, eyes, heart, somites, and limbs during mouse and zebrafish development (Söker *et al.,* 2008); however, *Eya3* homozygous mouse mutants are alive with no obvious defects in the eyes, ears, and kidneys (Söker *et al.,* 2008). *Eya4* encodes a protein which is important for continued function of the mature organ of Corti. Mutations in this gene are associated with progressive autosomal dominant hearing loss (Tóth *et al.,* 2004).

There are three subfamilies of *Six* genes (*Six1/2, Six4/5, Six3/6*) in vertebrates. *Six1/2* subfamily genes are important for sensory organogenesis. Mutations in *Six* gene family are associated with defects in multiple sensory organs derived from cranial placodes such as the olfactory epithelium (Ikeda *et al.,* 2007, 2010; Laclef *et al.,*2003), inner ear (Bricaud and Collazo, 2006; Kozlowski *et al.,* 2005; Laclef *et al.,* 2003; Li *et al.,* 2003; Ozaki *et al.,*2004; Xu *et al.,*1999; Zheng *et al.,* 2003; Zou *et al.,* 2004), epibranchial ganglia (Zheng *et al.,* 2003; Zou *et al.,* 2004), and defective development of taste papillae (Suzuki *et al.,*2010 and 2011). In humans, mutations of *SIX1* cause a severe auditory and renal disorder known as branchio-oto-renal syndrome (Kochhar *et al.,* 2008; Ruf *et al.,* 2004) similar to the one caused by *Eya1* mutation. On the other hand, *Six1* knockout mice show defects in the development of kidneys, salivary glands, and branchial organs such as the thymus and parathyroid gland (Kobayashi *et al.,* 2007; Laclef *et al.,* 2003; McCoy *et al.,* 2009; Ozaki *et al.,* 2004; Xu *et al.,* 2003). However, *Six1*$^{-/-}$*/Six4*$^{-/-}$ mice show defects in the trigeminal ganglion (Konishi *et al.,* 2006). Although *Six/Eya* mutants display cranial sensory organ developmental defects, no cranial placode induction defects have been reported. These observations suggest that the *Six/Eya* regulatory network is important for the specification of the PPR but is not the sole pathway.

Competence Transcription Factors (CTFs) versus Definitive Placodal Genes (DPGs)

Several studies highlight the importance of PPR genes for the induction of PPR; however, the first expression of each gene varies in time. Generally the CTFs are expressed first in the non-neural ectoderm, like *DLX* and *FOXI* gene family members. Their expression is initially broad and then regionalized in the prospective cranial placode (Khatri *et al*., submitted). Only later, DPGs, like *Six* and *Eya* gene families, are expressed (Khatri *et al.,* submitted). Moreover, knockouts of PPR genes have been studied extensively to understand their role in the establishment of PPR and cranial placode induction. Studies from knockout of different DPGs suggest an essential role of CTFs, like *Foxi* and *Dlx* gene family members, which have been shown to be important to induce general PPR genes (*Six* and *Eya*) within non-neural ectoderm. Competent non-neural ectoderm was also shown to be necessary for in vitro formation of PPR from embryonic stem cells (Leung *et al*., 2013).

It has been shown recently that all PPR genes regulate each other's expression, probably for a compensatory mechanism. However, there is evidence suggesting the importance of CTFs in the induction of specific cranial placodes. For example, *Foxi3* in mouse and chick (Khatri *et al.,* submitted) and *Foxi1* in zebrafish have been reported to be necessary for the induction of otic placode. Zebrafish embryos treated with *Foxi1-MO* showed severe defects in the induction of otic placode as well as otic specific marker gene, *pax2* (Nissen *et al.,* 2003). Similarly, down-regulation of otic specific genes (*Pax2* and *Foxg1*) was observed in chicks when *Foxi3* specific

morpholinos were expressed at earlier stages. In chick, otic induction was ceased when *Foxi3* morpholinos were electroporated, without affecting the expression of other PPR marker genes (Khatri *et al*., submitted).

Grafting and tissue culture experiments in chick showed the importance of PPR in the induction of generic cranial placodes. In these experiments, only PPR ectoderm, but not outside ectoderm was able to induce otic placode marker genes when cultured in the presence of growth factors like FGF (Martin and Groves, 2006; Schlosser, 2006). Similarly, another set of experiments showed the ability of naïve ectoderm to induce otic marker genes in the presence of FGF only when it was first grafted within PPR, promoting the expression of DPGs (Martin and Groves, 2006). As mentioned previously, the PPR expresses otic marker genes when exposed to FGF; therefore, it is possible that the naïve ectoderm in this experiment is specified as PPR. Indeed, naive ectoderm grafted in PPR was able to induce otic marker when cultured in the presence of FGF. Based on these experiments, we cannot conclude that DPGs are necessary for the induction of otic placodal genes. The same group showed competence of naïve ectoderm to express otic placode marker genes when grafted adjacent to the posterior hindbrain region without expressing first PPR genes (CTFs and DPGs) (Groves and Bronner-Fraser, 2000). Comparable experiments in chick showed that PPR ectoderm originates lens placode unless exposed to additional signals (Bailey *et al.*, 2006). Results from these experiments indicate the presence of a common placodal ground state shared throughout the preplacodal ectoderm. Afterwards, depending on surrounding signals received, various subregions of the PPR diverge gradually to become different placodes (Bailey *et al.,* 2006; Schlosser, 2006).

PPR genes discussed above are expressed at different stages during embryonic development. Moreover, the ability of a PPR gene to induce another PPR gene is stage dependent. *Dlx5* is expressed initially in the non-neural ectoderm dividing embryonic ectoderm into neural and non-neural domains. By Hamburger-Hamilton stage 4 (HH4) *Foxi3* is expressed as a band encircling the neural plate. By that time, *Dlx5* is also regionalized within *Foxi3*-positive domains. *Six* and *Eya* gene family members are expressed by HH5 (Figure 1). At this stage *Foxi3*, *Dlx5,* and *Six/Eya* can induce each other's expression when ectopically over-expressed (Khatri *et al*., submitted). PPR ectoderm at this stage is unspecified, and is able to become either lens (Bailey *et al*., 2006) or otic placode when cultured in vitro (Martin *et al*., 2006), depending on the external signals provided. By HH6-7, *Foxi3* and *Dlx5* show complementary expression domains. *Dlx5* covers the anterior and *Foxi3* the posterior PPR; instead, *Six/Eya* genes are localized in the entire PPR (Khatri *et al*., submitted). The ability of PPR genes to induce each other is reduced by this stage. *Foxi3* and *Dlx5* cannot induce each other but both can induce *Six/Eya* when over-expressed (Khatri *et al*., submitted). Moreover, anterior PPR ectoderm is able to induce lens placodal marker genes when cultured in vitro in the absence of external signals (Bailey *et al*., 2006). Nevertheless, posterior PPR ectoderm failed to induce lens but was able to induce otic placodal marker genes when cultured in the presence of FGF (Martin *et al*., 2006). These results indicate that PPR ectoderm is gradually committed to become individual placode. By HH7-8 stage, *Dlx5* and *Foxi3* expression is further segregated; by HH9-10 stages, *Dlx5* expression is restricted to olfactory and *Foxi3* to otic placodes, whereas *Six/Eya* are expressed through the entire PPR. By this time, genes specific to individuals placodes start their expression and are specified (Martin *et al*., 2006, Groves and Bronner-Fraser, 2000). A summary of gradual specification of cranial placodes is shown in Figure 2.

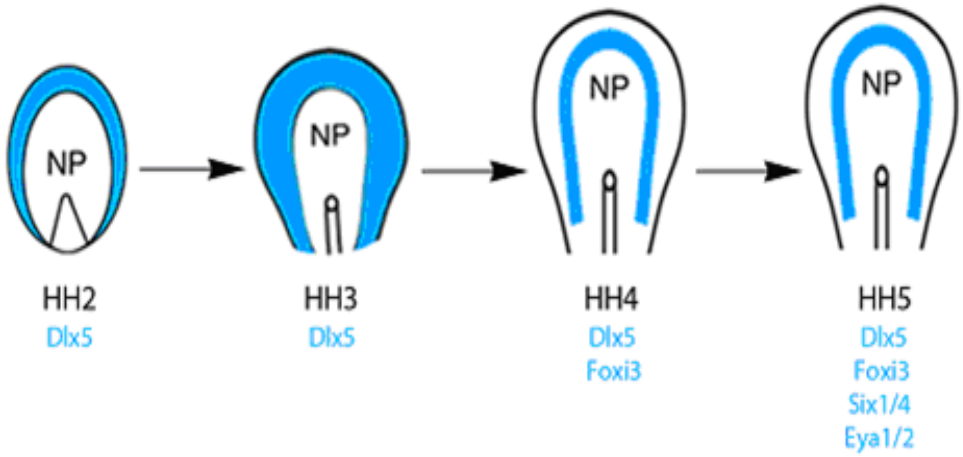

Figure 1: Scheme showing the expression pattern of PPR genes in chick. *Dlx5* is the first gene expressed in the non-neural field dividing embryonic ectoderm into neural and non-neural domains. Shortly afterwards, *Foxi3* is expressed in the presumptive PPR at HH4 stage. *Dlx5* is also localized in the PPR by that time. By HH5, *Six1/4* and *Eya1/2* genes are expressed in PPR. Blue color indicates expression domain of PPR genes. NP (Neural Plate), HH (Hamburger-Hamilton stages in chick).

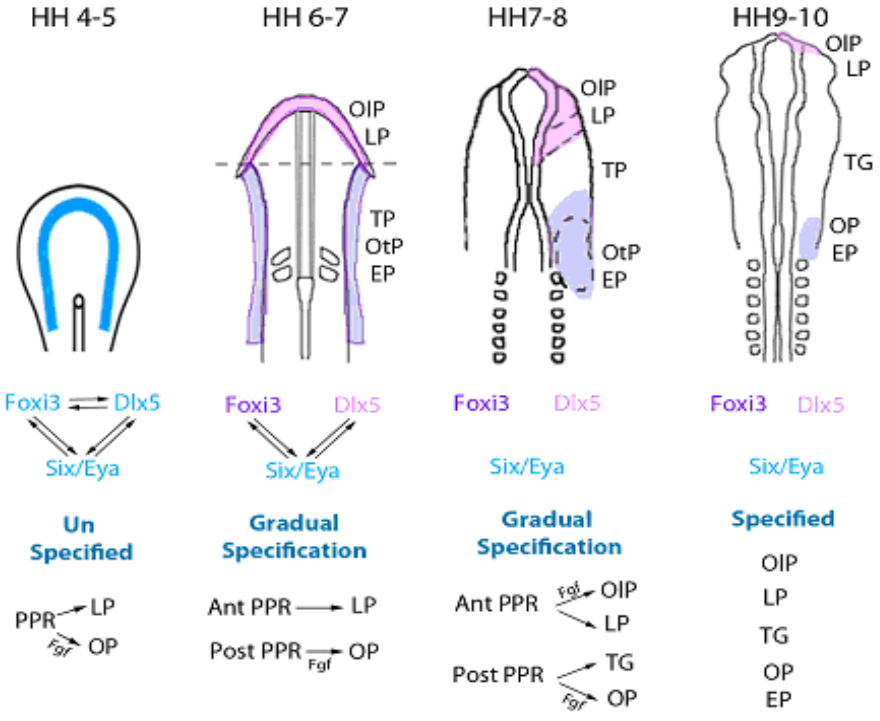

Figure 2: Schematic representation of gene-regulatory network inducing cranial placodes in the PPR. At HH4-5 stages, PPR genes are expressed and can regulate each others. At this stage precursors for cranial placodes are not completely committed and can either be lens placode or otic placode, depending on additional signals provided. By HH6-7, anterior and posterior placodes become gradually fated. *Dlx5* and *Foxi3* are expressed in complementary fashion whereas *Six/Eya* covers the entire PPR. At this stage *Foxi3*

and *Dlx5* cannot induce each other; conversely, each gene is able of inducing *Six/Eya* genes when over-expressed. By HH7-8 stage, *Dlx5* and *Foxi3* expression domains become more localized. At this stage PPR genes lose their ability to induce each other. By HH 9-10 stage, *Dlx5* expression is localized to the olfactory placode and *Foxi3* to the otic placode. Pink indicates *Dlx5,* and purple indicates *Foxi3* expression domains. OlP (Olfactory Placode), LP (Lens Placode), TP (Trigeminal Placode), OtP (Otic Placode), EP (Epibranchial Placode).

Conclusion

The induction of cranial placodes is a complex process and the progenitors initially are intermingled within the PPR. Several genes have been identified as PPR genes based on the expression pattern. Nevertheless, it is still unclear how various signaling cascades and competence factors cooperate to activate the transcription of placodal genes. Although CTFs have been shown to be sufficient to induce DPGs, the requirement of CTFs for DPGs has not been clearly demonstrated. Moreover, *Six* and *Eya* genes, referred as DPGs in different vertebrate species, not always show similar placodal deficiencies after their loss. Conversely, defective developmental processes shown by respective knockout studies suggest their role in placode specific differentiation and morphogenesis rather than in placodal induction.

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