Untersuchungen zur Frühentwicklung und Induktion neurogener Plakoden beim Krallenfrosch *Xenopus laevis*

Investigations concerning the early development and
induction of neurogenic placodes in the african clawed toad
Xenopus laevis

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Katja Ahrens

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1. Gutachter: Dr. Dr. Gerhard Schloßer
2. Gutachter: Prof. Dr. Dr. Gerhard Roth

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Vorwort

Plakoden sind embryonale Strukturen des Ektoderms, die aufgrund ihrer Lage im Embryo und der Zelltypen, die aus ihnen entstehen, weiter unterteilt werden können. So unterscheidet man in anteroposteriorer Reihenfolge folgende Plakoden: Adenohypophysen-, olfaktorische, Linsen-, Trigeminal- und Profundusplakode, otische Plakode und Seitenlinienplakoden, und Epibranchial- und Hypobranchialplakoden (reviewed in Webb und Noden, 1993; Baker und Bronner-Fraser, 2001). Aus diesen Plakoden gehen viele unterschiedliche Zelltypen, wie z.B. primäre und sekundäre Rezeptorzellen, Glia oder neurosekretorische Zellen hervor. Mit Ausnahme der Adenohypophysen- und Linsenplakode sind jedoch alle Plakoden neurogen, d.h. aus ihnen entstehen unter anderem Neuronen (reviewed in Baker und Bronner-Fraser, 2001). Plakoden sind daher nicht nur essentiell für die Bildung der meisten Sinnesorgane des Wirbeltierkopfes, sondern auch für deren Innervation. Im Gegensatz zur Neuralleiste, einer weiteren für die Entwicklung des peripheren Nervensystems wichtigen embryonalen Struktur, ist allerdings über die Entwicklung von Plakoden erst sehr wenig bekannt. Erst in letzter Zeit wird diesen Strukturen wieder mehr Beachtung geschenkt.

Es wird zum Teil noch immer kontrovers diskutiert, ob Plakoden separat voneinander induziert werden (Jacobson, 1963c; Jacobson and Sater, 1988) oder aber aus einer gemeinsamen Vorläuferregion (Wilson et al., 1997, Neave et al., 1997; Nguyen et al., 1998; Torres and Giraldez, 1998), einem panplakodalen Primordium, entstehen. Definitionsgemäß existiert ein panplakodales Primordium 1) wenn alle Plakoden aus einer gemeinsamen zusammenhängenden Region entstehen und 2) wenn diese Region bereits die Entwicklungstendenz hat, allgemeine plakodale Eigenschaften auszubilden, wie zum Beispiel die Fähigkeit, morphogenetische Bewegungen auszuführen oder Neuronen zu generieren. Inzwischen gibt es mehrere Hinweise darauf, dass ein solches panplakodales Primordium existiert. Zum einen werden bei Vertebraten Mitglieder der *Eya* und *Six* Genfamilien in einer Region um die Neuralplatte herum exprimiert und diese Expression setzt sich im Laufe der Entwicklung auch in den Plakoden und deren Abkömmlingen fort (Oliver et al., 1995; Esteve and Bovolenta, 1999; Pandur and Moody, 2000; Kobayashi et al., 2000; Ghanbari et al., 2001). Zum anderen weisen Mutanten in den entsprechenden Genen Fehlbildungen in der Plakodenentwicklung auf (Xu et al., 1999; Whitfield, 2002; Zheng et al., 2003; Li et al., 2003). Die Annahme einer gemeinsamen Vorläuferregion wird zusätzlich

durch Schicksalstudien unterstützt (Röhlich, 1931; Carpenter, 1937; Keller, 1975; Couly and Douarin, 1987, 1990; Eagleson and Harris, 1989; Eagleson et al., 1995; Baker and Bronner-Fraser, 2001).

Obwohl die Induktion einzelner Plakodentypen zum Teil recht gut untersucht ist (e.g. reviewed in Sheng und Westphal, 1999; Baker und Bronner-Fraser, 2001; Noramly und Grainger, 2002; Riley und Phillips, 2003), ist über die generische Induktion einer gemeinsamen Vorläuferregion nichts bekannt. Zur Induktion verschiedener ektodermaler Zelltypen wurden allerdings bereits einige Modelle vorgeschlagen (Albers et al., 1987; Neave et al., 1997; Nguyen et al., 1998; Marchant et al., 1998; Mayor und Aybar, 2001; Aybar und Mayor, 2002; Glavic et al., 2004). Ihnen gemeinsam ist die Grundannahme, dass die Induktion dieser unterschiedlichen Zelltypen gekoppelt ist. Diese Annahme gründet darauf, dass die diversen Zelltypen, wie Neuralplatte, Neuralleiste und Plakoden präzise in direkter Nachbarschaft voneinander angeordnet sind. Die Anwendbarkeit dieser verschiedenen Modelle auf die Entwicklung der Plakoden wurde allerdings noch nie überprüft. Die vorliegende Arbeit soll die Grundlage für eine solche Überprüfung legen, indem sie die Frühentwicklung und Induktion ektodermaler Plakoden untersucht. Um zunächst einen geeigneten Plakodenmarker zu finden, wurden in situ Hybridisierungen mit einigen vielversprechenden Genen durchgeführt und in Zusammenarbeit mit der Arbeitsgruppe um Prof. Dr. Doris Wedlich das panplakodale Markergen *Eya1* für *Xenopus laevis* kloniert und dessen Expressionsmuster beschrieben. Die Ergebnisse dieser notwendigen experimentellen Vorversuche, die sich auf die Expression unterschiedlicher plakodaler Markergene beziehen, sind bereits publiziert (David et al., 2001; Schlosser and Ahrens, 2004) und werden in der vorliegenden Arbeit in einem vorangestellten Kapitel (Part I: Molecular Anatomy of Placode Development in *Xenopus laevis*) kurz zusammengefasst.

Der Hauptteil der vorliegenden Arbeit (Part II: Tissues and Signals Involved in the Induction of Placodal *Six1* Expression) befasst sich mit den Ergebnissen einer Reihe von Transplantations- und Injektionsversuchen, die die Rolle sowohl von einzelnen Geweben als auch von verschiedenen Signalmolekülen bei der Induktion von Plakoden untersuchen sollten. Hierfür war es zunächst notwendig, mit Hilfe von Explantationen und Transplantationen das genaue Zeitfenster der Plakodeninduktion zu bestimmen. Anschließend wurden unterschiedliche Gewebetypen als mögliche Quelle plakodaler Induktoren untersucht. Die analysierten Gewebetypen liegen entweder in direkter Nachbarschaft zum panplakodalen Primordium (Neuralplatte, Endomesoderm) und sind daher als potentielle Quellen interes-

sant oder sie sind bereits als bedeutsam für die Induktion anderer ektodermaler Zelltypen bekannt (Organizer, Chordamesoderm, dorsal marginal zone, dorsal lateral marginal zone). Nach Bestimmung der für die plakodale Induktion notwendigen Gewebetypen, wurden Moleküle die diese induktive Aktivität vermitteln könnten (BMP (bone morphogenetic protein)-Inhibitoren, Fibroblastenwachstumsfaktoren), untersucht.

Die Ergebnisse dieser Arbeit erlauben schließlich, ein neues Modell zur Entstehung verschiedener ektodermaler Zelltypen vorzuschlagen.

Part I

Molecular Anatomy of Placode Development in *Xenopus laevis*

1. Identification of a panplacodal marker gene

When investigating the early development of ectodermal placodes, a necessary tool is a suitable marker gene. Such a specific marker gene has to meet two requirements. First, it has to be expressed in all neurogenic placodes and second it should not possess other ectodermal expression domains. At the beginning of this study no such gene was known in *Xenopus laevis*, so a first step had to be the characterization of a panplacodal marker gene in *Xenopus*. In zebrafish and amniotes, *Eya1* was known to be expressed in several domains amongst them many ectodermal placodes (Sahly et al., 1999; Xu et al., 1997; Abdelhak et al., 1997). For that reason, the expression pattern of *Eya1* in *Xenopus laevis* was investigated in cooperation with the lab of Prof. Dr. Doris Wedlich. *Eya1* is a transcription co-factor and belongs to the family of *Eya* genes, homologues of the *eyes absent* gene of *Drosophila*. *eyes absent* in *Drosophila* is part of a regulatory network, which is involved in the development of the eye (Bonini et al., 1993).

After two isoforms of the *Xenopus Eya1* gene were cloned in the lab of Dr. Doris Wedlich, I investigated the expression pattern by in situ hybridisation. The two isoforms differ in a 15 bp insertion at the N-terminus in the *Eya1-β* isoform, but their expression patterns are indistinguishable from each other. RT-PCR (performed at the lab of Dr. Doris Wedlich) reveals an expression of *Eya1* in *Xenopus* from stage 10 up to tadpole stages. In situ hybridisations show that *Eya1* is ectodermally expressed in a horseshoe shaped domain around the anterior neural plate at neural plate stages (lateral domain shown in Fig. 1 A, whole domain shown in Fig. 3 C). At neural fold stages, the continuous band of expression becomes separated in distinct parts. An anterior part, partly overlapping with the anterior neural folds will give rise to the adenohypophyseal and the olfactory placodes (ap in Fig. 1 B). Laterally the expression splits into two domains, one of which will give rise to the profundal and trigeminal placodes (pPrV in Fig. 1 B), whereas the other, posterior placodal domain most likely constitutes the precursor of the lateral line, otic and epibranchial placodes (pp in Fig. 1 B). During development, the expression domains further split into distinct domains corresponding to all neurogenic placodes (olfactory, profundus and trigeminal placodes, otic and lateral line placodes, epibranchial and hypobranchial placodes) and the adenohypophyseal placode (Fig. 1 C–E). As development proceeds, *Eya1* is expressed in placodal derivatives (Fig. 1 F). However, *Eya1* is neither expressed in the lens placode nor in the lens. With its expression in all neurogenic placodes and the adenohypophysis,

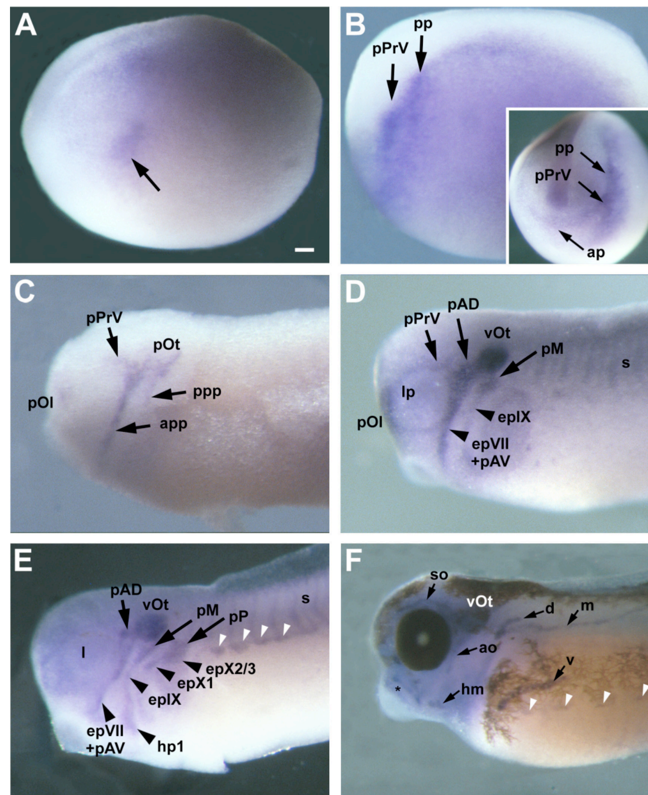

Fig. 1 Spatiotemporal expression of *Eya1* during *Xenopus* development (lateral views). **A** At neural plate stages (stage 14), an ectodermal domain (arrow) lateral to the neural folds expresses *Eya1*. **B** At neural fold stages (stage 18; insert: same specimen in oblique anterior view) this domain has separated into a profundal/trigeminal placodal area (pPrV) and a posterior placodal area (pp). The latter corresponds to the „dorsolateral placodal area" of Schlosser and Northcutt (2000), but has been assigned a more neutral label here, because *Eya1* expression suggests that it may be the precursor not only of lateral line and otic placodes but also of epibranchial placodes. *Eya1* also starts to be expressed in the anterior placodal area (ap) of the anterior neural folds, the precursor of adenohypophysis and olfactory placodes. **C** At early tailbud stages (stage 22), *Eya1* expression is maintained in the olfactory placodes (pOl), the developing adenohypophysis (not shown), and the profundal/trigeminal placodal area. Within the posterior placodal area of *Eya1* expression, it is now possible to distinguish the otic placode (pOt) and two ventral extensions. The anterior extension (app) is closely apposed to the profundal/trigeminal placodal area. It will give rise to the anterodorsal and anteroventral lateral line placodes and the facial epibranchial placode. The posterior extension (ppp) will broaden later and give rise to the remaining lateral line, epibranchial and hypobranchial placodes. **D** At mid tailbud stages (stage 26), *Eya1* continues to be expressed in the shrinking profundal and trigeminal placodes (pPrV), in the otic vesicle (vOt), in the anterodorsal (pAD) and anteroventral (pAV) lateral line placode, as well as in the facial epibranchial placode (epVII). The apparently contiguous *Eya1* expression domain anterior to the otic vesicle reflects the close apposition of these placodes (Schlosser and Northcutt, 2000). The middle (pM) lateral line placode and the developing glossopharyngeal (epIX) epibranchial placode but not the lens placode (lp) also express *Eya1*. Moreover, *Eya1* transcripts are now detectable in the somites (s). **E** At stage 30, *Eya1* is expressed in all neurogenic placodes, including the newly developed posterior lateral line placode (pP), vagal epibranchial placodes (epX1 and epX2/3), and hypobranchial placodes (hp1), in the somites and in the hypaxial muscle precursors (white arrowheads), but not in the lens (l). **F** At early tadpole stages (stage 41), *Eya1* expression persists in placodally derived structures such as the otic vesicle (vOt) and in the primordia of lateral lines derived from the anterodorsal (e.g., supraorbital line: so), anteroventral (e.g., hyomandibular line: hm, other lines: asterisk), middle (e.g., aortic lateral line: ao), and posterior lateral line placodes (dorsal, middle and ventral trunk lines: d, m, v). The *Eya1* positive hypaxial muscle precursors (white arrowheads) have migrated further ventrally. Bar in A: 0.1 mm (A-F).

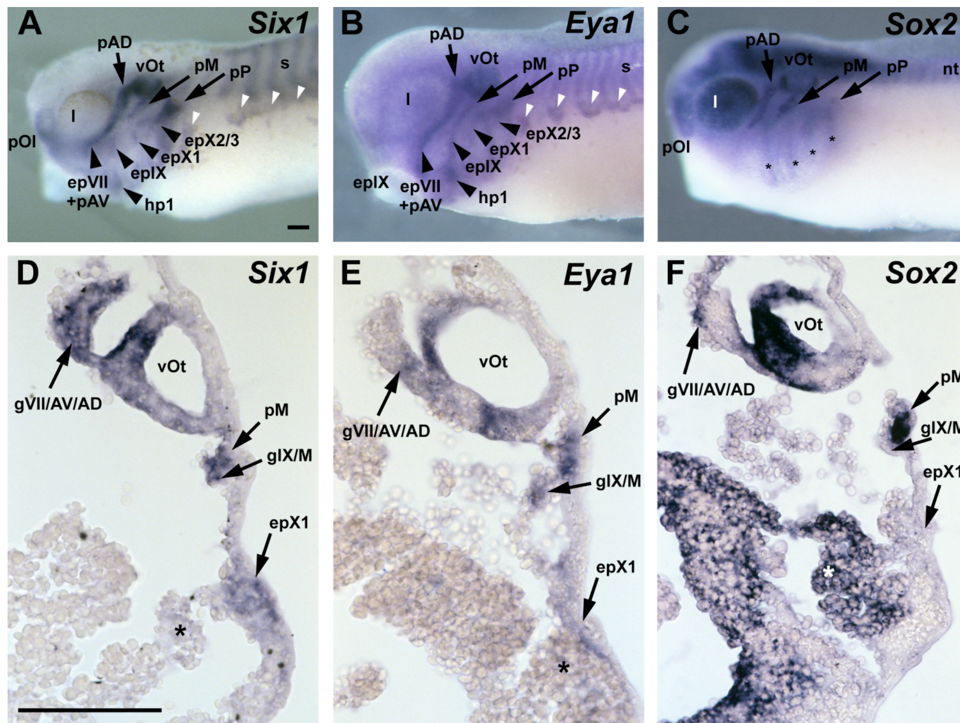

Fig. 2 Expression of *Eya1* (**B**,**E**) compared to other placodal markers *Six1* (**A**,**D**) and *Sox2* (**C**,**F**) in lateral views (**A**-**C**) and transverse sections at the level of the otic vesicle (**D**-**F**) of stage 30-34 *Xenopus* embryos. Patterns of expression of *Eya1* (**B**,**E**) and *Six1* (**A**,**D**) are largely identical in the adenohypophysis (not shown), olfactory placodes (pOl), the otic vesicle (vOt), lateral line placodes (pAV, pAD, pM, pP), epibranchial (epVII, epIX, epX1, epX2/3) and hypobranchial placodes (hp1). Coexpression of both genes is also observed in cranial ganglia, that have a placodally derived component, e.g., in the profundal-trigeminal ganglionic complex (not shown), in the fused ganglia of the facial, anteroventral and anterodorsal lateral line nerves (gVII/AV/AD in **D**,**E**), and in the fused ganglia of the glossopharyngeal and middle lateral line nerves (gIX/M in **D**,**E**). Additionally, *Eya1* and *Six1* are coexpressed in the somites (s in A,B), in hypaxial muscle precursors (white arrowheads in **A**,**B**) and weakly in the pharyngeal pouches (asterisks in **D**,**E**). Placodal expression of *Sox2* (**C**,**F**) overlaps with the expression of *Eya1* and *Six1* in the adenohypophysis (not shown), the olfactory placode, the otic vesicle and the lateral line placodes, as well as in some cranial ganglia (e.g., gVII/AV/AD in **F**). In contrast to *Eya1* and *Six1*, however, *Sox2* is not expressed in the profundal/trigeminal placodes or ganglia (not shown) and in the epibranchial placodes (**C**), whereas it is strongly expressed in the neural tube (nt), the lens (l), and the pharyngeal pouches (asterisks). Bar in A: 0.1 mm (A-C). Bar in D: 0.1 mm (D-F).

Eya1 fulfils the first demand for a suitable panplacodal marker gene. Moreover, *Eya1* has no other major ectodermal expression domains. Besides its expression in placodes, *Eya1* is only expressed in a few scattered cells in the brain and the retina, but has mesodermal expression domains in somites and hypaxial muscle precursors and endodermal expression domains in the pharyngeal pouches. The expression pattern of *Eya1* is more or less identical with the one described for *Six1* (Pandur and Moody, 2000; Ghanbari et al., 2001 and Fig. 2 A, B, D and E). Thus, *Eya1* as well as *Six1* are suitable marker genes for further investigations concerning the early development of ectodermal placodes and it is most likely that their expression in a horseshoe-shaped domain around the anterior neural plate at early neural plate stages demarcate a panplacodal primordium from which all placodes develop (see also Part II). However, this panplacodal domain is not a molecular homogenous tissue, as will be shown in the following sections.

The data presented in this section are already published in Mechanisms of Development (David et al., 2001).

2. Expression pattern of panplacodal markers at neural plate stages

At early neural plate stages, *Eya1*, *Six1* and additionally *Six4* are expressed in a horseshoe-shaped domain around the anterior neural plate (Figs. 3 A-C and 4 A-C). As discussed above, *Eya1* and *Six1* are expressed in all neurogenic placodes during subsequent development. The same is true for *Six4*. Besides the placodal defects seen in mutants and morphants for *Eya1* and *Six1* (see Part II), these expression patterns of *Eya* and *Six* genes argue for a panplacodal primordium, from which all placodes arise, which is demarcated at early neural plate stages by the expression of *Eya1*, *Six1* and *Six4*. Double in situ hybridisation and a combination of immunohistochemistry and in situ hybridisation were used to determine the precise position of this panplacodal primordium in relation to the neural plate and neural crest. Neural plate ectoderm was labelled with *Sox3* and neural crest with *FoxD3*. Double in situ hybridisations reveal that the *Six1* crescent is situated anterior to the neural plate domain of *Sox3* expression, but whereas anteriorly these two domains are in direct contact with each other, laterally there is a gap between the expression of *Six1* and *Sox3* (Fig. 5 E). This gap corresponds to *FoxD3* expressing neural crest cells (Fig. 5 D, F and H). In sagittal sections of embryos after wholemount in situ hybridisation, which were immunostained with a Sox3 antibody, it becomes visible that the dorsal expression domain of Sox3 expands anteriorly to the neural plate proper (Fig. 6 A), which is much more thickened, and that this anterior portion of dorsal Sox3 expression overlaps for a few cell diameter with the expression of *Six1* and *Eya1* (Fig. 6 D–I). In contrast, cross sections verify that laterally *Six1* expressing cells are separated from Sox3 expressing neural plate cells by *FoxD3* expressing neural crest cells (Fig. 7).

The data presented in this section are already published in Developmental Biology (Schlosser and Ahrens, 2004)

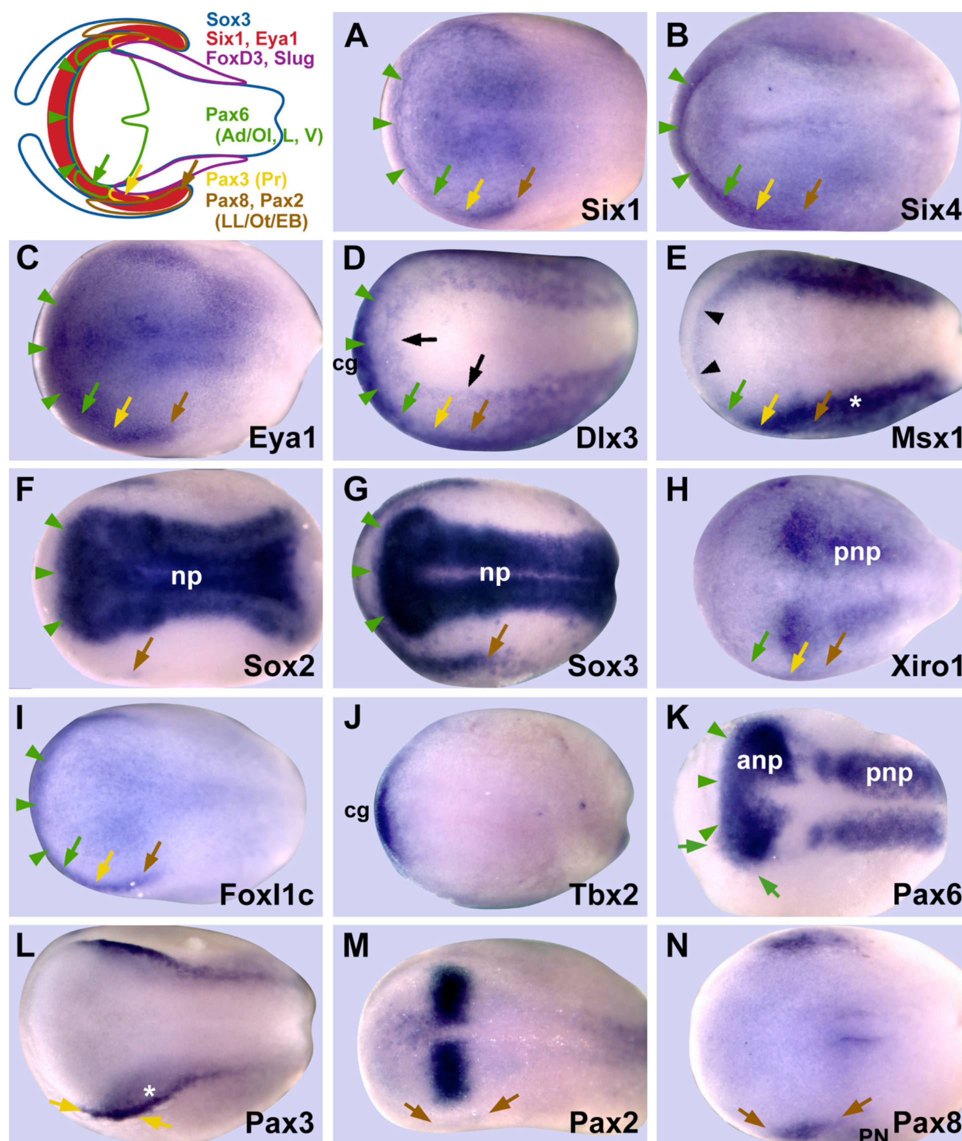

Fig. 3 Placodal gene expression patterns in neural plate stage *Xenopus* embryos (stages 13-14) in dorsal view (anterior is to the left, all embryos are equally oriented). To facilitate comparison of the placodal expression domains of different genes, color coded arrows and arrowheads corresponding to different placodal expression domains of *Pax* genes are used for the identification of comparable regions of gene expression (for more detailed labeling see Fig. 14). Colors are explained in the schematic drawing, which depicts the approximate relative position of some gene expression domains and lists the placodes to which they will give rise subsequently. Green arrowheads identify placodal gene expression immediately rostral to the anterior neural plate border (prospective adenohypophyseal placode medially and prospective olfactory placodes laterally), whereas green arrows identify placodal gene expression rostrolateral to the anterior neural plate (prospective lens and trigeminal placodes). Black arrows in **D** indicate the border of *Dlx3* expression in superficial ectoderm, which extends further medial than its border of expression in the deep ectodermal layer (colored arrows). Black arrowheads in **E** indicate *Msx1* expression in anterior neural plate. White asterisks in **E** and **L** indicate expression of *Msx1* and *Pax3*, respectively, in a region encompassing neural crest and lateralmost neural plate. For detailed description see text. Abbreviations: Ad/Ol: anterior placodal area, from which adenohypophyseal (Ad) and olfactory (Ol) placodes develop; anp: anterior neural plate; cg: cement gland; L: lens placode; LL/Ot/EB: posterior placodal area, from which lateral line (LL), otic (Ot) and epibranchial (EB) placodes develop; np: neural plate; PN: pronephros; Pr: profundal placode; pnp: posterior neural plate; V: trigeminal placode.

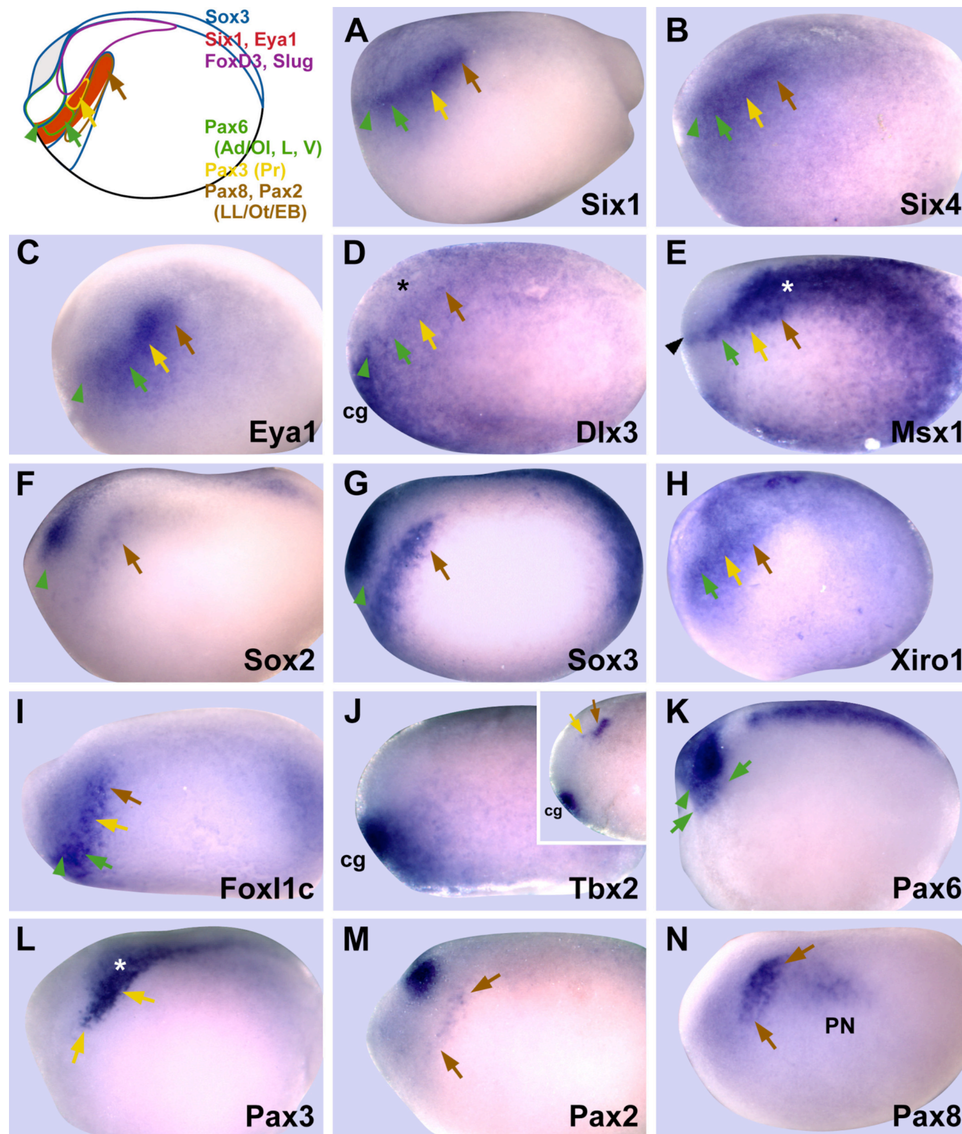

Fig. 4 Placodal gene expression patterns in neural plate stage *Xenopus* embryos (stages 13-14) in lateral view (anterior is to the left, all embryos are equally oriented). As in Fig. 3, color coded arrows and arrowheads are used for the identification of comparable regions of gene expression as indicated in the schematic drawing. Green arrowhead corresponds to the lateral green arrowhead in Fig. 3 (prospective olfactory placode). Black asterisk in **D** indicates region, where *Dlx3* is expressed only in the superficial ectodermal layer. Black arrowheads in **E** indicate *Msx1* expression in anterior neural plate.White asterisks in **E** and **L** indicate expression of *Msx1* and *Pax3*, respectively, in a region encompassing neural crest and lateralmost neural plate. Insert in **J**, shows incipient placodal *Tbx2* expression at a slightly later (neural fold) stage. For detailed description see text. For abbreviations see Fig. 3.

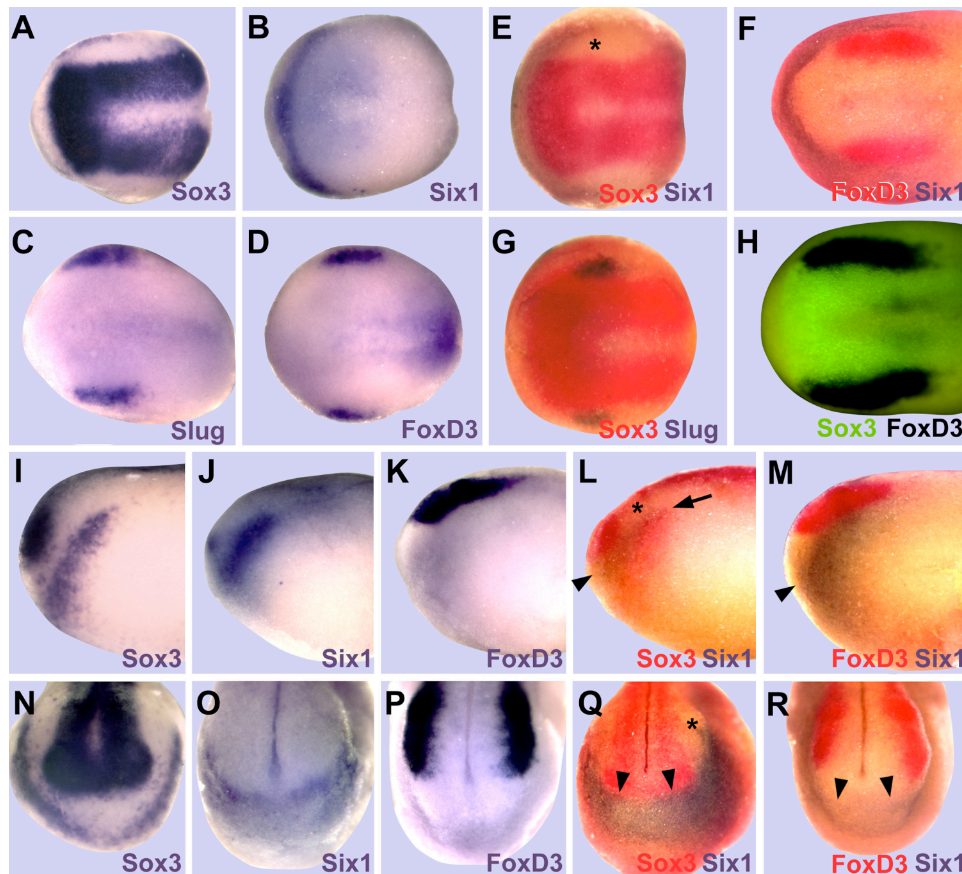

Fig. 5 Positioning of placodal *Six1* and *Eya1* expression domains relative to neural plate and neural crest as revealed by wholemount double staining procedures. **A**-**H** Dorsal views of neural plate stage *Xenopus* embryos (anterior is to the left). **I**-**R** Lateral (**I**-**M**, anterior to the left) and frontal (**N**-**R**) views of neural fold stage *Xenopus* embryos. Expression of *Sox3*, *Six1*, *Slug* and *FoxD3* is shown individually (**A**-**D**, **I**-**K**, **N**-**P**) and after double in situ hybridization (**E**-**G**, **L**-**M**, **Q**-**R**) or combination of in situ hybridization for *FoxD3* with *Sox3* immunostaining (**H**). The crescent of *Six1* expression is shown to be closely apposed to the neural plate domain of *Sox3* rostrally (**E**, **L**, **Q**) and to the *FoxD3*-positive neural crest domain, laterally (**F**, **M**, **R**). *Slug* and *FoxD3* expressing neural crest cells are located in the lateral outer neural folds adjacent to the *Sox3* positive neural plate (**C**, **D**, **G**, **H**). Asterisks in **E**, **L** and **Q** indicate a gap between *Sox3* and *Six1* expression, corresponding to this *Slug* and *FoxD3* positive neural crest domain. Arrowheads in **L**, **M** and **Q**,**R** emphasize position of rostral *Six1* expression, which is shown to be located between the neural plate domain and the lateral domain of *Sox3* expression. However, posteriorly, *Six1* expression overlaps broadly with the lateral domain of *Sox3* expression as indicated by the arrow in **L**.

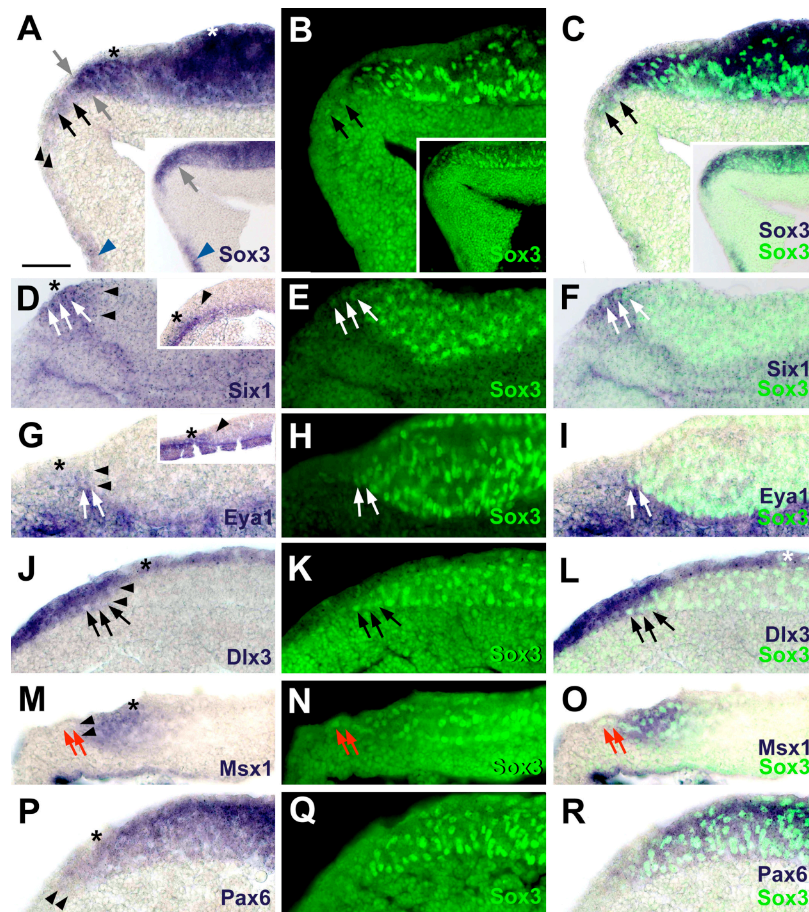

Fig. 6 Positioning of gene expression domains at the anterior neural plate border as revealed by double staining procedures in sagittal sections (anterior is to the left) of neural plate stage *Xenopus* embryos (stage 13-14). For each gene analyzed, a first panel depicts gene expression at the anterior neural plate border, a second panel shows the pattern of nuclear Sox3 immunostaining in the same section (apparent non-nuclear staining in the endoderm is an artifact due to autofluorescence of yolk granules), and a third panel presents a superposition of the first two panels. **A-C** Distribution of *Sox3* expression (**A**) and Sox3 immunostaining (**B**). The anterior border of the neural plate is indicated by grey arrows. The superficial ectodermal layer is indicated by asterisks. *Sox3* expression and immunostaining is found in the superficial (in the domain marked by a white asterisk) and deep ectodermal layers. The rostral limit of expression in the deep ectodermal layer (black arrowheads) extends beyond the neural plate border. Cells within this area of *Sox3* expression rostral to the neural plate border are also immunopositive for Sox3 (black arrows). However, no Sox3 immunopositive cells are yet discernible in the lateral crescent shaped domain of *Sox3* expression (identified by blue arrowheads in **A**) as is particularly evident in the inserts of **A**-**C** depicting sections of the same specimen at a more lateral level. **D-R** Comparison of the expression pattern of *Six1* (**D**-**F**), *Eya1* (**G**-**I**), *Dlx3* (**J**-**L**) *Msx1* (**M**-**O**) and *Pax6* (**P**-**R**) at the anterior neural plate border with Sox3 immunostaining. Inserts in **D** and **G** show *Six1* and *Eya1* expression patterns, respectively, in additional specimens. The borders of expression of each gene in the deep ectodermal layer are indicated by arrowheads. Doublelabeled cells, which show expression of the respective gene as well as Sox3 immunostaining are identified by black or white arrows. Because *Pax6* expression and Sox3 immunoreactivity are largely coextensive, doublelabeled cells are not specifically marked in **P-R**. Black asterisks indicate the superficial ectodermal layer (which is artifactually missing in **D**). White asterisks in **L** indicate cells in the superficial ectodermal layer that express *Dlx3* and are Sox3 immunopositive (posterior border of *Dlx3* expression is immediately to the right of panel). Red arrows in **M**-**O** indicate Sox3 immunopositive cells located rostral to the domain of *Msx1* expression in the anteriormost neural plate. Bar in A: 100μm (for all panels).

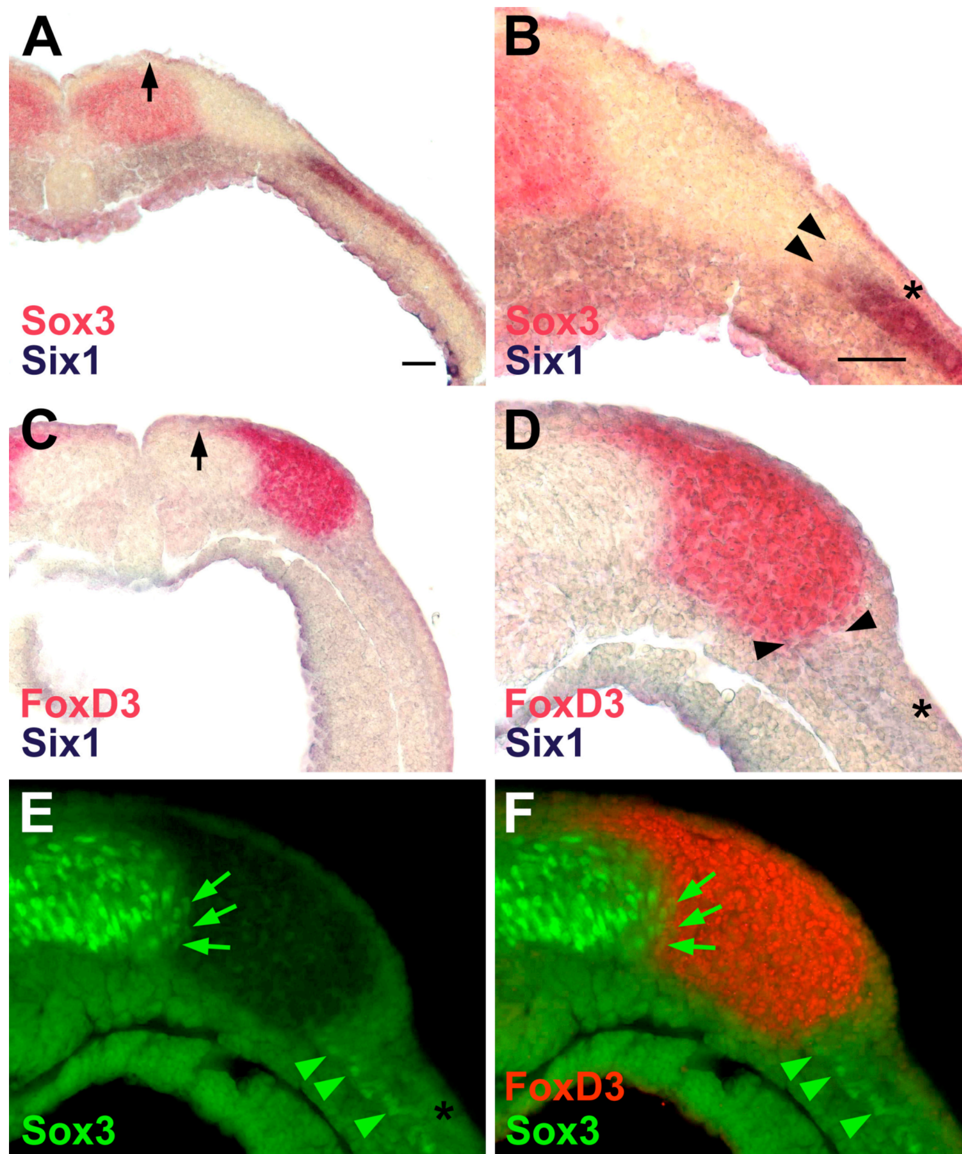

Fig. 7 Positioning of gene expression domains at the lateral neural plate border as revealed by double staining procedures in transverse sections through the cranial neural folds of neural fold stage *Xenopus* embryos (stage 16-17). **A**, **B** Double in situ hybridization reveals gap between the neural plate domain of *Sox3* expression and the lateral crescent of *Six1* expression. Arrow in **A** indicates tip of the neural folds. Arrowheads in **B** (representing higher magnification of **A** indicate medial border of *Six1* expression in the deep ectodermal layer (superficial ectodermal layer marked by asterisk). **C**-**F** Double in situ hybridization for *FoxD3* (red) and *Six1* (blue; medial border of expression marked by arrowheads in **D** combined with Sox3 immunohistochemistry (**E**) reveals the position of the *FoxD3* expressing neural crest relative to the Sox3 immunopositive neural plate and the lateral, placodal region, which expresses *Six1* and is immunoreactive for Sox3. Arrow in **C** indicates tip of the neural folds. Arrowheads in **D** (representing higher magnification of **C** indicate medial border of *Six1* expression in the deep ectodermal layer (superficial ectodermal layer marked by asterisk). Green arrows in **E** and **F** (showing a superposition of a fluorescent image of *FoxD3* expression with **E** indicate that Sox3 immunopositive nuclei of the lateral neural plate are located within the domain of *FoxD3* expression. Green arrowheads in **E**, **F** indicate Sox3 immunopositive nuclei in the deep ectodermal layer of the lateral, placodal domain, which also expresses *Six1* (compare with **D**), but does not show clear overlap with *FoxD3* expression. Bar in A : 100μm (for **A**, **C**). Bar in B: 100μm (for **B**, **D**-**F**).

3. Early distinction between different placodes by combinations of transcription factors

Expression patterns of several transcription factors within the panplacodal primordium suggest that it is already subdivided in parts differently biased for distinct types of placodes at neural plate or early neural fold stages. Besides *Six1*, *Six4* and *Eya1*, the transcription factor *FoxI1c* is also expressed in a crescent shaped domain around the anterior neural plate (Fig. 3 I), but in contrast to *Six* and *Eya* genes, *FoxI1c* becomes restricted to the profundal and trigeminal placodes and the lateral line and epibranchial placodes during further development (Fig. 4 I, Figs. 8-10 I).

Additional transcription factors, viz. *Dlx3* (Fig. 3 D) and *Msx1* (Fig. 3 E) are expressed in the panplacodal primordium, but their expression is not restricted to this region of ectoderm. *Dlx3* and *Msx1* are also widely expressed in the epidermis, however the dorsal boundary of *Dlx3* expression in the inner ectodermal layer coincides with the expression domain of *Six1*, *Six4* and *Eya1* at neural plate stages, although its expression in the outer ectodermal layer extends further medially (Fig. 4 D). Expression of *Dlx3* in the inner ectodermal layer shows a similar overlap with Sox3 expression as *Six1* and *Eya1* (Fig. 6 J-L). After neural fold closure, expression of *Dlx3* is partly downregulated and becomes restricted to the dorsal part of the otic vesicle and the olfactory placodes at late tailbud stages (Figs. 8–10 D). *Msx1* is expressed in the lateralmost neural plate and in prospective neural crest at neural plate stages (Figs. 3 E and 4 E). Its expression overlaps with the dorsal part of the crescent shaped panplacodal primordium laterally, whereas it is absent in anterior parts. Sagittal sections reveal that *Msx1* is expressed in the anteriormost neural plate, but in contrast to *Dlx3* is not expressed in the Sox3 positive domain anterior to the neural plate proper, which has an overlap with the *Six1* and *Eya1* domains (Fig. 6 M-O). Placodal *Msx1* expression persists in the prospective profundal and trigeminal placodes and the dorsal aspect of the posterior panplacodal area, giving rise to the otic placode and parts of the lateral line during further development (Fig. 8-10 E).

Sox2, *Sox3* and *Xiro1* are also expressed in a crescent shaped domain around the anterior neural plate in addition to a dorsal expression domain in the neural plate itself (Fig. 3 F-H). The crescent shaped expression domain outside the neural plate coincides with the expression domain of *Six1*, *Six4* and *Eya1* in posterior parts, but in anterior parts the expression of *Sox2*, *Sox3* and *Xiro1* is interrupted and lies ventral to the expression of *Six1*,

Six4 and *Eya1* (Fig. 4 F-H, Fig. 5 E, I-J, L, N-O). Expression of *Sox2* and *Sox3* in the dorsal domain does not coincide exactly with the anterior boundary of the neural plate but extends slightly further anteriorly. In agreement with that, during subsequent development *Sox2* and *Sox3* are expressed in the olfactory and adenohypophysial placodes (Figs. 8-10 F,G). In contrast, *Xiro1* is expressed in the profundal and trigeminal placodes at later stages (Figs. 8-10 H). All three transcription factors are expressed in the posterior placodal area, although from mid-tail bud stages on, they become restricted to dorsal parts of this area (Figs. 8-10 F-H).

Pax6 is also expressed in the anterior neural plate with an anterior boundary extending beyond the anterior border of the neural plate proper similar to that of the expression of *Sox2* and *Sox3* (Figs. 3 K and 4 K, Fig. 6 P-R, Fig. 11 A). Laterally the expression of *Pax6* also extends into placodal domains (Figs. 3 K and 4 K, Fig. 11 A-C). After neural fold closure, expression of *Pax6* is maintained in the adenohypophyseal, olfactory, lens and trigeminal placodes, but expression in the latter declines at late tailbud stages (Figs. 8-10 K).

Immediately posterior to the lateral expression domain of *Pax6* at neural plate stages lies the placodal expression domain of *Pax3* (Figs. 3 L and 4 L), most likely corresponding to the profundal placode, in which *Pax3* is expressed throughout development (Figs. 8-10 L, Fig. 11 D-F). Comparison of expression of *Pax3* and *Pax6* in the postorbital region suggests that these genes mediate a distinction between ectoderm biased to form the profundal placode from ectoderm biased to form the trigeminal placode. Additionally, *Pax3* is expressed in the most lateral neural plate and the prospective neural crest at neural plate stages (Figs. 3 L and 4 L).

In addition to an expression domain in the midbrain-hindbrain boundary, *Pax2* is placodally expressed in a region posterior and lateral to the profundal expression domain of *Pax3* (Figs. 3 M and 4 M). At neural plate stages, *Pax8* shows a placodal expression pattern that is very similar to that of *Pax2* (Figs. 3 N and 4 N). Its expression coincides with posterior parts of the panplacodal primordium which express *Six1*/*Eya1* as well as *Sox3* (Fig. 11 G-I). This domain corresponds to the posterior placodal area, which is visible as a distinct ectodermal thickening as described by Schlosser and Northcutt (2000) and from which otic as well as lateral line placodes and the epibranchial placodes appear to develop.

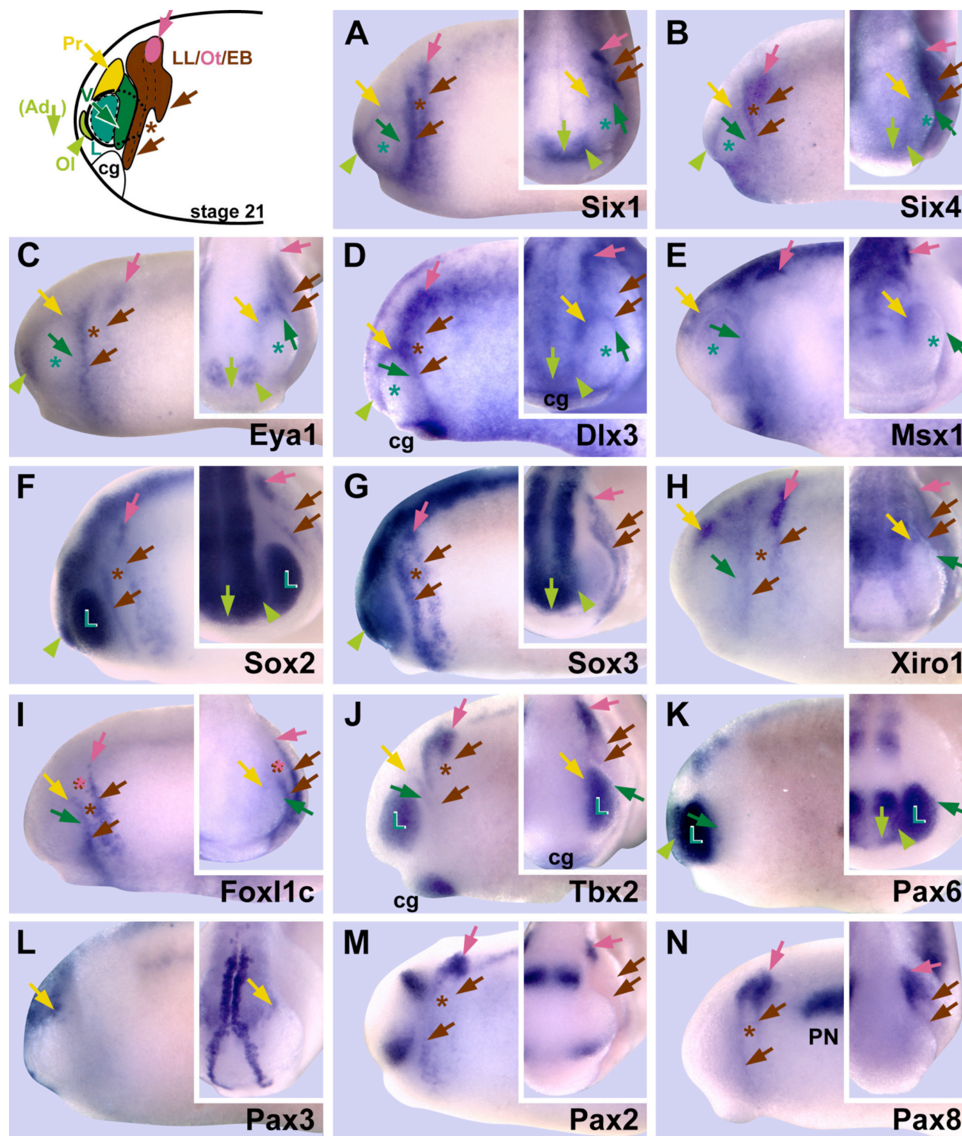

Fig. 8 Placodal gene expression patterns in early tailbud *Xenopus* embryos (stages 21-22) in lateral views (anterior is to the left) and right frontal views (inserts). Color coded arrows and arrowheads are used for the identification of comparable regions of gene expression. Expression in the prospective lens placode (which is not yet thickened at this stage) is indicated by L. Colors are explained in the schematic drawing, which shows the distribution of placodes in a stage 21 embryo (modified after Schlosser and Northcutt, 2000; see Fig. 15 for detailed explanation). The unpaired adenohypophyseal placode is located medial to the ventral part of the olfactory placode and is, thus, hidden behind the olfactory placode in this lateral perspective (indicated by light green arrow and Ad in brackets). Various shades of green identify placodes (including the prospective lens placode) expressing *Pax6*, yellow identifies the profundal placode expressing *Pax3*, and brown and pink jointly identify the posterior placodal area expressing *Pax2* and *Pax8*, with pink being reserved for the subregion, which will form the otic vesicle. Bluegreen asterisks indicate downregulation of the respective genes in the region of the prospective lens placode, whereas brown asterisks indicate downregulation of the respective genes in a region intervening between the anterior and posterior subregions of the posterior placodal area. The pink and brown asterisks in **I** indicate downregulation of *FoxI1c* expression in the dorsal part of the posterior placodal area. For additional abbreviations see Fig. 3.

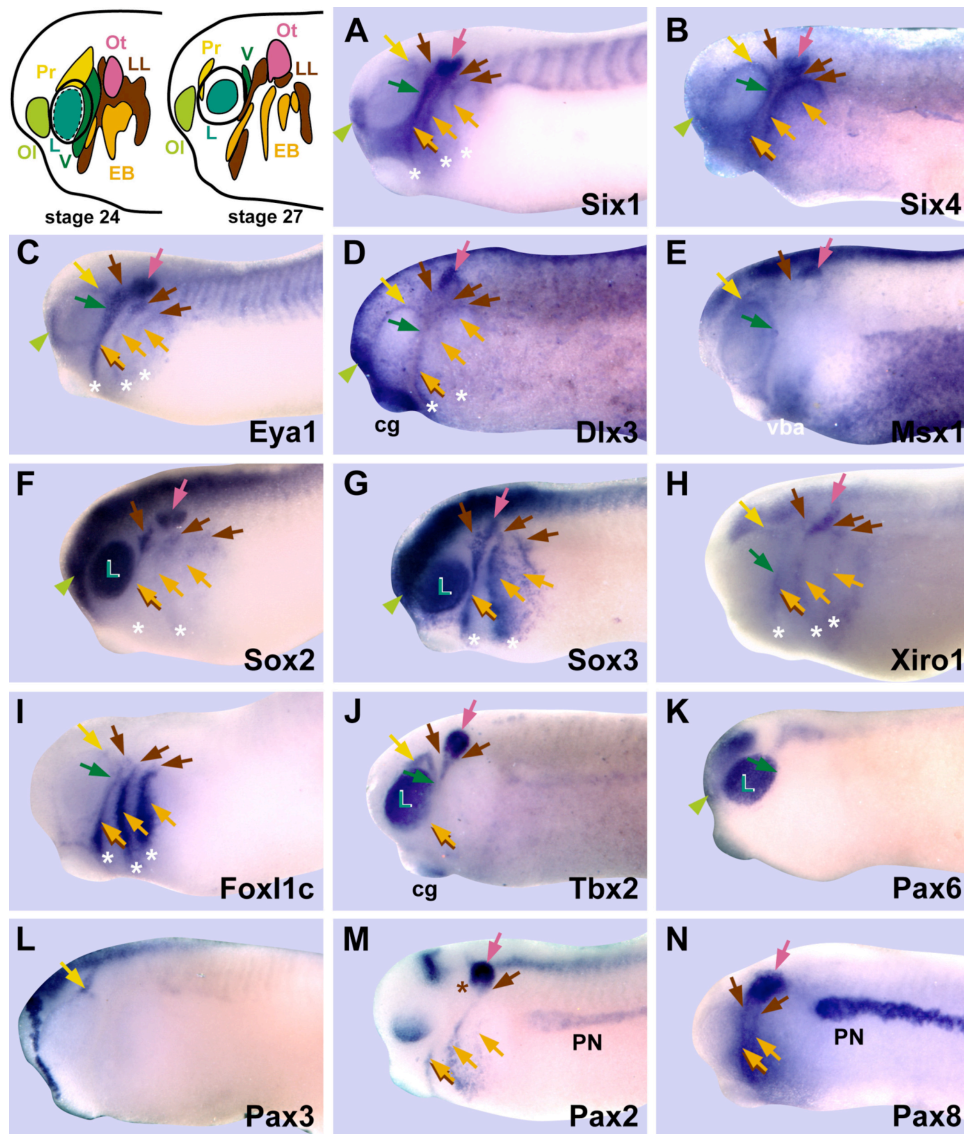

Fig. 9 Placodal gene expression patterns in mid tailbud *Xenopus* embryos (stages 24-27) in lateral views (anterior is to the left). As in Fig. 8 color coded arrows and arrowheads are used for the identification of comparable regions of gene expression. Expression in the lens placode is indicated by L. Colors are explained in the schematic drawings, which show the distribution of placodes in stage 24 and stage 27 embryos (modified after Schlosser and Northcutt, 2000; see Fig. 15 for detailed explanation). By stage 24 the posterior placodal area has extended caudally, the formation of the otic vesicle by invagination of the otic placode has begun and epibranchial placodes (orange) begin to become recognizable in its ventral part (Schlosser and Northcutt, 2000). By stage 27 the posterior placodal area has broken up into distinct placodes, while invagination of the otic vesicle is continuing (Schlosser and Northcutt, 2000). The lens placode has appeared as a focal thickening. The orange and brown double arrows indicate expression of the respective genes in a region encompassing facial epibranchial placode and anteroventral lateral line placode, because both placodes are closely apposed and, thus, cannot be differentiated from each other. White asterisks indicate gene expression in the pharyngeal pouches (which can be clearly distinguished from placodal expression domains in transverse sections). The brown asterisk in **M** indicates downregulation of *Pax2* in the region of the anterodorsal lateral line placode. For detailed description see text. Abbreviations: EB: epibranchial placodes; LL: lateral line placodes; Ot: otic placode or vesicle; vba: ventral branchial arch region (all three germ layers). For additional abbreviations see Fig. 3.

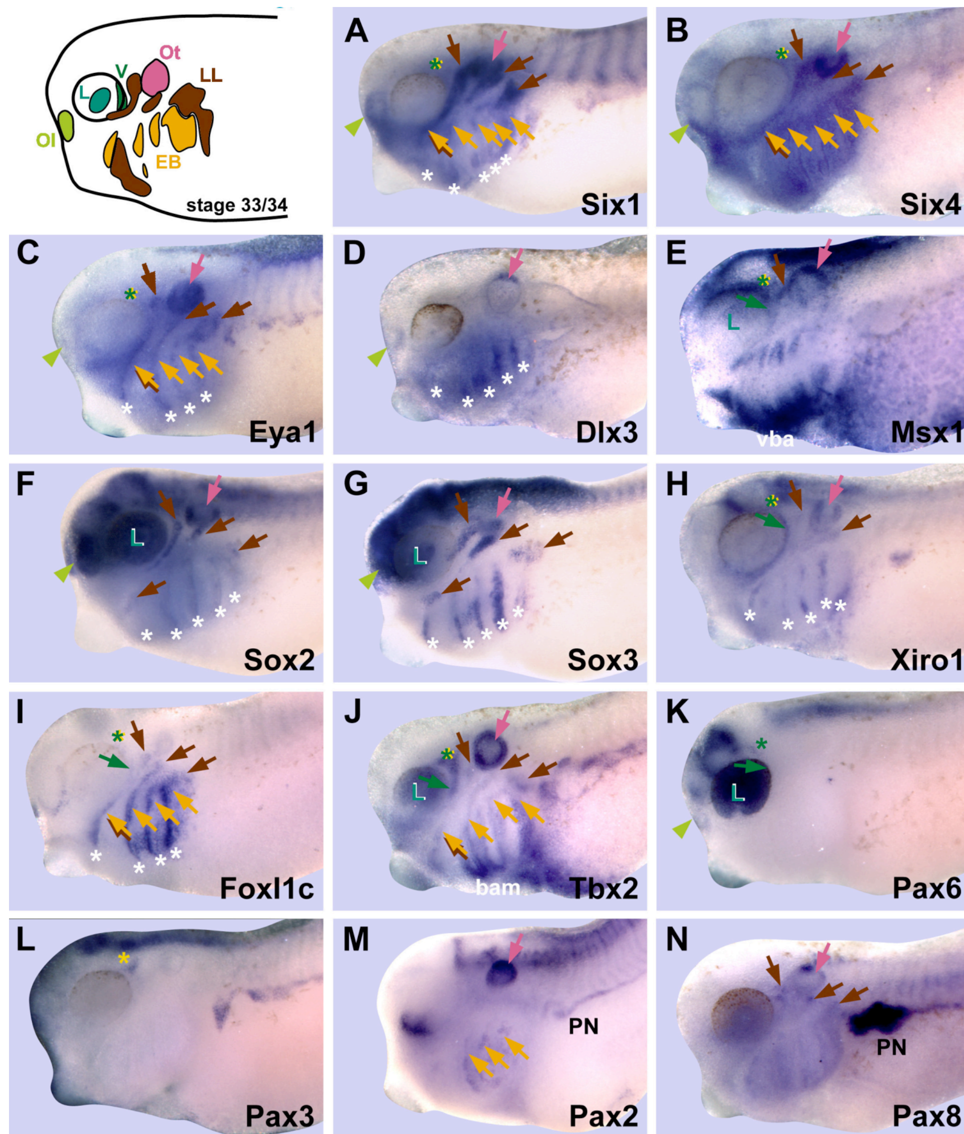

Fig. 10 Placodal gene expression patterns in late tailbud *Xenopus* embryos (stages 32-34) in lateral views (anterior is to the left). As in Fig. 8 color coded arrows and arrowheads are used for the identification of comparable regions of gene expression. Expression in the lens is indicated by L. Colors are explained in the schematic drawing, depicting the distribution of placodes in a stage 33/34 embryo (modified after Schlosser and Northcutt, 2000; see Fig. 15 for detailed explanation). By stage 33/34 the profundal placode has disappeared, the trigeminal placode is shrinking rapidly, lens placode and otic placode have completely invaginated to form the lens and the otic vesicle, respectively, and lateral line primordia have begun to extend from the lateral line placodes (Schlosser and Northcutt, 2000). Again, orange and brown double arrows indicate expression of the respective genes in a region encompassing facial epibranchial placode and anteroventral lateral line placode. White asterisks indicate gene expression in the pharyngeal pouches. Green and yellow asterisks indicate gene expression in the trigeminal and profundal ganglia (which are fused proximately), respectively. Abbreviations: bam: branchial arch mesenchyme; L: lens; Ot: otic vesicle; vba: ventral branchial arch region (all three germ layers). For additional abbreviations see Fig. 3.

From early tailbud stages on, *Pax2* expression begins to decline in lateral line and afterwards in epibranchial placodes, but is still strongly expressed in the dorsomedial part of the otic vesicle (Figs. 8-10 M), whereas *Pax8* expression begins to decline in epibranchial placodes and later on in lateral line placodes (Figs. 8-10 N). Thus, *Pax2* and *Pax8* seem to demarcate a region in the panplacodal primordium that corresponds to a common precursor of the otic, lateral line and epibranchial placodes.

Tbx2 expression is located in the cement gland anlage at early neural plate stages (Figs. 3 J and 4 J) but in early neural fold stages it is additionally expressed in a domain, which coincides with the dorsocaudal part of placodal *Pax3* and *Pax8* expression. During development, placodal expression of *Tbx2* further expands rostrally and encompasses the prospective lens placode, profundal and trigeminal placodes and the posterior placodal area (Figs. 8-10 J).

The presented data suggest that the panplacodal primordium, which is demarcated by the expression of *Six1*, *Six4* and *Eya1* (Figs. 3 A-C and 4 A-C, Fig. 12 C, D, F, G) is subdivided in regions biased to form specific types of placodes, demarcated by overlapping expression domains of several transcription factors (summarized in Figs. 13, 14 and 15). With the exception of *Pax3*, which specifically labels the profundal placode, diverse transcription factors are expressed in subsets of placodes. These observations are in agreement with a multistep model for the induction of placodes, which postulates that generic placodal properties may be promoted by *Six* and *Eya* genes, but that different types of placodes are specified via an induction of several transcription factors, which work in unique combinations to form a distinct type of placodes.

The data presented in this section are already published and discussed in more detail in Developmental Biology (Schlosser and Ahrens, 2004).

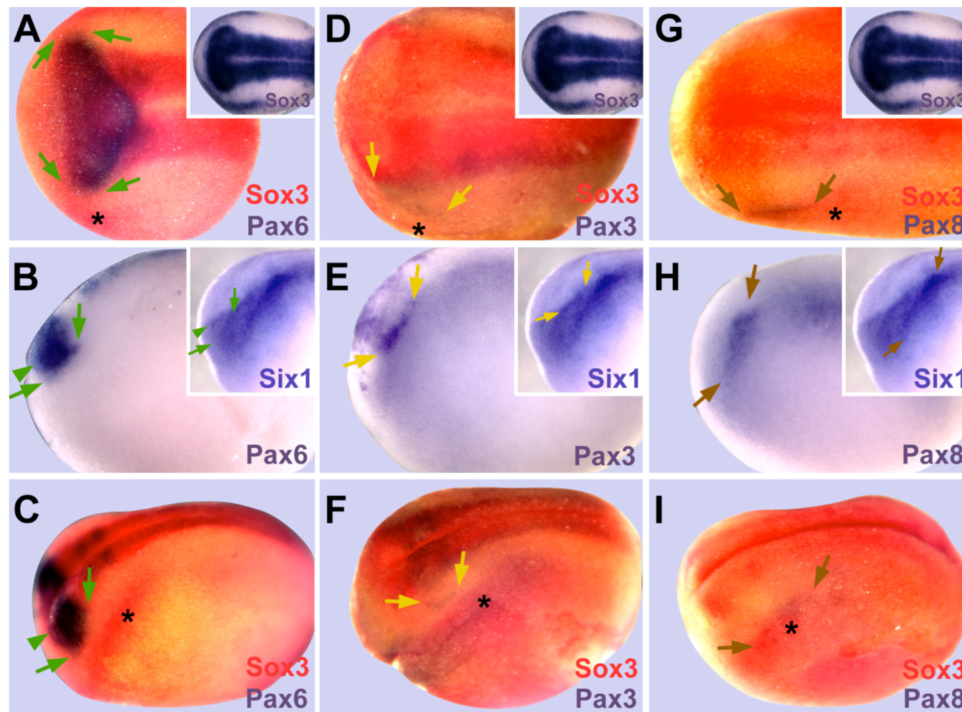

Fig. 11 Positioning of *Pax* gene expression domains relative to *Sox3* as revealed by wholemount double in situ hybridization. Dorsal views (**A**, **D**, **G**) of neural plate stage *Xenopus* embryos (stage 14; anterior to the left) and lateral (**B**, **E**, **H**) and oblique dorsolateral (**C**, **F**, **I**) views of neural fold stage *Xenopus* embryos (stage 16; anterior to the left) demonstrating the position of *Pax6* (**A-C**), *Pax3* (**D-F**), and *Pax8* (**G-I**) expression domains relative to *Sox3* expression. Inserts in **A**, **D**, and **G** show *Sox3* expression individually for clarification. Asterisks indicate lateral domain of *Sox3* expression. Arrows indicate anterior and posterior borders of placodal expression domains for *Pax6* (green arrows: prospective lens and trigeminal placodes, **A-C)**, *Pax3* (yellow arrows: prospective profundal placode, **D-F**), and *Pax8* (brown arrows: posterior placodal area, **G-I**). The green arrowhead in **A-C** indicates *Pax6* expression in the lateral part of the anterior placodal area (prospective olfactory placode). Inserts in **B**, **E**, and **H** identify corresponding subdomains of *Six1* expression.

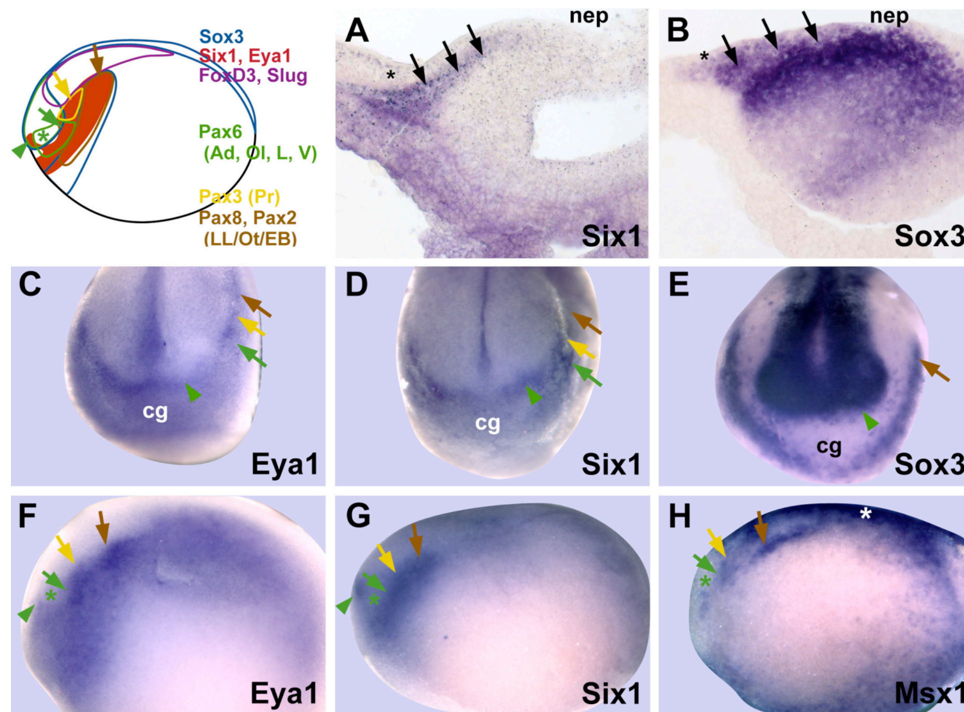

Fig. 12 Changes in placodal gene expression patterns during neurulation in *Xenopus* embryos (stages 16-18). As in Fig. 3, color coded arrows and arrowheads are used for the identification of comparable regions of gene expression indicated in the schematic drawing. Green arrowhead indicates prospective olfactory placode. The green asterisk indicates an anterior subregion of the lateral *Pax6* expressing domain, where panplacodal genes such as *Eya1* and *Six1* are downregulated during neurulation (note indentation in the red domain) and which will likely give rise to the lens placode. Green arrow indicates posterior subregion of the lateral *Pax6* expressing domain (prospective trigeminal placode). **A**, **B** Sagittal sections through the anterior neural folds reveal expression of *Six1* (**A**) and *Sox3* (**B**) in the deep ectodermal layer of the outer neural folds (black arrows). The outer ectodermal layer is indicated by black asterisks. **C**-**E** Frontal views of gene expression domains in neural fold stage *Xenopus* embryos. Note the separation of the anterior domain of *Eya1* and *Six1* expression (arrowheads) from the lateral domain (arrows) due to the elevation of the neural folds. **F**-**H** Lateral views of gene expression domains in neural fold stage *Xenopus* embryos. Note downregulation of *Eya1* (**F**), *Six1* (**G**) and *Msx1*(**H**) in a region, which will likely give rise to the lens placode (green asterisk). The white asterisk in **H** indicates expression of *Msx1* in a region encompassing neural crest and lateral-most neural plate. Abbreviations: cg: cement gland; nep: anterior neuropore. For additional abbreviations see Fig. 3.

Ad/Ol L V Pr LL/Ot/EB | Ad Ol L V Pr LL/Ot/EB | Ad Ol L V Pr LL Ot EB | Ad Ol L V Pr LL Ot EB | Ad Ol L V Pr LL Ot EB

st 13-14 | st 16-18 | st 20-22 | st 24-26 | st 32-34

Six1 ?

Six4 ?

Eya1 ?

Dlx3 ?

Msx1

Sox2 ?

Sox3 ?

Xiro1 ?

Foxl1c

Tbx2

Tbx3

Pax6 ?

Pax3

Pax2

Pax8

Ngnr1

NeuroD

Fig. 13 Summary of gene expression patterns in the placodal ectoderm during *Xenopus* development. Borders of prospective placodal areas are indicated with grey lines as soon as these areas are defined by expression of a distinct combination of transcription factors (although not evident from the genes analyzed here, the prospective adenohypophyseal placode can be distinguished molecularly from the prospective olfactory placode at neural fold stages due to its expression of *Pitx3*; see Pommereit et al., 2001). Borders are indicated by solid lines as soon as placodes become also morphologically recognizable (by ectodermal thickenings or interruption of basement membranes). While the lens placode is not thickened before stage 26, it is bounded by the thickenings of olfactory and trigeminal placodes from stage 20 on. At stage 32-34, when lens placode and otic placode have completely invaginated and the profundal and trigeminal placodes cease to contribute cells to the profundal and trigeminal ganglion, L, Ot, Pr, and V refer to the derivatives of these placodes, i.e., to lens, otic vesicle, profundal ganglion, and trigeminal ganglion, respectively. Faint colors indicate weak expression. In some cases, spatial restriction of gene expression to parts of a placode is indicated by corresponding partial coloring. In a few instances, gene expression in a particular placode at a particular stage could not be determined unequivocally (question marks). Except for stages 13-18, data for *Ngnr1* and *NeuroD* are taken from Schlosser and Northcutt (2000). Abbreviations: Ad/Ol: anterior placodal area, from which adenohypophyseal (Ad) and olfactory (Ol) placodes develop; L: lens placode; LL/Ot/EB: posterior placodal area, from which lateral line (LL), otic (Ot) and epibranchial (EB) placodes develop; Pr: profundal placode; V: trigeminal placode.

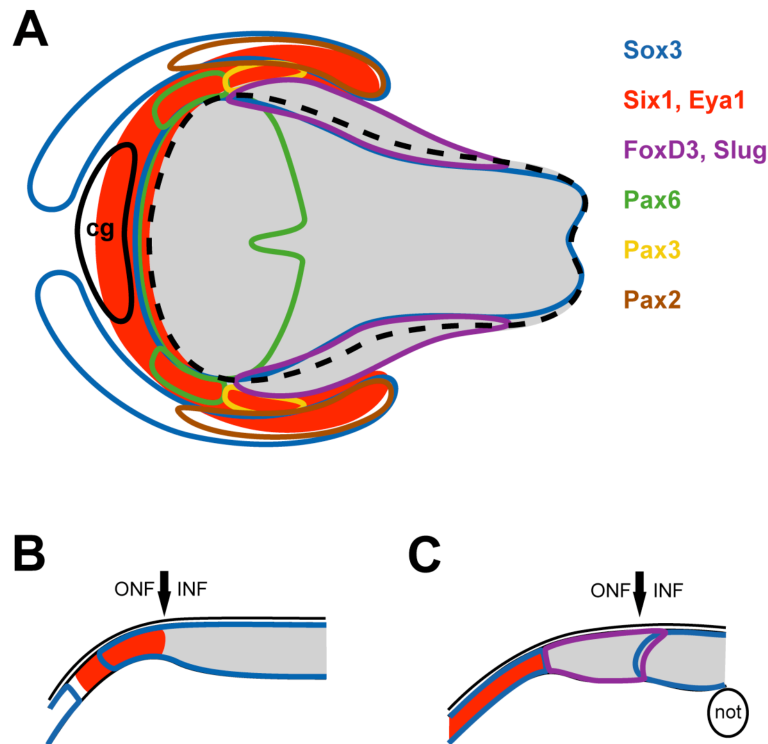

Fig. 14 Schematic drawing summarizing the relative position of ectodermal thickenings and gene expression domains around the *Xenopus* neural plate. **A** Dorsal view. **B** Sagittal section through anterior neural plate border. **C** Transverse section through lateral neural plate border in cranial region. Only expression domains in deep ectodermal layer are represented in **B** and **C**. Gene expression domains are shown in colored outlines. The position of the panplacodal primordium as defined by the expression of *Six1* and *Eya1* is shown in solid red. The grey area represents a region of prominently thickened ectoderm including the neural plate proper (border indicated by dashed black line in **A** and by arrows in **B** and **C**) and the neural crest arising from the *FoxD3* and *Slug* expressing region. The border of the neural plate proper (defined as the region of ectoderm contributing to the neural tube) is drawn laterally to the domain of *Sox3* expression, because previous studies (Bellefroid et al., 1998) indicate that the primary sensory cells of the dorsal neural tube arise from a region lateral to *Sox3* expression. The inner neural folds (INF) originate from neural plate ectoderm immediately centrally adjacent to the dashed black line, whereas the outer neural folds (ONF) arise from ectoderm immediately peripherally adjacent to the dashed black line. Thus, the ONF are located in a region of thin ectoderm, which expresses *Six1* and *Eya1* anteriorly (**B**), but in a region of thick ectoderm expressing *FoxD3* and *Slug*, laterally (**C**). Abbreviations: cg: region of prospective cement gland (arising from deep ectodermal layer; see Drysdale and Elinsons, 1992); not: notochord.

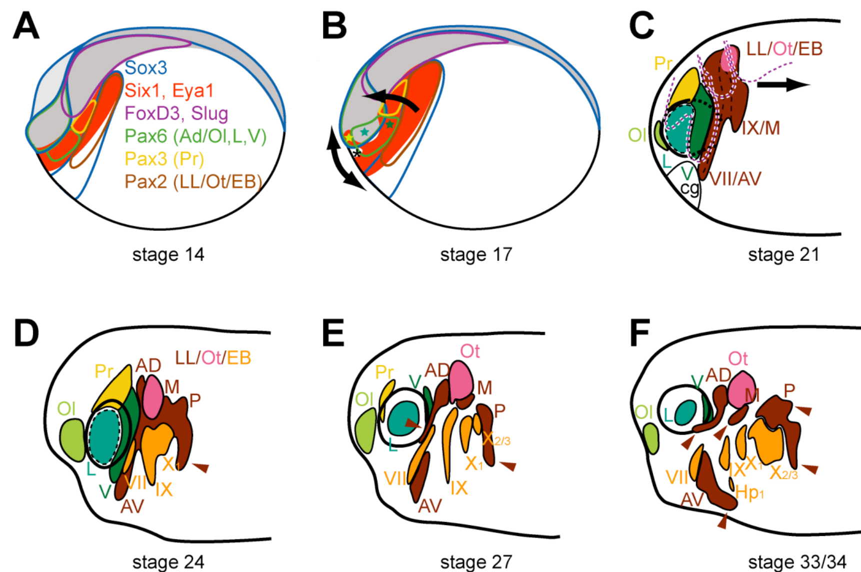

Fig. 15 Summary of placodal development in *Xenopus laevis* in lateral views. **A**,**B** Gene expression domains during neural plate (**A**) and neural fold stages (**B**) are shown in colored outlines. The position of the panplacodal primordium as defined by the expression of *Six1* and *Eya1* is shown in solid red. The grey area represents a region of prominently thickened ectoderm including the neural plate proper and the neural crest (see Fig. 14). Arrows in **B** indicate positional shifts of placodal expression domains due to neurulation (dorsal shift) and the wedge like expansion of the anterior ectoderm accompanying the formation of the optic vesicles. Green stars denote three areas of *Pax6* expression that will contribute to (from anterior to posterior) adenohypophyseal and olfactory placode (light green), lens placode (blue green) and trigeminal placode (dark green). Note downregulation of *Six1* and *Eya1* expression in the region of the prospective lens placode and the developing cement gland (black asterisk). **C**-**F** Development of placodes after neural tube closure until late tailbud stages. Drawings are based on reconstructions of ectodermal thickenings based on serial sections (modified after Schlosser and Northcutt, 2000). Only the adenohypophyseal placode, which is located medial to the ventral part of the olfactory placode is not shown in these lateral views.Various shades of green identify placodes (including the prospective lens placode) expressing *Pax6*, yellow identifies the profundal placode expressing *Pax3*, and brown, pink and orange jointly identify the posterior placodal area expressing *Pax2* and *Pax8*, with pink being reserved for the subregion forming the otic placode/vesicle and orange for the subregions forming the epibranchial placodes. For stages 21 and 24, 5 μm plastic sections described in Schlosser and Northcutt (2000) were reanalyzed in order to obtain a more precise mapping for the posterior placodal area. Moreover, additional information on gene expression was included in order to define the region of the prospective lens placode (not yet thickened; hatched outline) and to distinguish the regions of profundal and trigeminal placode. At stage 21, the posterior placodal area is divided into an anterior and a posterior subregion of thickened ectoderm, separated ventrally by an indentation and dorsally by a region of thinner ectoderm (between hatched black lines), while the region of the prospective otic placode (pink) is identifiable as a particularly prominent thickening. The violet dotted lines in **C** indicate the position of neural crest streams as reconstructed from serial sections (Schlosser and Northcutt, 2000). The arrow in **C** indicates the posterior extension of the posterior placodal area at early tailbud stages. Brown arrowheads in **D**-**F** indicate developing lateral line primordia. Abbreviations: Ad/Ol: anterior placodal area, from which adenohypophyseal (Ad) and olfactory placodes (Ol) develop; AV: anteroventral lateral line placode; cg: cement gland; Hp_1: first hypobranchial placode; L: prospective lens placode (hatched outline), lens placode or lens (invagination of placode between stage 27 and 33/34); LL/Ot/EB: posterior placodal area, from which lateral line (LL), otic (Ot) and epibranchial (EB) placodes develop; M: middle lateral line placode; Ol: olfactory placode; Ot: otic placode or vesicle (invagination of placode between stage 24 and 33/34); P: posterior lateral line placode; Pr: profundal placode; V: trigeminal placode; VII: facial epibranchial placode; IX: glossopharyngeal epibranchial placode; X_1: first vagal epibranchial placode; $X_{2/3}$: second and third vagal epibranchial placodes (fused).

Part II

Tissues and Signals Involved in the Induction of Placodal *Six1* Expression

1. Introduction

Placodes are specialized regions of the embryonic ectoderm that give rise to various non-epidermal cell types. Most placodes are visible as thickenings of the inner, i.e. sensory layer of the ectoderm, but this is not true for all placodes. Moreover, not all transient thickenings of the ectoderm are regarded as placodes. Thus, a more detailed definition of placodes would characterize them as regions of ectoderm that are "either thickened and/or display a disruption of the basement membrane and the formation of mesenchymal cells" (Schlosser and Northcutt, 2000).

Different types of placodes can be distinguished according to their location in the embryo and to the derivatives they give rise to (reviewed in Webb and Noden, 1993; Baker and Bronner-Fraser, 2001). Thus, in anteroposterior order one can distinguish the olfactory placodes, the adenohypophyseal and lens placodes, the profundal and trigeminal placodes, the otic, lateral line and epibranchial placodes, and - so far only reported for *Xenopus laevis* and *Eleutherodactylus coqui* - the hypobranchial placodes (Schlosser et al., 1999; Schlosser and Northcutt, 2000).

The OLFACTORY PLACODE forms at the most anterior part of the embryo and gives rise to the primary receptor cells of the olfactory and vomeronasal epithelia in addition to nonneural supporting cells (reviewed Farbman, 1994; Baker and Bronner-Fraser, 2001) and thus forms the olfactory, vomeronasal and terminal nerves (reviewed in Demski, 1993). Moreover, in contrast to all other types of placodes, the olfactory placode gives rise to glia cells, which form the myelin sheaths of the nerves it gives rise to (Couly and Le Douarin, 1985; Marin-Padilla and Amieva, 1989; Chuah and Au, 1991; Norgren et al., 1992; reviewed in Ramón-Cueto and Avila, 1998). Additionally, neurons secreting gonadotropin-releasing hormone, which migrate into the tel- and diencephalon, develop from the olfactory placode (Muske, 1993; reviewed in Baker and Bronner-Fraser, 2001.

The ADENOHYPOPHYSEAL PLACODE, located ventral to the diencephalon, forms Rathke´s pouch which gives rise to the adenohypophysis (reviewed in Treier and Rosenfeld, 1996; Watkins-Chow and Camper, 1998; Kioussi et al., 1999; Sheng and Westphal, 1999; Baker and Bronner-Fraser, 2001).

The LENS PLACODE develops into the lens of the eye (Ashery-Padan et al., 2000; Walter et al., 2004; reviewed in Baker and Bronner-Fraser, 2001).

The TRIGEMINAL AND PROFUNDAL PLACODES form dorsally and posteriorly from the eye and give rise to neurons that contribute to the respective ganglia of the Vth cranial nerve (Schlosser and Northcutt, 2000; reviewed in Baker and Bronner-Fraser, 2001).

LATERAL LINE PLACODES are unique to aquatic anamniotes and form receptor organs containing secondary sensory cells, i.e. neuromasts and electroreceptive ampullary organs in addition to sensory neurons of the lateral line ganglia innervating these receptor organs (Northcutt et al., 1994; Northcutt and Brändle, 1995; reviewed in Baker and Bronner-Fraser, 2001; Schlosser, 2002).

Closely related to the lateral line system is the OTIC PLACODE, which gives rise to the entire inner ear, including the hair cells, the neurons of the ganglion of the VIIIth cranial nerve innervating them, all supporting cells and the otoliths or otoconia (Fekete, 1999; Fekete and Wu, 2002; Fritzsch et al., 1999; Whitfield et al., 2002; Torres and Giraldez, 1998).

The EPIBRANCHIAL PLACODES, located dorsal to the pharyngeal pouches, give rise to neurons, which contribute to the ganglia of the facial (VIIth), glossopharyngeal (IXth) and vagal (Xth) nerves, respectively (Begbie et al., 1999; reviewed in Baker and Bronner-Fraser, 2001).

HYPOBRANCHIAL PLACODES, located ventral to the pharyngeal pouches in anurans, form neurons of the hypobranchial ganglia (Schlosser and Northcutt, 2000; Schlosser, 2003).

It has long been thought that placodes are exclusive to vertebrates because they are involved in the formation of the paired sense organs of vertebrates and the associated nerves, and thus, together with the neural crest form the basis for many evolutionary novelties of the vertebrate head (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Recently, it has been suggested that homologues of specific types of placodes (otic and adenohypophyseal placodes) also exist in non-vertebrate chordates (reviewed in Baker and Bronner-Fraser, 1997; Shimeld and Holland, 2000; Manni et al., 2001; Holland and Holland, 2001). It has been shown that several genes involved in the induction of placodes in vertebrates are expressed in comparable fashion in urochordates (Wada et al., 1998; Boorman and Shimeld, 2002). Additionally, cells of the ascidian neurohypophysial duct are able to undergo an epithelial-to-mesenchymal transformation like placodes (Manni et al., 1999). Thus, it has been suggested that specific placodes originated with the evolution of chordates (Wada et al., 1998; Manni et al., 1999, 2001; Shimeld and Holland, 2000; Graham and Begbie,

2000; Begbie and Graham, 2001; Boorman and Shimeld, 2002). Additional types of placodes may be subsequently elaborated from these types of placodes by the recruitment of additional signalling molecules and transcription factors (Schlosser and Ahrens, 2004).

The induction of some specific types of placodes is well investigated. In particular the induction of the olfactory, lens and otic placodes had already been the subject of many classical studies because they form structures, which are easily recognizable even without the availability of specific molecular markers. In the meantime, specific markers have been cloned for different kinds of placodes; and molecules and tissues involved in the induction of these placodes have been partly elucidated. For the induction of the olfactory placode, for example, the anterior mesendoderm has been shown to play an important role (reviewed in Baker and Bronner-Fraser, 2001; Jacobson, 1963b). Molecules, shown to be involved in the induction of the olfactory placode, include for example the transcription factors *Otx2* (Matsuo et al., 1995; Rhinn et al., 1998) and *Pax6* (Grindley et al., 1995**;** Quinn et al., 1996; reviewed in Baker and Bronner-Fraser, 2001). In contrast epibranchial placodes have been shown to be induced by the pharyngeal pouches mediated by the signalling molecule BMP7 (Begbie et al., 1999), whereas FGF signalling from the adjacent hindbrain is necessary for the induction of the otic placode (reviewed in Noramly and Grainger, 2002).

Because placodes give rise to a diverse array of cell types and different kinds of molecules and tissues have been shown to be involved in the induction of specific placodes, it has been suggested that placodes are not a coherent group of structures, but can rather be separated into functional sub-groups (Northcutt and Brändle, 1995; Graham and Begbie, 2000; Begbie and Graham, 2001). However, placodes also share some common developmental characteristics. On the one hand, all except the lens and adenohypophyseal placodes are neurogenic, i.e. they have neurons among their derivatives. On the other hand, placodes undergo cell shape changes, because they either invaginate (olfactory, adenohyphophysis, lens and otic placodes) or they give rise to delaminating cells (epibranchial, lateral line, otic, hypobranchial) (reviewed in Webb and Noden, 1993). Moreover, recently transcription factors of the *Six* and *Eya* family have been shown to be expressed in all neurogenic placodes at least until tailbud stages (Oliver et al., 1995; Esteve and Bovolenta, 1999; Pandur and Moody, 2000; Kobayashi et al., 2000; Ghanbari et al., 2001; David et al., 2001). In early neural plate stages these genes are expressed in a horseshoe-shaped domain around the anterior neural plate and it has been proposed that all placodes arise from this

region, which thus demarcates a panplacodal primordium (Baker and Bronner-Fraser, 2001; Schlosser and Ahrens, 2004). This proposal is also in agreement with existing fate mapping studies (Carpenter, 1937; Röhlich, 1931; Keller, 1975; Eagleson and Harris, 1989; Eagleson et al., 1995; Couly and Douarin, 1987; Couly and Douarin, 1990; reviewed in Baker and Bronner-Fraser, 2001). Moreover, *Six* and *Eya* mutants show defects in the development of placode derived structures, indicating that they play a functional role in the development of placodes. In *Six1* deficient mice the inner ear and the nasal cavity are malformed and the ganglia of the VIIIth and IXth cranial nerves are absent (Laclef et al., 2003**;** Zheng et al., 2003 Li et al., 2003; Ozaki et al., 2004). Similar results have been obtained in *Eya1* deficient mice and zebrafish (Xu et al., 1999; Whitfield et al., 2002). Also in *Xenopus*, *Eya1* plays a role in the development of neurogenic placodes as has been shown in our lab (Völker, 2003), because it influences the expression of the neuronal differentiation marker *NeuroD*.

It seems likely, that Six and Eya proteins are essential for developmental processes and properties that are common to all placodes, i.e. morphogenetic movements and neurogenesis. Thus, induction of placodes seems to involve a generic step of placode induction, during which panplacodal identity is induced and additional steps, during which the specific identity of a distinct type of placode is induced by the interplay of specific signalling molecules and transcription factors. In contrast to the induction of different specific types of placodes, not much is known about the generic induction of placodes. However, it has been proposed that the induction of placodes relies on a common mechanism shared with the induction of the neural plate and the neural crest, because these different ectodermal cell types are located at precise positions immediately adjacent to each other (Mayor, 1999).

The competence model suggests that the induction of different ectodermal cell types relies on the competence of the responding ectoderm (Albers, 1987; reviewed in Nieuwkoop and Albers, 1990). According to this model, different ectodermal cell types are induced by a common inductor, which spreads from dorsal to ventral through the embryo during development starting from the dorsal midline. Tissue that is in contact with this common inducer at early developmental stage will become neural plate, whereas older tissue has lost the competence to form neural plate and will become neural crest (or placodes) instead, whereas even older ectoderm will form epidermis, because it has lost the competence to either become neural plate or neural crest or placodes.

Another model proposed for the patterning of the ectoderm is the BMP (bone morphogenetic protein) gradient model, which suggests that different kinds of ectodermal cell types form in dependence of a common inducer, viz. BMP. According to this model, different threshold levels of BMP are responsible for the induction of the neural plate, the neural crest and placodes, respectively. At early stages, BMP is expressed ubiquitously in the embryo (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). A gradient of BMP is then suggested to be established during gastrulation by BMP inhibitors (noggin, chordin, follistatin) emanating from the organizer and its derivatives (Mayor et al., 1995; Knecht and Harland, 1997; Jones and Smith, 1998). According to the BMP gradient model, neural plate will form where BMP is strongly inhibited and epidermis develops in ventral parts of the embryo with a high amount of BMP, whereas intermediate levels of BMP are responsible for the formation of the neural crest and placodes (Mayor et al., 1995; Marchant et al., 1998; Glavic et al., 2004). Indeed, it could be shown, that neural crest can be induced in ectodermal explants by an intermediate concentration of BMP (Marchant et al., 1998). However, these models have never been tested for the induction of placodes.

In the present study, the generic induction of a common panplacodal primordium, i.e. an area of ectoderm biased for placodal fate, is investigated. Placodal bias was visualized by the expression of the transcription factor *Six1* as a panplacodal marker gene. As a basis for further experiments, the time course of placodal induction was first elucidated. Timing of specification and commitment, the presence of ectodermal competence as well as the presence of inducing signals for *Six1* expression was investigated using explantation and transplantation experiments.

In a next step, tissues involved in the induction of *Six1* were identified. Because of the location of the panplacodal primordium around the anterior neural plate, two candidate tissues were investigated. First, the neural plate, being localized immediately adjacent to the panplacodal domain, was tested for its sufficiency and necessity for the induction of *Six1* expression, using different kinds of transplantation and extirpation experiments. Moreover, the importance of the endomesoderm underlying the panplacodal primordium for the induction of *Six1* was investigated using grafting and extirpation experiments.

As it has been proposed for neural crest induction (Mayor, 1999) that the anterior neural plate may merely mimick the effects of the earlier acting organizer, the dorsal blastopo-

re lip and its derivative, viz. chordamesoderm were also tested for their ability to induce *Six1* expression by means of transplantation and conjugation experiments.

After identifying the anterior neural plate and the anterior endomesoderm as tissues inducing placodal *Six1* expression, further experiments were performed in order to elucidate the molecular nature of these inducing activities. A combination of micro-injections (RNA, DNA and morpholino antisense oligonucleotides), incubation assays, bead implantations and grafting experiments was used to investigate three kinds of candidate molecules.

Because the BMP gradient model suggests that placodes are induced by an intermediate BMP level, the inductive activity of the BMP inhibitor *noggin* was investigated. Furthermore, the necessity of BMP inhibition for the induction of placodal *Six1* expression was determined.

As a second candidate inducing molecule *FGF8* (fibroblast growth factor 8) was investigated. Fibroblast growth factors have been shown to be involved in several developmental processes, including gastrulation, limb bud formation, neural crest induction, development of the telencephalon and formation of the midbrain-hindbrain boundary (Nutt et al., 2001; Frazzetto et al., 2002; Crossley et al., 1996; Mizuseki et al., 1998; Sasai et al., 2001; Mayor et al., 1995; Monsoro-Burq et al., 2003; Eagleson and Dempewolf, 2002; Lupo et al., 2002; Dono, 2003). Because of its expression in the anterior neural plate (Christen and Slack, 1997), *FGF8* was investigated as a specific candidate molecule mediating the inductive acitivity of the anterior neural plate.

Finally, *Wnt8* (vertebrate homologue of *Drosophila wingless*) was investigated concerning its potential role in restricting the panplacodal domain to an anterior part of the embryo. *Wnt8* is expressed in *Xenopus laevis* embryos in posterior dorsal, lateral and ventral mesoderm (Smith and Harland, 1991). Villanueva et al. (2002) showed that neural crest markers could be induced by several posteriorizing factors including Wnts in anterior neural plate border regions where normally no neural crest would form. While such posteriorizing molecules seem to be required for restricting neural crest to more posterior regions of the neural plate border, they may negatively regulate placodal markers, thus restricting them to rostral parts of the neural plate border.

The results obtained in this study are not compatible with the currently favoured BMP gradient model for the induction of different kinds of ectodermal cell types and allow me to propose a new model for the induction of placodes, which explains the precise registration of the panplacodal primordium with the neural crest and the neural plate by a different mechanism.

2. Materials and Methods

2.1. Animals and in vitro fertilization

This study was carried out on embryos of the African clawed toad *Xenopus laevis* (Amphibia, Anura, Pipidae). *Xenopus laevis* is native to sub-Sahara Africa and thrives in temperatures from 15° to 27°C (www.anapsid.org/xenopus.html). It can mostly be found in calm stagnant water like grassland ponds but also populates rapidly flowing streams. *Xenopus laevis* is a fully aquatic frog but comes to surface to breathe (Nieuwkoop and Faber, 1967; Kaplan, 1995).

For this study, adult female animals were kept in pairs in aquariums (20 l) covered with dark pasteboard. Adult male frogs were kept in dark plastic basins (100 l) in groups of up to eight individuals. All animals were kept in a climatic chamber at a temperature from 19 – 21°C, with a light/dark cycle of 12/12 hours. The animals were fed twice a week with bovine heart muscle meat supplied with a multivitamin preparation (Multimulsin N, Mucos). The day after feeding water was changed using stale water.

Xenopus laevis is a well established model organism especially for developmental studies. Its embryos are relatively large and can therefore easily be manipulated. *Xenopus laevis* normally does not mate in captivity but can be induced to egg laying by hormon injections. This is advantageous because experiments can be timed. Six days before desired egg laying, female frogs were primed with an injection of 50 units of human chorionic gonadotropin (Sigma) into the dorsal lymph sac. The evening before egg laying, pigmented frogs were injected 750 units and albino frogs 500 units of human chorionic gonadotropin due to size differences. Animals were kept at 18°C to prevent early onset of egg laying overnight. To induce egg laying the next day, each female frog was transferred to a single plastic basin (3 l) filled with a high salt solution, 1xMBSH (100 ml 10xMBSsalts, 7 ml 0,1 M $CaCl_2$, 4 ml 5 M NaCl, 889 ml H_2O bidest; 10xMBSsalts (Modified Barth´s Saline salts): 51,3 g NaCl, 0,75 g KCl, 2 g $MgSO_4x7H_2O$, 23,8 g HEPES, 2 g $NaHCO_3$ ad 1 l, pH 7,8). Eggs were collected in Petri dishes at least every hour.

Female frogs were used for egg laying at most every two months to give them time to recover.

To gain testes, male frogs were anesthetized in 2% MS 222 (tricaine methanesulfonate, Sigma) for 20 minutes. The frog was layed on its back and the skin of the belly

was folded back. Two slits were taken at each side of the ventral midline. The yellowish fat body to which the testes are attached was pulled out using blunt forceps. The testes were separated from the fat body and stored in 1xMBSH at 4°C during the day.

For in vitro fertilization, the medium was sucked off and every egg was touched with a smashed piece of testes. The eggs were floated with 0,1x MBS (1xMBS: 100 ml 10xMBSsalts, 7 ml 0,1M $CaCl_2$, 893 ml H_2O bidest). The sperms were now motile and the eggs were fertilized. 20 minutes past fertilization the eggs were dejellied by 2% cysteine in tap water (pH 8,0). After several washes, the fertilized and dejellied eggs were allowed to develop in 0,1x MBS at 14°C. During cysteine treatment and washing steps, the eggs were shaken very gently to avoid disturbing gradients of maternal mRNAs, which may lead to secondary axis formation.

Embryos were staged according to Nieuwkoop and Faber (1967).

2.2. Injections

In order to manipulate embryonic development or to differentiate between host and donor tissue, different kinds of mRNA or DNA were injected into the embryo. These injections were performed using a nanoliter injector (WPI) and selfmade micropipettes (tipdiameter 10 - 15μm) that were drawn from glass capillaries (3,5 nl, WPI) at a horizontal puller (Sutter Instrument, Model P87, two cycles: 1. cycle: heat 606, pull 10, velocity 20, time 120; 2. cycle: heat 583, pull 10, velocity 30, time 200). For injection, the embryos were transferred to a self-made injection dish. To prepare this injection dish, a Petri dish was filled with 4% hot agar and a piece of table tennis racket coating was laid on top of it. After hardening, the piece of coating was removed. The surface of the agar was now covered with tiny holes. For injection each embryo was placed in such a hole and the medium was sucked off. Volume of the injections was always 5 nl. Injections were carried out under a stereomicroscope using a micromanipulator. Afterwards the embryos were allowed to heal for several hours in 5% Ficoll (Sigma) in 0,1x MBS at 14°C. At blastula stages the embryos were washed and transferred into 0,1x MBS, as Ficoll may interfere with normal gastrulation and lead to exogastrulation defects.

2.2.1. mRNA and DNA

Candidate molecules were tested concerning their ability to induce ectopic *Six1* expression and their necessity for the formation of the normal panplacodal *Six1* expression. For that reason, mRNA of the respective proteins was injected into the embryos. DNA in form of plasmid DNA was sometimes injected instead of mRNA to delay its proteinsynthesis, thus avoiding earlier effects on embryonic development. In embryos, which served as donors for tissue grafts, for a better distribution, the mRNA or DNA was injected into the animal part of each of four blastomeres at the four cell stage or into corresponding regions at the two cell stage of the embryo. Concentration of the injected *BMP4*, *noggin*, *FGF8* and *dnFGFR4* mRNA was 50ng/μl, volume was 5 nl. These concentrations were also used in co-injections of *FGF8* and *noggin* mRNA and co-injections of *FGF8* and *dnFGFR4*, as well as of *FGF8* and *BMP4*. mRNA of a dominant negative form of the BMP receptor (*dnBMPR-IA*) was injected into each of four blastomeres at the four cell stage at a concentration of 100ng/μl, volume 5 nl.

In order to overexpress *FGF8* or *dnFGFR4*, mRNA or DNA was injected unilaterally at the two cell stage. Different amounts of mRNA (*FGF8*: 250 pg, 31,25 pg; *dnFGFR4*: 1ng, 125 pg) and DNA (*FGF8*: 25 pg; *dnFGFR4*: 50 pg) were injected.

To test whether Wnt proteins play a role in restricting the panplacodal *Six1* expression domain to the anterior part of the embryo, DNA of a dominant negative form of Wnt8, dnWnt8, was coninjected with *noggin* or *FGF8* mRNA alone or with a combination of *noggin* and *FGF8* mRNA, respectively. dnWnt8 DNA was injected into each of four blastomeres at the four cell stage at a concentration of 5ng/μl, volume 5nl.

2.2.2. Morpholinos

In the living embryo, translation of a certain transcript can be inhibited by injecting morpholino antisense oligonucleotides (morpholinos). Morpholinos consist of four different subunits with the genetic bases Adenine, Cytosine, Guanine and Thymine, respectively, attached to a 6-membered morpholine ring. This morpholine ring replaces the ribose or desoxyribose of RNA or DNA nucleotides. 18-25 subunits form a morpholino being linked by non-ionic phosphorodiamidate intersubunits (www.gene-tools.com). Morpholinos bind

to complementary mRNA, thereby blocking translation of a gene. The advantages in comparison to RNA or DNA antisense oligonucleotides are mainly their stability against nucleases. As morpholinos are uncharged, they are not recognized by cellular nucleases. Thus, the antisense effects are stable for a much longer time period and can be studied over a long time of embryonic development. Morpholinos, in addition, are very specific and they bind to their antisense target mRNA very effectively.

Morpholinos complementary to a specific subsequence of a gene can be ordered from GeneTools. To block translation of a gene effectively, the sequence of a morpholino must be complementary to a region of the spliced mRNA between the 5`terminus and about 25 bp towards the 3´terminus of the AUG start codon. The FGF8 morpholino for this study was designed to bind to the sequence of *Xenopus FGF8* published in GenBank, Accession Number Y10312. The sequence of the FGF8 morpholino and the region it binds to in the *FGF8* gene sequence is shown in Figure 1. Specifity of the morpholino for *FGF8* in *Xenopus laevis* was confirmed by BLAST search.

FGF8 morpholino was injected into one blastomere at the two cell stage at a concentration of 500 μM, volume 5 nl. To distinguish between experimental and control side, *lacZ* was coinjected with the morpholino.

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5′ ccaggatggaggtgatgtagttcat 3′
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B

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 01 atgaactaca tcacctccat cctgggctat ctgttactgc acctgtttgt catctgccta
 61 caagcccagc atgtgaggga gcagagcctg gtgaccgacc aactaagccg acggctcatc
121 cgaacctacc agttgtacag ccggaccagc ggcaagcatg tgcaaatcct ggcgaacaag
181 aagattaacg ccatggcaga agacggcgac ccacacgcca agttaatcgt ggaaacagat
241 acgtttggaa gcagagttcg cattaaaggt gcggagactg gttactacat ctgcatgaac
301 aaaaaaggga agctgattgg gaagactaat ggaaggggca aagactgcgt cttctcggaa
361 attgtccttg aaaacaacta cacagctctg cagaatgtca agtacgaagg ctggtttatg
421 gctttcacaa gaaggggtcg cccaaggaaa gggtcgaaga caaggcaaca tcaaagagaa
481 gtccacttca tgaagaggtt gccaaaggga caccacacca cagaacctca taaacgtttt
541 gagtttatta attacccttt caatagaaga agtaaaagaa ctcgatattc aagttctcgg
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Fig. 1 A Sequence of the FGF8 antisense morpholino oligonucleotide used in this study. **B** Bases the FGF8 morpholino binds to (underlined) in the FGF8 gene of *Xenopus laevis* as confirmed via BLAST alignment.

2.2.3. Lineage tracer

To distinguish between host and donor tissue, mRNA for green fluorescent protein (GFP) was injected in both blastomeres at the two cell stage at a concentration of 50 ng/μl, volume 5 nl. In living embryos, GFP is clearly visible during development as a bright green colour under a fluorescence stereomicroscope equipped with a GFP filter set. However, in fixed embryos GFP is no longer visible. The GFP used in this study possessed a c-myc tag that could be detected by immunohistochemistry in fixed embryos after in situ hybridisation.

To distinguish between experimental and control side of manipulated embryos in case of morpholino or mRNA injection, *lacZ* mRNA was coinjected as a lineage tracer at a concentration of 50 ng/μl, volume 5 nl. *LacZ* encodes the bacterial enzyme β-galactosidase. After injection, the manipulated side of the embryo can be easily recognized by histochemical reactions involving the enzymatic activity of β-galactosidase (see 2.5 below).

2.3. Grafting Procedure

Transplantations were performed under a stereomicroscope in a plasticin covered Petri dish filled with 1xMBSH supplemented with 2 mM $CaCl_2$ and 400 mg/l gentamycine (Sigma). The vitelline membrane of the embryos was carefully removed with watchmaker forceps. Embryos were positioned in small grooves and fixed with plasticine. Pieces of tissue were cut out using flamesharpened tungsten needles and replaced by donor tissue. Pigmented embryos served as donors and albino embryos served as hosts. The grafts were held in position by a piece of glass coverslip and were allowed to heal in for one to two hours. Afterwards, the embryos were transferred to 0,1xMBS containing 25 mg/l gentamycine, 400 mg/l penicillin (Sigma) and 400 mg/l streptomycine sulfate (Sigma). The embryos were allowed to survive up to various stages, fixed in 4% paraformaldehyde in 0,1 M phosphate buffer (PB: 28,48 g $Na_2HPO_4x2H_2O$, 5,44 g KH_2PO_4 ad 2 l, pH 7,38) overnight, washed three times with 0,1 M PB and stored in 70% ethanol at –20°C.

Embryos of *Xenopus laevis* were relatively easy to manipulate. Nevertheless, success of transplantation was strongly dependent on the quality of the eggs. Embryos of poor quality sometimes already died during the healing phase of the experimental procedure,

although they were treated with antibiotics. In contrast, embryos of good quality survived transplantation or explantation of large pieces of tissue. Even after removing almost a quarter of the covering ectoderm, embryos survived and developed perfectly. Grafts healed in within 20 minutes. However, ease of experimental handling and healing was also dependent on the age of the manipulated embryos. Younger stages (neural plate stages) were easier to manipulate than older stages (neural fold stages), because the different germ layers were easier to separate. In older stages these germ layers tended to stick together and it was harder to explant only the desired germ layer without inadvertently removing cells belonging to other germ layers. Additionally, older embryos were not as tolerant as younger ones in accepting transplants. Healing sometimes took up to two hours or transplants did not heal in at all. Only embryos with perfectly integrated transplants were analyzed.

2.3.1. Transplantation series

In order to determine the time window of placode induction, several series of transplantation experiments were performed.

In a first series, commitment for the expression of *Six1* was investigated. A tissue is considered as committed when it follows its fate in the presence of other inducing molecules. In order to check commitment, a piece of ectoderm adjacent to the lateral neural folds of the head region (indicated in Fig. 2), where normally placodal *Six1* is expressed, was cut out from pigmented embryos at stages 14 to 20 and transferred to the belly of albino host embryos at stages 14 to 17. After fixation, expression of *Six1* and additionally *FGF8* within the graft was investigated.

It was also important to know at what time of development signals that induce placodal *Six1* expression are present. To check for the presence of placodal *Six1* inducing signals, pieces of unspecified ectoderm immediately adjacent to the lateral neural folds or belly ectoderm of pigmented embryos at stage 13 was grafted into the ectodermal region of placodal *Six1* expression adjacent to the lateral neural folds of albino host embryos at stages 13 and 16. The grafts were analysed for *Six1* induction.

In another series of transplantation experiments, competence to respond to these inducing signals was tested by grafting belly ectoderm of pigmented embryos at stages 13 to 22 into ectoderm adjacent to the lateral neural folds (indicated in Fig. 3) of albino host em-

bryos at stage 13, when inducing signals are present as previously shown. Again *Six1* expression was checked within the grafts. Additionally, animal cap ectoderm was grafted adjacent to the lateral neural folds and checked for the expression of *Six1* and *Sox3*.

To investigate what kinds of tissue are sufficient and/or necessary for the induction of *Six1*, several series of transplantations were performed.

In a first transplantation assay, the dorsal blastopore lip, the so called organizer of amphibians of a pigmented stage 11 embryo was grafted into the belly of an albino host embryo and ectopic expression of *Six1* was checked to test whether the organizer is capable of inducing placodal tissue.

In a second series of transplantations the inducing potential of tissue that developed from or had been induced by the organizer was investigated.

The neural plate that is induced by the organizer was checked for its ability to induce *Six1* expression. Separate experiments were carried out with anterior and posterior neural plate and transplantations were performed at two different stages (stage 12 and stage 13), to analyze spatial and temporal differences concerning inducing activities. Pieces of the neural plate were grafted into the belly of a stage 13 embryo. Conversely, belly ectoderm of stage 13 donor embryos was grafted to substitute for the anterior neural plate unilaterally in stage 13 host embryos. The embryos were analyzed for the expression of *Six1*, *Eya1*, *Sox3* or *FGF8*.

In order to determine whether those parts of the neural plate that are sufficient to induce ectopic *Six1* are also necessary for this induction, the anterior neural plate and the anterior neural ridge, respectively, were removed unilaterally at stage 13 and the placodal expression pattern of *Six1* was examined.

The dorsal marginal zone and the lateral marginal zone of the organizer give rise to mesoderm. Thus, several parts of the mesoderm were tested for their involvement in the induction of *Six1*. Whether the organizer derived chordamesoderm is necessary for the induction of *Six1* was investigated by removal of the chordamesoderm either alone or together with the overlying ectoderm at stage 13 leaving the edges of the neural plate intact.

Also lateral parts of the mesoderm were tested concerning their importance for the induction of the placodal *Six1* expression. To remove the underlying endomesoderm, the ectoderm situated adjacent to the lateral neural folds was folded back and after extirpation of the endomesoderm was put back in place and allowed to heal. Again, the *Six1* expres-

sion pattern of this manipulated side of the embryos was compared with the normal control side of the embryos.

Lateral endomesoderm was also grafted into the belly of host embryos to check whether it is sufficient to induce *Six1* expression.

2.3.2. Animal cap assays

The animal cap of blastula stage embryos consists of prospective yet undifferentiated ectodermal cells. Animal caps were localized by the blastocoel shining through the thin layer of tissue and dissected at stage 9. In order to investigate the potential role of several proteins in the induction of placodal *Six1* expression, animal caps of embryos injected with the respective mRNAs or DNAs were grafted into host embryos.

Animal caps of embryos injected with *noggin*, *FGF8*, *dnBMPR-IA*, or co-injected with *noggin* + *FGF8*, *dnWnt8* + *noggin*, *dnWnt8* + *FGF8* and *dnWnt8* + *noggin* + *FGF8*, respectively, were grafted into the belly of stage 13 host embryos. After reaching at least stage 23, the embryos were analysed concerning ectopic expression of *Six1* and *Sox3*.

As a control, animal caps of uninjected embryos were grafted into belly ectoderm to show that although they express *Sox3*, they do not induce *Six1* in adjacent belly ectoderm in a significant amount.

Animal caps of *BMP4* mRNA injected donor embryos and uninjected animal caps were grafted into ectoderm adjacent to the lateral neural folds of stage 13 embryos where normally placodal *Six1* expression would occur, to see whether the normal panplacodal *Six1* expression is disturbed by BMP.

For rescue experiments, animal caps of *FGF8* and *BMP4*, as well as *FGF8* and *dnFGFR4* co-injected embryos were grafted unilaterally into the neural plate and the host embryos were analyzed for the expression of placodal *Six1*.

2.3.3. Explants and Conjugates

Explantations or conjugations were performed under the same conditions as tranplantations, but afterwards the tissue was transferred to microtiter plates with 0,5xMBS containing

1% BSA and 25mg/l gentamycine. The microtiter plates had been preincubated with the same solution for at least two hours to avoid that embryos glued to the plastic plates. Sibling embryos served as stage controls. Explants and conjugates were fixed in 4% paraformaldehyde for 2 hours. Explants and conjugates had to be relatively large otherwise they disaggregated. Additionally, in situ hybridisation turned often out to be not as strong on explants and conjugates as on wholemount embryos. These small parts of tissue often developed relatively strong background staining. In the following, only explants and conjugates were considered, that were in perfect shape and where labelled cells could be distinguished with no doubt from background staining.

In order to investigate the time of specification for placodal *Six1* expression, a series of explantations were performed. A tissue is said to be specified when it follows its fate in the absence of any other inducing molecules. To test this, a piece of ectoderm lateral and immediately adjacent to the lateral neural folds (indicated in Fig. 2), where normally placodal *Six1* expression occurs, was explanted at stages 13 to 20 and held in culture. Whole embryos served as stage controls. The fixed explants were analysed for the expression of *Six1*.

The dorsal marginal zone (DMZ, about 45-60° wide dorsal to blastopore) and the dorso-lateral marginal zone (DLMZ, about 45-60° wide and immediately lateral to DMZ), respectively, of stage 10 embryos were conjugated with animal caps to test their ability to induce *Six1*. These four to five cell layers consist of prospective migrating mesodermal cells, in contrast to the epithelial cell layers that will give rise to endodermal cells.

2.3.4. Beads

Beads provide the opportunity to test direct effects of proteins or other molecules in the absence of tissues that could be a source of other not wanted molecules. Beads can be soaked with the respective molecule and implanted in the embryo. In the embryo the bead then represents a source of this molecule that can diffuse and affect neighbouring tissue.

Different types of beads vary in their suitability for the treatment with different proteins. In vivo fibroblast growth factors bind to heparin. Interaction with heparin or heparin sulfate proteoglycans makes them stable against thermal denaturation and proteolysis (Ornitz and Itoh, 2001). For this study, recombinant mouse bFGF8 protein (R&D Systems) was applied using heparin acrylic beads (Sigma).

Recombinant human BMP4 (R&D System) and recombinant mouse noggin (R&D systems) proteins, in contrast, were bound to Affi-gel blue beads (BioRad). Affi-gel blue beads consist of a crosslinked agarose gel with covalently attached Cibachron BlueF3GAdye. A large variety of proteins bind to this agarose gel (www.biorad.com). For coincubations with noggin and FGF8, Affi gel blue beads were also used.

Both kinds of beads were treated equally during the experimental procedure. In order to soak beads with the respective molecule, 10 μl of beads were washed 3x5 minutes with PBS (137 mM NaCl, 50 mM Na_2HPO_4, pH 7,2) and 0,1% bovine serum albumine (BSA). During washing steps, liquids were carefully pipetted avoiding loss of beads. The beads were incubated in 5 μl 0,5-1 mg/ml protein solution in PBS + 0,1% BSA for 2 hours at room temperature or overnight at 4°C. During the experimental implantation procedure, the beads were stored at 4°C. Control beads were treated identically except for incubation merely in PBS containing 0,1% BSA.

The FGF inhibitor SU5402 (see below) was applied using AG 1-X2 resin beads (BioRad). Beads were washed 3x5’ with methanol, dried at 37°C and incubated in 0,5 mM SU5402 (diluted in PBS from a 300mM stock solution in DMSO) overnight at 4°C. For grafting, 5 embryos were placed into plasticin holes and 0,5 μl beads were pipetted into the transplantation dish. A small slit was made with a flame-sharpened tungsten needle into the epidermis of the embryo and a tunnel leading to the desired position of the bead was formed with a blunt tungsten needle. By means of this blunt tungsten needle one middle sized bead was then placed along the tunnel at the desired position just below the epidermis. The slit was covered with a piece of glass coverslip to avoid remigration of the bead during healing. After 5 implantations new beads were pipetted into the dish avoiding possible elution of the proteins. Control beads were treated identically except for incubation merely in PBS.

Beads that were incubated with either noggin or FGF8 protein, alone or with a combination of both proteins were implanted into the belly of stage 13 embryos. Ectopic expression of *Six1* in the vicinity of the bead was investigated. Control beads had no effect.

Beads incubated with BMP4 or SU5402 protein were implanted into the panplacodal primordium of stage 13 embryos. These manipulated embryos were investigated concerning their placodal *Six1* expression pattern. Control beads had no effect.

Beads had the tendency to migrate downwards within the embryo and dislocate to the mesoderm during development of the embryo. Only those embryos were taken into account that retained a clearly visible bead situated right under the epidermis.

2.4. Incubation

Another method to manipulate embryonic development is the incubation assay. Embryos are incubated in solutions of the examined molecule for a certain time period.

Here, embryos were treated with the FGF inhibitor SU5402 (3-[(3-(2-carboxyethyl)-4-methylpyrrol-2-yl)methylene]-2-indolinone, Calbiochem). Effects of FGFs are mediated by transmembrane receptors (FGFR-1, -2, -3, -4) that belong to the protein receptor tyrosin kinases. The FGF inhibitor SU5402 is specific for FGF receptors and directly interacts with their catalytic domain. Thus, SU5402 inhibits FGF signalling not via competition with the substrate but via binding at the ATP binding site, thus inbiting the kinase activity of FGF receptors (Mohammadi et al., 1997).

Incubation was performed in glass vials in the dark. For better permeability, the vitelline membrane of the embryos was removed using forceps. Incubations were performed at a SU5402 concentration of 60 μM. The stem solution (300 mM in DMSO) was diluted in 0,1x MBS, except for stages earlier than neural plate stages, where 0,5x MBS was used. As the stem solution of SU5402 contains DMSO, control incubations were carried out with DMSO at the same concentration in 0,1x MBS or 0,5x MBS, respectively.

2.5. X-gal staining

LacZ was coinjected as a lineage tracer. To differentiate between experimental and control side, the enzymatic activity of this protein can be visualized as a light blue colour in X-gal reactions which were performed in fixed embryos prior to in situ hybridisation. After fixation these embryos were washed as usual in PB but were not transferred to 70% ethanol as alcohol decelerates the staining reaction. After two additional 5 minutes washes with 0,1M phosphate buffer (pH 6,3), the embryos were stained in prewarmed 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution (2 ml 0,1M phosphate buffer, pH

6,3 + 6,6 mg Potassium Ferricyanide, 8,4 mg Potassium Ferrocyanide and 3 mg X-gal in 20 μl DMSO) at 37°C. After developing a bright blue colour, embryos were washed 3x5 minutes with 0,1 M MOPS buffer (10,47 g MOPS, 1,03 g NaOAc, 5 ml 0,5 M EDTA ad 500 ml) to stop the reaction and stored in 70% ethanol.

2.6. In situ hybridisation

In order to visualize gene expression in fixed embryos, wholemount in situ hybridisation was carried out after Harland, 1991. The procedure prior to RNase treatment at day 2 had to be performed under RNase free conditions. For that purpose, all solutions had been treated with DEPC (Diethylpyrocarbonate, Sigma, 1ml/l) followed by autoclaving, or had been prepared with DEPC treated aqua bidest, and gloves were worn during these steps. At the first day, embryos were prepared for probe hybridisation. Embryos were rehydrated and washed 3x5 minutes in Ptw (1xPBS + 0,1% Tween). To increase permeability of cell membranes embryos were treated with proteinase K (Sigma) in Ptw (10μg/ml) for 4-10 minutes. The embryos were rinsed twice in 0,1M TEA (Triethanoalamine, Sigma) and treated with acetic anhydride in 0,1 M TEA (2,5 μl/ml) to neutralize positive charges that could interact with nucleic acids (in this case the applied probe) and lead to unspecific staining. This step was followed by 2x5 minutes washes in Ptw. Afterwards, the embryos were refixed in MEMFA (10 ml 1 M MOPS, 1 ml 200 mM EGTA, 100 μl 1M $MgSO_4$, 79 ml DEPC-H_2O, 10 ml formaldehyde solution, min 37%) for 20 minutes. MEMFA was washed away by 5x5 minutes washes with Ptw. After that, embryos were softly transferred into hybridisation buffer (50 ml formamide, 25 ml 20xSSC, 100 mg torula RNA, 10 mg Heparin, 1 ml Denhardts solution, 0,1 ml Tween 20, 0,1 g Chaps, 5 ml 0,2 M EDTA). All but 1ml of Ptw was removed and 250 μl hyb buffer was added. After embryos had settled, hybridisation buffer was removed completely and exchanged by another 250 μl. The embryos were incubated for 10 minutes at 60°C. Again the hybridisation buffer was exchanged and embryos were preincubated for 6 hours at 60°C. The digoxigenin labelled antisense probe (see below) was applied at a concentration of 1 μg/ml in hybridisation buffer and allowed to hybridize at 60°C overnight. Probes were reused three times.

At the second day of in situ hybridisation, unspecific binding of the probe was washed away and embryos were prepared for immunohistochemical detection of the hybridized

probe. For this purposes, probe solution was removed and embryos were washed additional 10 minutes in hybridisation buffer at 60°C. Afterwards embryos were washed 3x20 minutes with 2xSSC (20xSSC: 87,65 g NaCl, 44,1 g NaCitrat ad 500 ml, pH 7,0) at 60°C and treated with RNase A (20 μg/ml, Roche) and RNase T1 (0,1 μl/ml, Roche) which cut single stranded RNA, i.e. unhybridized probe molecules thus reducing background (30 minutes at 37°C). RNase was washed away by a 10 minute incubation in 2xSSC. This was followed by 2x30 minutes high stringency washes at low salt concentration (0,2x SSC) and high temperatures (60°C) to remove unspecifically bound probe. To prepare for antibody detection, embryos were then washed with 1xMAB (maleic acid buffer: 29 g maleic acid, 21,9 g NaCl ad 1 l, pH 7,5) and then incubated in 1xMAB with 2% BBR (Boehringer blocking reagent) for one hour. After preincubation in 2% BBR and 20% heat inactivated (30 minutes at 60°C) normal goat serum (Vektor) diluted in 1xMAB, embryos were incubated with an antibody against digoxigenin (anti-digoxigenine(DIG)-AP Fab fragments, Roche, 1:1000) coupled with alkaline phosphatase at 4°C overnight.

At the last day, gene expression was made visible via a colour reaction catalysed by alkaline phosphatase (AP). The embryos were washed for 5 hours with 1x MAB and 2x5 minutes with AP buffer (5 ml 1M Tris (pH 9,5), 2,5 ml 1 M $MgCl_2$, 1 ml 5 M NaCl, 50 μl Tween 20, 41,5 ml H_2O bidest, 1,2 mg/ml levamisole). For staining, embryos were incubated for 0,5 – 3 days in 2ml AP buffer with 9 μl NBT (nitro blue tetrazolium, 75mg/ml in dimethylformamide, Boehringer Mannheim) and 7 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate, 50mg/ml in dimethylformamide, Boehringer Mannheim). Alkaline phosphatase catalyses the reaction of these chromogenes into a dark blue colour product.

2.7. Immunohistochemistry

In order to detect myc-GFP immunohistochemically, the embryos were washed 2x30' with TBS buffer (12,11 g Tris base, 9 g NaCl ad 1 l, pH 7,5). The first antibody (anti-c-myc-9E10-mouse IgG, Developmental Studies Hybridoma Bank) was diluted 1:1 with normal goat serum and 5% DMSO and embryos were incubated in cryovials overnight at 4°C. After 5 hours washes in TBS, embryos were incubated overnight at 4°C in the secondary antibody (goat-anti-mouse IgG1, fluoresceine (FITC) Conjugate, Southern Biotechnology

Associates) diluted 1:100 with TBS buffer and 5% DMSO. After 5 hour washes in TBS, embryos were stored in fluorescent mounting medium (DAKO).

Embryos were analysed using a stereomicroscope (Zeiss, Axioplan 2 microscope) with a GFP Filter set (band pass excitation filter BP 450–490, barrier emission filter LP 520). To locate ectopic expression more precisely, transverse paraffin sections were made of some GFP labelled embryos (see below). Pictures were taken with a 3 chip RGB camera (Hamamatsu Photonic System, Bridgewater)

In order to look at spatial relationships between *FGF8* expression and *Sox3* expression as a marker for the neural plate, distribution of Sox3 protein was detected on vibratome sections (30-40 μm) after in situ hybridisation. For that purpose, sections were rehydrated in PBS 3x10 minutes. The sections were preincubated 30 minutes in 3% BSA in PBS and 30 minutes in 3% BSA in PBS supplemented with normal goat serum (1/67). The first antibody, a polyclonal rabbit anti-Sox3 antibody (Zhang et al., 2003) was diluted 1:1000 in PBS with 3% BSA and 5% dimethylsulfoxide (DMSO) and incubated overnight at room temperature with soft constant rocking. The sections were washed 3x10 minutes in PBS and incubated with the secondary FITC-conjugated anti-rabbit-IgG1 antibody (F0382, Sigma) that was diluted 1:80 in PBS and 0,1% Triton X-100 for two hours. Sections were again washed 3x10 minutes with PBS and coverslipped with fluorescent mounting medium (DAKO).

Sections were analysed using a fluorescent microscope with a Zeiss filter set (excitation 450-490 nm, emission 515-565 nm).

2.8. Vibratome sections

To have a closer look at the spatial relationship between *FGF8* gene expression and neural plate ectoderm, some individuals were cut sagitally on a vibratome after in situ hybridisation. Embryos were rehydrated and washed 3x5 minutes with 0,1 M phosphate buffer. Before embedding into 4% agar in 0,1 M PB, embryos were dried carefully with a cellulose tissue. The embryos were cut on a vibratome at 30-40 μm and mounted on chrome alum-gelatinized slides. Afterwards, sections were immunohistochemically analyzed for Sox3 protein (see above).

2.9. Paraffin sections

Serial sections of some individuals were cut on a microtome (10 μm) after in situ hybridisation and immunohistochemical detection of myc-GFP. These sections helped to ascertain the definite location of ectopic *Six1* expression. Embryos were dehydrated in an ascending ethanol series (30’ per step) and transferred into 100% ethanol. After 2x30’ incubation in 100% ethanol, embryos were transferred into a mixture of ethanol and butanol (30’ incubation in a dilution 1:3, 30’ dilution 1:2 and 30’ dilution 3:1) followed by 2 x 30’ incubation in 100% butanol. Embryos were transferred into the first paraffin bath and incubated overnight at 60°C. The next morning, paraffin was exchanged and the embryos were incubated for additional 2x1 hour. Embryos were embedded in paraffin and cut at 10 μm. Immediately after drying on slides (SuperFrost Plus, Menzel-Gläser), sections were covered to protect fluorescent signals from light. Slides were dried overnight in a drying chamber at 40°C and deparaffinated 3x5 minutes with xylene to remove paraffin. Sections were embedded in fluorescent mounting medium (DAKO) and stored at 4°C. Sections were analysed using a fluorescent microscope with a Zeiss filter set (excitation 450-490 nm, emission 515-565 nm).

2.10. Midiprep

To obtain probes for in situ hybridisation or mRNA for microinjection, plasmids at first had to be multiplied via midiprep (Plasmid Midi Kit, Qiagen). This was done in S1 laboratories as it implied working with genetically modified organisms. For this procedure, bacteria (*E. coli* XL1 competent cells, Stratagene) had to be transformed with the respective plasmids. This was done by heat shock. Bacterial cells that were stored at –70°C were softly defrosted on ice and split into 100 µl aliquots. 1,7 µl β-mercaptoethanol was added to increase transformation efficiency. The bacteria were incubated on ice for 10 minutes with gentle shaking every two minutes. Then 2,5 µl plasmid DNA (approximately 5 ng/µl) were added to the bacterial cells and incubated on ice for 30 minutes. Afterwards heat shock was applied for 45 seconds at 42°C. During heat shock, bacterial cells open their membranes und incorporate the plasmid. Heat shock was given for a very short time to avoid destruction of bacterial cells. 900 µl Luria Broth culture medium (Sigma) was added and bacteria were incubated at 37°C for one hour. After this time of recovery, the bacterial culture was plated on Luria Broth agar (Sigma) dishes supplied with 0,1 mg/ml ampicillin (Sigma). The plasmids carried a gene for resistance to the antiobiotic ampicillin. When plated out on ampicillin dishes only transformed bacteria will grow. After overnight incubation at 37°C, one of the transformed colonies was picked with a sterilized tip and transferred into 25 ml Luria Broth containing 0,1 mg/ml ampicillin and again allowed to grow overnight at 37°C with constant rocking. On the next day, the bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C and subjected to midiprep procedure following the protocol supplied with the Qiagen kit. During this procedure the plasmid DNA was isolated via an Anion-Exchange-Resin tip. The bacterial cells were lysed by a modified alkaline lysis procedure and afterwards applied to the column followed by several washes. A medium salt wash removed RNA, proteins and low molecular weight impurities. A high salt buffer wash eluted the plasmid DNA (Plasmid Midi Kit, Qiagen handbook). The plasmid DNA was precipitated by 100% isopropanol. The pellet was washed with 70% ethanol and resuspended with 100 µl RNase free water. After photometrically determining the concentration of the yield, the plasmid DNA was diluted in RNase free water to a concentration of 1 µg/µl.

2.11. Linearising plasmid DNA

Plasmid DNA had to be linearised to be suitable for in vitro transcription. For this purpose, 10 μg (corresponding to 10 μl) plasmid DNA was mixed with 5 μl of a specific restriction endonuclease that cuts at a defined position at the 5´end of the insert for α-sense transcripts, or at the 3`end of the insert for sense transcripts, respectively, and 5 μl of the appropriate buffer. This assay was filled up to a volume of 50 μl with RNase free water and incubated at 37°C overnight. The linearised DNA was purified using the Qiaquick PCR Purification Kit (Qiagen). For that purpose, the linearised sample was applied to a Qiaquick column. The column consists of a silica-gel membrane to which double and single stranded DNA binds. A following washing step removes salts, enzymes and unincorporated nucleotides (Qiaquick PCR Purification Kit handbook). The linearised plasmid DNA was eluted with 10 mM Tris-Cl (pH 8,5) and stored at –20°C.

Linearization of the plasmid DNA was verified via gel electrophoresis. 1 μl plasmid DNA was diluted with 9 μl loading buffer and loaded on a 1% agarose gel in 1xTBE (Tris/Borat/EDTA, pH 8,2-8,5). The gel was run at 100 V for 90 minutes and dyed with ethidiumbromid. A single band was visible if the plasmid was correctly linearised.

2.12. In vitro transcription

α-sense probes were synthesized from the following plasmids: *FGF8* (pCS2+XFGF8; Christen and Slack, 1997), *Six1* (pBSIISK-XSix1; Pandur and Moody, 2000), *Sox3* (pBK-CMV-XSox3; Penzel et al., 1997). 1 μg template DNA was used in an in vitro transcription assay (DIG RNA Labeling Kit, SP6/T7, Roche) containing 2 μl 10x NTP labeling mixture, 2μl 10x transcription buffer, 1 μl RNase inhibitor (20 u/μl) and 2 μl of the appropriate polymerase in order to gain anti-sense RNA probes for in situ hybridisation. The labeling mixture contains digoxigenin labelled desoxyuridintriphosphate molecules that are incorporated in the synthesized RNA stochastically. The in vitro transcription assay was incubated for two hours at 37°C. Afterwards the template DNA was hydrolysed by adding 2 μl of supplied DNase1 and incubation for 15 minutes at 37°C. This reaction was stopped with 2 μl 0,2 M EDTA. Transcribed RNA was precipitated by adding 2,5 μl ammonium acetate and 60 μl 100% ethanol on ice for 15 minutes and centrifuged at 15000 rpm for 30

minutes. The RNA was washed in 70% ethanol and resuspended in 10 µl RNase free water. After photometric determination of the concentration, the RNA was diluted to a concentration of 100 µg/ml, stored at –20°C and could now be applied as a probe during in situ hybridisation.

mRNAs for microinjections were synthesized from the following linearized plasmids: *myc-GFP* (pCMTEGFP; kindly provided by Doris Wedlich), *noggin* (pNog_5'-CS2+; Eimon and Harland, 1999), a *truncated BMP-receptor* (pSP64T-tBMPR; Graff et al., 1994), *BMP4* (pGEM7-xBMP4; Hemmati-Brivanlou and Thomsen, 1995), a *dominant-negative FGFR4* receptor (pSP64T-_FGFR-4a; Hongo et al., 1999), and *FGF8* (pCS2+XFGF8; Christen and Slack, 1997) with the mMessage mMachine in vitro transcription Kit (Ambion). In contrast to probes made for in situ hybridisation, plasmids had to be transcribed in vitro into sense mRNA that is capable of being translated within the embryo after injection. For this purpose 1 µg of template DNA was mixed with 2 µl 10x transcription buffer, 10 µl 2x ribonucleotide mix (10 mM ATP, CTP and UTP, 2 mM GTP and 8 mM 5´-7-methylguanosine-cap-analogon) and 2 µl 10x enzyme mix containing RNA polymerase and RNase inhibitor. This assay was incubated for 2 hours at 37°C. Template DNA was removed by addition of 1 µl of DNase followed by incubation for 15 minutes at 37°C. After stopping the reaction with 15 µl 5 M ammonium acetate and 115 µl RNase free water, the mRNA was cleaned using the RNeasy Mini Kit for RNA Cleanup (Qiagen). The in vitro transcription assay was mixed with a highly denaturating buffer containing guanidine isothiocyanate that inhibits RNases and ethanol to obtain appropiate binding conditions. This sample was applied to a column consisting of a silica-gel based membrane. RNA binds to the column and buffers and other contents were removed by centrifugation and two washing steps. The mRNA was diluted with 30 µl RNase free water. Concentration of the yield was determined photometrically and mRNA was diluted to a concentration of 0,25 µg/µl.

3. Results

3.1. Time window for the induction of placodal *Six1* expression

To provide a basis for experiments concerning the induction of placodal *Six1* domain, the time window for the induction of placodal *Six1* expression was first investigated. In a first series of transplantations experiments, pieces of the ectoderm that lie immediately adjacent to the lateral neural folds of the head region of the embryo, corresponding to the placodal *Six1* expressing region, were cut out at different stages, held in culture until control embryos had reached at least neural fold stages, and analyzed for *Six1* expression by in situ hyridisation. When held in culture, the explants could develop in the absence of any inducing molecules. If the explants express *Six1* under these conditions they are regarded as specified (Fig. 2 A).

When explanted at stage 13, i.e., early neural plate stage, none of the explants (n=12) show *Six1* expression. 27% of the explants at stage 14 (n=11) express *Six1*. At stages 15 – 16 already 78% of the explants (n=19) show expression of *Six1* and at stage 20 all the explants (n=9) are *Six1* positive (Fig. 2 B).

To determine the stage of commitment of the placodal *Six1* expressing region, pieces of ectoderm adjacent to the lateral neural folds where transplanted into the belly of a stage 13 host embryo (Fig. 2 C). In this ectopic environment the prospective placodal ectoderm was exposed to different inducing factors. If the transplants nevertheless express *Six1* they are considered to be committed.

When transplanted at stage 14 none of the grafts (n=11) show *Six1* expression. In 39% of all grafts (n=23) transplanted at stages 15 – 16 *Six1* expression is visible. 37% of all transplantations performed at stage 17 (n=19) are *Six1* positive. At stage 20, all specimens (n=7) show *Six1* expression within the graft (Fig. 2 D).

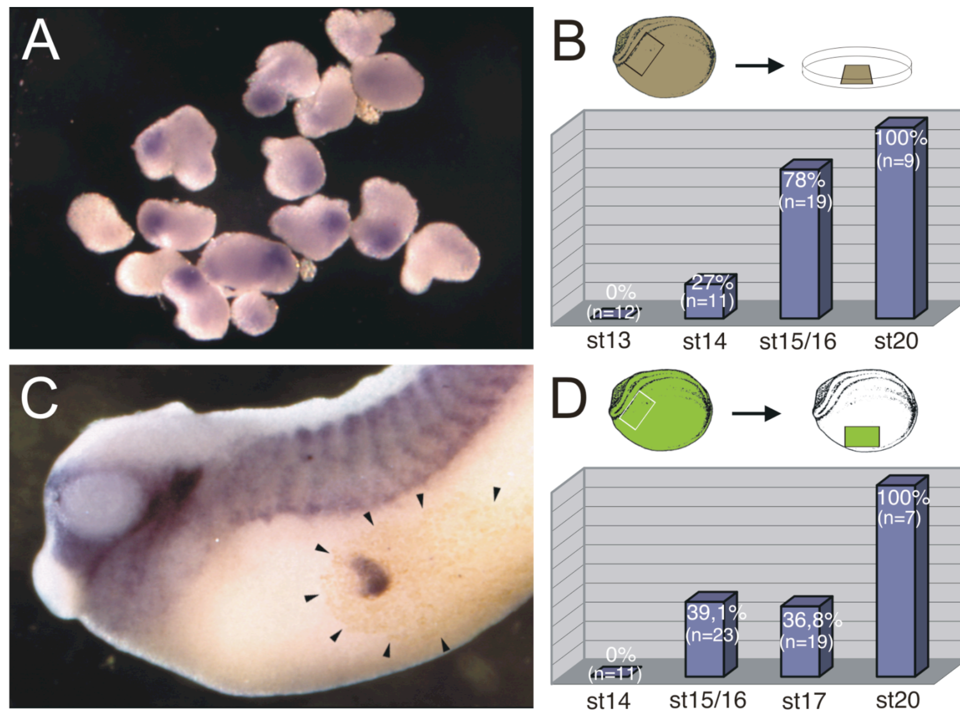

Fig. 2 Time course for placodal *Six1* induction. **A** The majority of ectodermal explants is specified to express *Six1* when explanted at stage 16. Explanted region and percentage of explants expressing *Six1* at different stages is indicated in **B**. **C** *Six1* positive pigmented graft (arrowheads) in the belly of an albino host embryo, transplanted isochronically at stage 15. Grafted region and percentage of grafts expressing *Six1* after transplantation at different stages into stage 13-15 belly ectoderm are indicated in **D**.

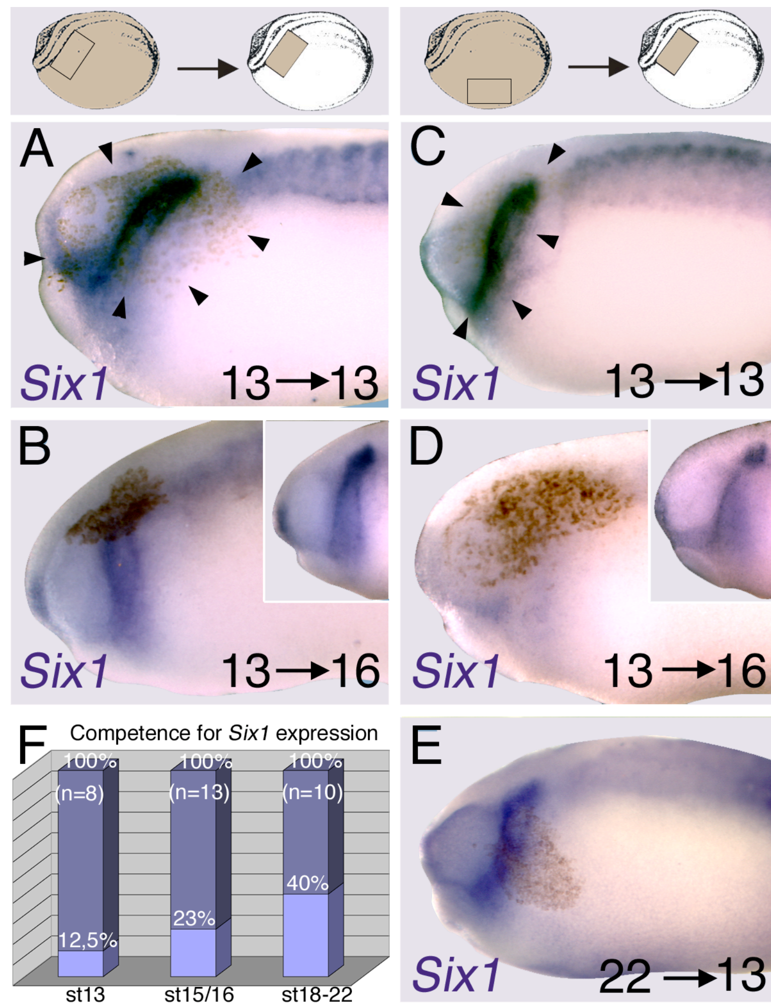

Fig. 3 Ectodermal competence and availability of signals for placodal *Six1* induction. After orthotopic transplantations (**A**,**B**), stage 13 placodal donor ectoderm expresses *Six1* when grafted isochronically (**A**), but fails to do so when grafted into stage 16 hosts (**B**). Heterotopic transplantations (**C-E**) reveal that belly ectoderm is competent to express *Six1* when grafted into the placodal region of stage 13 hosts from stage 13 (**C**) up to at least stage 22 (**E**), but does not express *Six1* when grafted into stage 16 hosts (**D**). **F** Percentage of grafts expressing *Six1* after transplantation of belly ectoderm at different stages into stage 13 placodal ectoderm. Light blue bars indicate the percentage of grafts exhibiting a disturbed pattern of *Six1* expression.

It was also important to know at what time signals for the induction of placodal *Six1* expression were present. In order to address this question, prospective placodal *Six1* expressing tissue of stage 13 donor embryos, which is still unspecified, was grafted into ectoderm adjacent to the lateral neural folds of stage 13 or stage 16 host embryos.

When orthotopically transplanted into a stage 13 donor (n=4), expression of *Six1* within the placodal domain is present and its pattern looks quite normal (Fig. 3 A), indicating that the grafting procedure itself does not perturb placodal *Six1* expression. But when transplanted into a stage 16 host, this is not the case (n=3). Placodal *Six1* expression in the latter cases is reduced and the expression pattern is disturbed (Fig. 3 B). In two cases the otic vesicle is more or less lost on the experimental side. The third case shows an ectopic expression domain of *Six1* behind the graft that might be an ectopic ear.

Additional experiments were carried out, in which belly ectoderm of stage 13 embryos was transplanted into the placodal *Six1* expressing region of stage 16 embryos (n=11). In these cases the graft completely misses *Six1* expression (Fig. 3 D).

In order to investigate ectodermal competence for the induction of placodal *Six1* expression, belly ectoderm at stages 13 (n=8), 15 – 16 (n=13) and 18 – 22 (n=10) was grafted lateral to the neural folds into the region of normal *Six1* expression of a stage 13 embryo. As shown before, at this stage signals that induce placodal *Six1* expression are present. If grafts show *Six1* expression it indicates that the ectoderm is competent to respond to those inducing signals. At all different stages analysed, belly ectoderm shows *Six1* expression after transplantation into the *Six1* expressing domain of stage 13 embryos (Figs. 3 C, E and F). However, the expression pattern sometimes is disturbed compared with the normal placodal expression of *Six1*. The ratio of disturbed pattern seems to increase slightly with age (Fig. 3 F). Only severe disturbances of the ectodermal *Six1* expression pattern were taken into account, viz. complete or major loss of an expression domain, corresponding to one type of placode. With these criteria, 12,5%, viz. 1 out of 8 of the embryos transplanted at stage 13 show a disturbed *Six1* expression pattern. When transplanted at stage 15/16 disturbance in the expression of placodal *Six1* is visible in 23%, viz. 3 out of 13 cases. 40% of the embryos, viz. 4 out of 10 possess a disturbed expression pattern of placodal *Six1* when transplanted at stages 18 – 22.

In contrast, young animal cap ectoderm that was grafted into the placodal region of stage 13 host does not express *Six1* in most of the cases (6/7), but intensely expresses *Sox3*

(n=7), indicating that ectoderm of such an early embryo still possesses competence for neural induction (Fig. 7 H and I).

With these experiments, the time window for the induction of *Six1* expression in its placodal domain was determined. Ectoderm is specified to express placodal *Six1* at early neural fold stages and committed to do so at late neural fold stages. Signals for the induction of placodal *Six1* expression are present at stage 13 and decrease afterwards. The ectodermal competence to respond to these signals remains at least until neural tube closure.

The following experiments were performed at neural plate stages when signals for the induction of placodal *Six1* expression are present and placodal *Six1* expression has not yet been specified.

3.2. Tissues involved in the induction of placodal *Six1* expression

Several candidate tissues were tested for their ability to induce ectopic *Six1* expression and their necessity for the induction of placodal *Six1*. Mesodermal and ectodermal structures as well as their progenitors or inducers were taken into account.

3.2.1. Organizer, dorsal marginal zone, dorsal lateral marginal zone

Different regions of prospective mesoderm were investigated for their potential role in the induction of placodal *Six1*. In a first series of experiments the organizer of stage 11 embryos was transplanted into the belly of stage 13 embryos. In seven of nine cases the organizer induced ectopic *Six1* expression in the surrounding host ectoderm (Fig. 4 A, Tab. 2). In four of these cases, the ectopic expression domain is localised at the anterior border of the graft. In all seven cases, the induced ectopic expression domain lies in the anterior part of the host embryo. These experiments show that the organizer is able to induce ectopic *Six1* expression in anterior belly ectoderm.

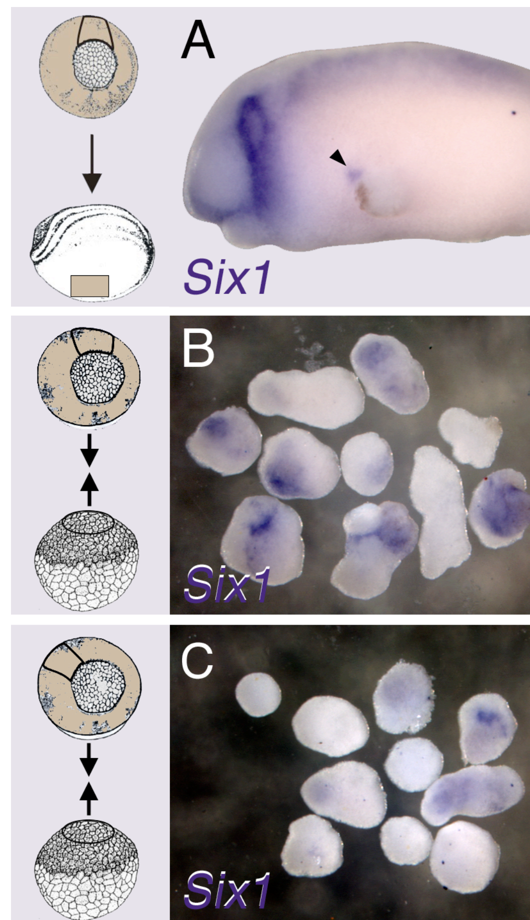

Fig. 4 *Six1* induction by organizer and gastrula mesoderm. **A** Induction of *Six1* expression (arrowhead) in host belly ectoderm after grafting a stage 11 organizer into stage 13 belly ectoderm. **B** Conjugates of stage 11 dorsal marginal zone and stage 9 animal caps show strong ectodermal *Six1* expression. **C** Conjugates of stage 11 dorsolateral marginal zone and stage 9 animal caps, in contrast, show weaker expression of *Six1* in fewer cases.

The dorsal marginal zone (DMZ) and the dorsal lateral marginal (DLMZ) zone from stage 11 embryos were conjugated with animal caps, i.e. non-specified ectoderm. In all cases (n=10) the DMZ induced *Six1* expression in the animal cap part of the conjugate (Fig. 4 B, Tab. 1). Six cases show a very strong and broad expression of *Six1*, whereas four cases express *Six1* more weakly.

The DLMZ was also able to induce *Six1* but only in 50% of the cases (n=10) (Fig. 4 C, Tab. 1). Two cases showed a strong expression of *Six1* and three only a very weak one. So while both DMZ and DLMZ seem to be capable of inducing *Six1* expression, the inducing activity of the DMZ is more effective.

Table 1

Expression of *Six1* in animal cap ectoderm after conjugation with mesodermal tissue

conjugated tissues	probe	number of experiments	number of conjugates	induction of expression
DMZ + AC	*Six1*	1	10	10
DLMZ + AC	*Six1*	1	10	5

3.2.2. Neural plate

It has been shown that the neural plate is able to induce ectopic expression of the neural crest marker *slug* in adjacent belly ectoderm (Mancilla and Mayor, 1996). Thus, in a next series of transplantations it was tested whether the neural plate is also able to induce ectopic *Six1* expression.

Anterior neural plate of stage 13 embryos was grafted into the belly of stage 13 host embryos. When hybridised with a probe for the neural plate marker *Sox3*, the graft shows an intensive label in all seven cases analyzed (Fig. 5 A, Tab. 2), indicating that the neural plate was already committed at time of transplantation. It could not be clarified whether *Sox3* expression is restricted to the graft or whether adjacent host ectoderm also shows *Sox3* positive cells.

Analysed for the expression of *Six1*, it could be demonstrated that the anterior neural plate is able to induce ectopic expression of *Six1*. This ectopic expression ranges from

spots of *Six1* positive cells to stripes of ectopic expression up to open rings situated around the graft (Fig. 5 B). In 28 of 38 cases the anterior neural plate induced an ectopic expression domain of *Six1* (Tab. 2). To analyse whether the ectopic expression of *Six1* was induced within host or donor tissue or both as reported for the neural crest marker *slug* (Mancilla and Mayor, 1996), some (n=6) individuals were cut on a vibratome. The donor tissue was identified by antibody detection of GFP. Vibratome sections were cut at 30 μm. At a first glance, it appeared as if *Six1* was expressed in a part of the GFP positive transplant. But this apparently GFP-positive part of the transplant always showed a very weak GFP label. Upon closer inspection, it seemed likely that *Six1* expression and GFP label are in fact complementary in these embryos, but because of the thickness of the vibratome sections, it was not possible to distinguish unambiguously between colabelling and complementary label of GFP and *Six1*. In order to verify the hypothesis that *Six1* and GFP are complementarily expressed, paraffin sections of 3 additional embryos were made at 10 μm. Paraffin section turned out to be a suitable instrument. GFP label was not affected by the experimental procedure and tissue was preserved much better than with vibratome sections. When analysed in paraffin sections, it became clear that GFP label and *Six1* expression are always complementary, indicating that the ectopic expression domains of *Six1* indeed lie outside but immediately adjacent to the graft, i.e., exclusively within host tissue (Figs. 5 C-E).

As another panplacodal marker gene, ectopic expression of *Eya1* could also be induced by transplanting the neural plate of stage 13 embryos into the belly of stage 13 hosts. 3 out of 8 embryos show an ectopic *Eya1* expression domain around the graft (Tab. 2). *Eya1* is a much weaker probe than *Six1* is and it is harder to detect ectopic domains, which may be the reason why only 37% of the embryos show obvious ectopic expression of *Eya1* in contrast to 74% of all embryos analysed that show ectopic *Six1* expression.

To test whether other parts of the neural plate are also able to induce placodal *Six1* expression, the most anterior part of the neural plate, the anterior neural ridge of stage 13 donors was transplanted into the belly of host embryos. In all cases (n=13), *Six1* expression is visible in the adjacent ectoderm (Fig. 5 F, Tab. 2). Boundaries of the grafts were determined by pigment of the donor tissue. In at least 7 cases there is additional *Six1* expression within the graft itself (insert in graft Fig. 5 F). This is most likely due to the fact that the anterior border of grafts not only included neural plate tissue but also placodal tissue immediately anterior to the neural plate. *Six1* expression outside the graft is comparable to

that observed after transplanting the anterior neural plate as described above. A stripe of ectopic expression of *Six1* can be detected at the border of the graft.

Posterior neural plate was also transplanted into the belly of host embryos in 10 cases but was never able to induce ectopic expression of *Six1* (Fig. 5 G, Tab. 2).

Thus, the neural plate is sufficient to induce *Six1* expression in adjacent belly ectoderm but there are spatial differences. Whereas anterior parts of the neural plate are capable of inducing ectopic *Six1* expression, posterior parts are not.

In order to determine possible temporal differences in the ability of the neural plate to induce *Six1* expression, prospective anterior neural plate of donor embryos at stage 12 was then transplanted into the belly of stage 13 host embryos. For that purpose, a piece of ectoderm slightly anterior to the dorsal blastopore lip as indicated in Fig. 5 H was cut out and transferred to the belly of the host embryo. Out of nine cases only two show a very weak ectopic expression domain at the border of the transplant (Fig. 5 H, Tab. 2). In contrast to stage 13 neural plate, which is able to induce strong ectopic *Six1* expression in 64% of the transplanted embryos, neural plate at stage 12 was only able to induce weak ectopic expression in 29% of the cases. Thus, ability of the neural plate to induce ectopic *Six1* expression not only shows spatial differences but also changes with time, as the neural plate acquires the ability to induce placodal *Six1* expression at the end of gastrulation.

The just mentioned transplantation series ascertained that anterior neural plate of stage 13 embryos is sufficient to induce ectopic *Six1* expression. However, they do not show, whether anterior neural plate is also necessary for this induction. To determine whether the anterior neural plate is also necessary for the induction of the placodal expression of *Six1*, parts of the anterior neural plate were removed leaving the underlying mesoderm intact. In a first step, the most anterior part of the neural plate, the ANR was removed and the placodal expression pattern of *Six1* was investigated. This was done in 16 embryos. 50% of the manipulated embryos show reduction in anterior placodal *Six1* expression, but still express placodal *Six1* (Fig. 5 I, Tab. 3). Thus, the ANR may be necessary for the induction of only a part of the placodal *Six1* expression domain.

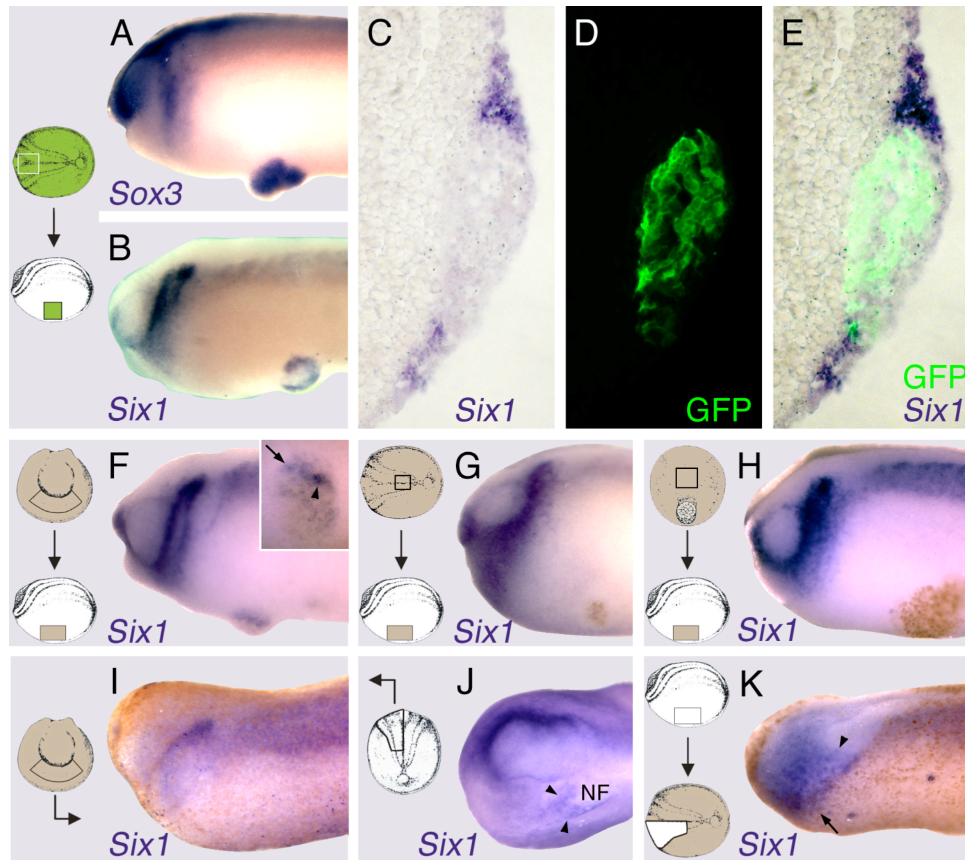

Fig. 5 Role of neural plate in placodal *Six1* induction. Embryos are shown in lateral (**A**,**B**,**F**,**G**,**H**,**I**), dorsal (**J**) or dorsolateral views (**K**) with anterior to the left. **A-E** Grafts of stage 13 anterior neural plate into stage 13 belly ectoderm. Grafts are neurally committed as indicated by strong *Sox3* expression (**A**) and induce a ringshaped domain of *Six1* in the surrounding ectoderm (**B**). **C-E** Cross section of a neural plate graft demarcated by green GFP label (**D**,**E**) shows that expression of *Six1* (**C**,**E**) is confined to host ectoderm. **F** Induction of *Six1* after grafting stage 13 anterior neural ridge into stage 13 belly ectoderm. *Six1* is expressed in adjacent ectoderm (arrow) as well as in the graft (arrowhead) as shown at higher magnification in the insert. **G** Stage 13 posterior neural plate does not induce *Six1* when grafted into stage 13 belly ectoderm. **H** Anterior neural plate of stage 12 embryos is typically not able to induce *Six1* in stage 13 belly ectoderm, although a small domain of *Six1* is induced in the specimen depicted. **I** Reduction of anterior but not posterior domains of placodal *Six1* expression after extirpation of the anterior neural ridge at stage 13. **J** Placodal *Six1* expression is lost after unilateral extirpation of the anterior neural plate except for residual *Six1* expression (arrowheads) at remnants of neural folds (NF). **K** Broad expression of *Six1* (arrowhead) in stage 13 belly ectoderm grafted unilaterally into stage 13 anterior neural plate. *Six1* expression extends beyond graft boundaries into host ectoderm (arrow).

In a next series of transplantation assays, the complete anterior neural plate was removed unilaterally. All 18 embryos that miss a neural plate on one side show pronounced defects in the placodal *Six1* expression on this experimental side. 7 embryos still express *Six1* in the region of normal placodal *Six1* expression with a disturbed pattern, but the remaining 11 embryos almost completely lost placodal *Six1* expression (Fig. 5 J, Tab. 3). In all cases of residual *Six1* expression, residues of the anterior neural folds were still visible.

When the anterior neural plate was removed unilaterally and replaced by a piece of belly ectoderm, 11 of 12 cases express *Six1* within the graft. 5 of these showed a very strong expression of *Six1* (Fig. 5 K). In 8 cases, the *Six1* expression on the experimental side is restricted to the graft. The other 3 embryos show additional expression of *Six1* within the host ectoderm (Tab. 2). In 2 of these cases this expression in the host ectoderm lies adjacent to residues of the neural folds and thus may be due to the fact that the neural plate had not been extirpated completely. Nevertheless, one case shows *Six1* expression ventral to the graft in host ectoderm, but it is important to mention that boundaries of the graft were determined just by pigment of the donor tissue and not via lineage tracers.

3.2.3. Axial Mesoderm and lateral Endomesoderm

As mentioned above, the organizer is able to induce ectopic expression of *Six1* in the belly of a host embryo. The DMZ and to some extend the DLMZ are also able to induce *Six1* expression in animal caps. These marginal zones give rise to mesodermal structures. Two different parts of the mesoderm, axial mesoderm and lateral endomesoderm were, therefore, tested for their contribution to placodal *Six1* induction.

Chordamesoderm was removed at stage 13 either alone (n=10) or together with the overlying central neural plate (n=6) but the edges of the neural plate have been kept intact. Embryos that underwent this kind of explantation, did not develop a completely closed neural tube, and chordamesoderm apparently had not regenerated. Nevertheless, the *Six1* expression pattern of these embryos looks quite normal (Fig. 6 C, Tab. 3).

In a second explantation assay, the lateral endomesoderm that lies directly beneath the placodal region of *Six1* expression was removed. The overlying placodal ectoderm was not damaged but was folded back and afterwards allowed to heal in again. It was not possible to test necessity of the mesodermal tissue alone, because at early neural plate stages when

these explantations were performed it was not possible to separate mesoderm and endoderm from each other as they tended to stick together. Embryos from which the lateral endomesoderm was removed (n=15) exhibit a strongly reduced placodal *Six1* expression (n=14) or even lost this expression domain completely (n=1) (Fig. 6 B, Tab. 3). Thus, in contrast to chordamesoderm, lateral endomesoderm is necessary for the induction of panplacodal *Six1* expression.

By transplanting lateral endomesoderm into the belly of a host embryo, its ability to induce ectopic expression of *Six1* was tested. Nine embryos received such a graft but none of these show ectopic expression of *Six1* in the belly ectoderm (Fig. 6 A). Thus, the lateral endomesoderm is necessary but not sufficient for the induction of placodes.

Table 2

Expression of *Six1*, *Eya1*, *Sox3* and *FGF8* after grafting different tissues

grafted tissue (st13, if not otherwise indicated	place of implantation (st13)	probe	number of experiments	number of individuals	induction of expression in host	reduction or loss of expression in host	expression within the graft
organizer (st11)	belly	*Six1*	2	9	7	0	0
anterior neural ridge	belly	*Six1*	2	13	13	0	?
anterior neural plate	belly	*Six1*	6	38	28	0	0
		Eya1	1	8	3	0	0
		Sox3	1	7	?	0	7
		FGF8	1	6	?	0	6
posterior neural plate	belly	*Six1*	1	10	0	0	0
stage 12 ant. neural plate	belly	*Six1*	1	9	2	0	0
belly	lateral anterior neural plate	*Six1*	3	12	3	0	11
endo-mesoderm	belly	*Six1*	2	9	0	0	0
lateral pan-placodal domain	belly	*FGF8*	1	10	0	0	2

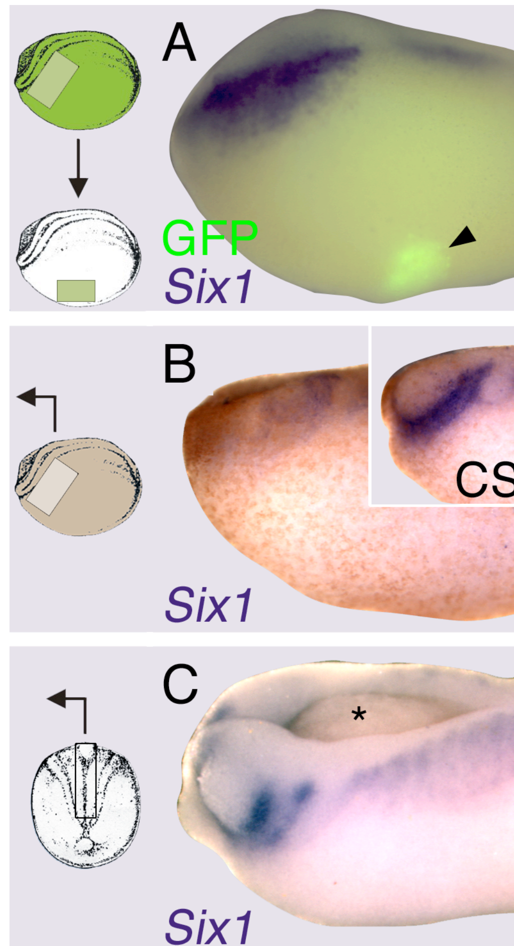

Fig. 6 Role of endomesoderm in placodal *Six1* induction. Embryos are shown in lateral (**A,B**) or dorsolateral views (**C**) with anterior to the left. **A** No ectopic *Six1* expression is induced after grafting lateral endomesoderm (demarcted by green GFP label) into belly ectoderm isochronically at stage 13. **B** Loss of placodal *Six1* expression after removal of the underlying lateral endomesoderm at stage 13. Control side (CS) of same embryo is shown in insert for comparison. **C** Normal *Six1* expression after extirpation of axial and paraxial mesoderm together with the overlying neural plate at stage 13. The neural tube did not close completely (asterisk).

Table 3

Expression of *Six1* after extirpation of specific ectodermal and mesodermal tissues

extirpated tissue (st13)	probe	number of experiments	number of individuals	induction of expression	reduction or loss of expression
chorda	*Six1*	2	16	0	0
dorsolateral endomesoderm	*Six1*	3	15	0	15
anterior neural ridge	*Six1*	2	16	0	8
lateral anterior neural plate	*Six1*	3	18	0	18

3.3. Molecules involved in the induction of placodal *Six1* expression

Different kinds of molecular signals were tested concerning their importance for the induction of placodal *Six1* expression. In a first series of transplantations, the role of BMP, a member of the transforming growth factor β family and its antagonists were investigated. As a second group of molecules fibroblast growth factors (*FGFs*) were tested. Wnt proteins play a role in anterior-posterior patterning. Thus, it was also investigated whether Wnts may play a role in restricting the placodal *Six1* expression domain to anterior parts of the embryo.

3.3.1. BMP inhibitors

The role of BMP and its antagonists for the induction of placodes was investigated. First the role of BMP antagonists using the candidate molecule noggin was analysed. BMP antagonists are known to be expressed in several tissues involved in placode induction, e.g. the organizer, the neural plate and the lateral endomesoderm and thus could contribute to the inductive activity of these structures. To test this, two different approaches were used. In the first approach, *noggin* injected animal caps were transplanted into the belly of 29

host embryos. In 23 of these cases, the normal placodal *Six1* expression domain of hosts is broadened, and expression extends posteriorly to the anterior border of the grafts (Fig. 7 B, Tab. 4). Thus, *noggin* injected animal caps were able to induce ectopic *Six1* expression, but only in the vicinity of the normal placodal *Six1* expression domain and only adjacent to their anterior border. This is in contrast to the ectopic expression induced by grafts of the neural plate, which were able to induce ectopic expression of *Six1* in belly ectoderm located adjacent to the graft also at the posterior border.

When hybridised with a probe for the neural plate marker *Sox3* it became clear that all *noggin* injected animal caps (n=17) thus analysed showed strong expression of *Sox3* within the graft. This suggests that the *noggin* injected animal cap had been neuralised by the BMP antagonising activity of noggin (Fig. 7 A). In 12 cases there is also a small *Sox3* positive region in adjacent host ectoderm (Tab. 4). In contrast to ectopic *Six1* expression induced by *noggin* injected animal caps in host ectoderm, these ectopic expression domains of *Sox3* are not restricted to the anterior border of the graft. In at least 2 cases, there are some *Sox3* positive cells in host ectoderm posterior to the graft. Additionally, ectopic *Sox3* expression in host ectoderm is rather patchy and not as homogenous as *Six1* expression. Sometimes, only individual cells are *Sox3* positive. In contrast to the ectopic *Six1* expression that is induced immediately adjacent to the graft, small *Sox3* expression domains may occur in a certain distance to the graft (n=4).

In order to test whether ectopic induction of *Six1* by *noggin* injected animal caps is due to direct action of noggin diffusing from the graft or whether it is merely due to the fact that noggin neuralizes the graft, *dnBMPR-IA* injected animal caps were transplanted in the belly of host embryos. DnBMPR-IA is a dominant negative form of a BMP receptor, thus inhibiting BMP signalling. DnBMPR-IA is capable of cell-autonomously blocking signalling by BMP2, BMP4 and BMP7 (Eimon and Harland, 1999). By this inhibition, the animal cap should be neuralised as well, wihtout acting as a source of diffusible BMP antagonists in contrast to *noggin* injecteted animal caps. Neural character of the graft was revealed by in situ hybridisation with a probe for *Sox3*. The 12 cases investigated all show expression of *Sox3* within the graft, indicating that the transplanted animal cap had been neuralised (Fig. 7 C, Tab. 4). In contrast to *noggin* injected animal caps where *Sox3* expression is always homogenous, *Sox3* expression within *dnBMPR-IA* injected animal caps in four cases is rather spotty. This may be due to the fact that the dominant negative BMP receptor is not diffusible so that only cells that inherit sufficient dnBMPR-IA from the in-

jected blastomeres will be neuralized. Additionally, in six cases *Sox3* expression is visible outside the graft. This ectopic expression outside the graft extends from the normal placodal *Sox3* expression domain towards the graft. In two of these cases the normal placodal expression domain is slightly broadened, whereas in the four cases mentioned with spotty *Sox3* label within the graft the ectopic expression outside the graft is a broad and relatively homogenous domain.

Hybridisation with *Six1* showed that only 8 out of 34 *dnBMPR-IA* injected animal caps were able to induce ectopic *Six1* expression in adjacent belly ectoderm (Tab. 4). Three of these cases possess a broadened placodal *Six1* expression that reaches the anterior part of the graft. Five cases show a weak ectopic expression of *Six1* around the graft. To summarise, *dnBMPR* injected animal caps did induce ectopic expression of *Six1* only rarely, while in most cases no ectopic *Six1* expression is visible (Fig. 7 D). Furthermore, in cases that show ectopic expression of *Six1*, this expression is very weak in comparison to that induced by *noggin* injected animal caps. These results suggest that neuralization of the graft in fact plays some role, but the stronger effect of *noggin* injected animal caps in comparison with *dnBMPR* injected animal caps indicate that diffusibility of BMP antagonists is necessary to get a strong ectopic expression of *Six1*.

To test the potential of noggin to induce *Six1* expression directly in the absence of other possible signalling molecules emanating from the neuralized animal caps, beads that had been soaked with noggin protein were implanted in the belly of host embryos. In 14 of these cases the bead ended up in an appropriate position. None of these embryos showed ectopic *Six1* expression in the area of the implanted bead (Fig. 7 E, Tab. 5). These experiments show that diffusible BMP inhibitors such as noggin are important for the induction of *Six1* but are not sufficient to induce *Six1* ectopically by themselves.

Nevertheless, these experiments indicate that BMP inhibition might play a role in placodal *Six1* induction. In order to test whether elevated levels of BMP also interfere with normal placodal *Six1* induction, in a next transplantation series, *BMP4* injected animal caps were grafted into placodal *Six1* expressing ectoderm adjacent to the lateral neural folds of host embryos. In seven embryos the graft lies within or just beside the normal placodal *Six1* expressing region. In all of these cases, placodal *Six1* expression is reduced on the experimental side in comparison to the control side. The graft is surrounded by a white ring completely free of *Six1* expression (Fig. 7 F, Tab. 4).

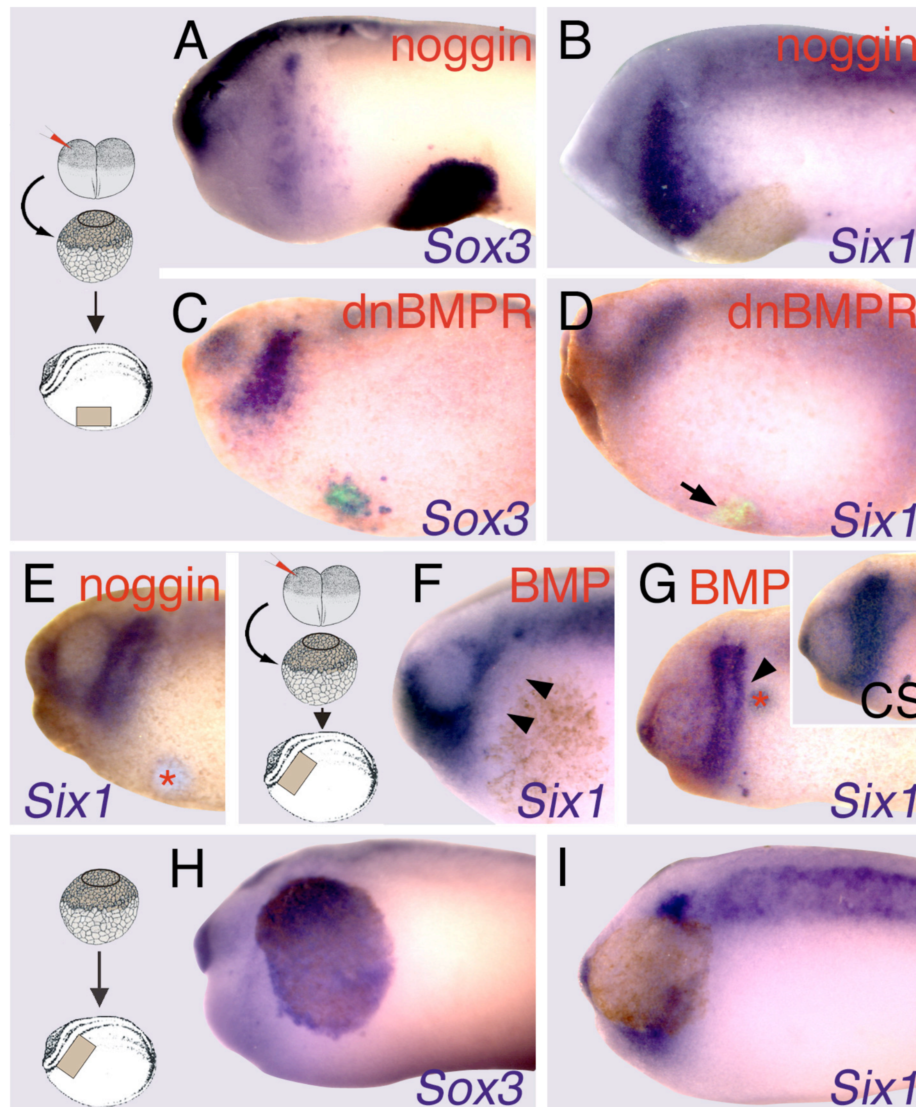

Fig. 7 Role of BMP inhibition in placodal *Six1* induction. Embryos are shown in lateral views with anterior to the left. **A, B** After transplanting animal caps from *noggin* injected embryos into belly ectoderm at stage 13, strong *Sox3* expression is observed in the graft (**A**) and placodal *Six1* expression of the host is broadened towards the anterior border of graft (**B**). **C, D** After transplanting animal caps from *dnBMPR* injected embryos (as indicated by green GFP label, arrow) into belly ectoderm at stage 13, strong *Sox3* expression is observed in the graft (**C**) but *Six1* expression in host ectoderm is not broadened (**D**). **E** Noggin-soaked beads (red asterisk) do not induce ectodermal *Six1* expression. **F** Reduction of placodal *Six1* expression (arrowheads) around the graft after transplanting an animal cap from a *BMP4*-injected embryo into the prospective placodal region at stage 13. **G** Reduction of placodal *Six1* expression (arrowhead) in vicinity of bead soaked with BMP4 (red asterisk) grafted into prospective placodal domain at stage 13. Insert shows control side (CS) for comparison. **H, I** After transplanting animal caps from an uninjected embryo into the prospective placodal domain at stage 13, grafts strongly express *Sox3* (**H**) but do not express *Six1* (**I**), while normal *Six1* expression is seen in adjacent host ectoderm.

Again, the activity of this protein was next tested in the absence of other possible inducing molecules. Beads soaked with BMP4 protein were implanted in the region of normal placodal *Six1* expression of stage 13 embryos. In only two cases out of 5 the beads were clearly visible. In both cases, placodal expression of *Six1* is reduced in the neighbourhood of the implanted bead (Fig. 7 G, Tab. 5). These experiments show that high levels of BMP as they are known to be present in the placodal ectoderm (Fainsod et al., 1994) are not compatible with placodal *Six1* expression. Therefore, inhibition of BMP plays a role in the normal induction of placodal *Six1* expression.

3.3.2. Fibroblast growth factors

The role of FGF signalling in placodal *Six1* induction was investigated by several approaches.

FGF signalling was inhibited using the FGF-Inhibitor SU5402, which inhibits the kinase activity of FGF receptors. SU5402 was initially applied by incubating embryos in a solution of the inhibitor. Incubation assays turned out to be difficult to interpret, because they give variable results and do not always show the same strong effects. This has already been reported earlier by other authors (e.g. Lombardo et al., 1998) and is most likely due to the fact that molecules do not penetrate sufficiently.

Embryos incubated in SU5402 during gastrulation, i.e. from stage 8 until 12 could not be analyzed, because they did not complete gastrulation and failed to neurulate. Embryos that were incubated for a time period from stage 12 until 20 (n=24), showed a clearly disturbed expression pattern of placodal *Six1* in comparison to control embryos (n=15) (Fig. 8 A-C). In 6 cases embryos did not close their neural tube. The remaining embryos proceeded in their development but also did not completely close their neural tube at the posterior end. Additionally, the anterior-posterior axis is slightly shortened in the latter cases. In all cases, there is still some *Six1* expression left but the placodal *Six1* expression pattern is reduced and disturbed.

When incubated in SU5402 for a time period from stage 18 until stage 25 (n=18), 10 embryos look normal with *Six1* expression in the placodal region (Fig. 8 C). The other 3 embryos possess a weak *Six1* expression not only in the placodal region but also in

somites. Similarly, also control embryos show reduction of *Six1* expression in 2 cases (n=17).

A more localised inhibition of FGF signalling was realised by grafting beads that had been soaked with SU5402 into the region of placodal *Six1* expression. In nine cases the bead ended up in the right position and in eight of these cases the expression of *Six1* is reduced in the ectoderm just above the implanted bead, while the surrounding *Six1* expression is still very strong (Fig. 8 D and E, Tab. 5).

As a promising candidate of fibroblast growth factors responsible for placodal *Six1* induction, *FGF8* was investigated. *FGF8* is first expressed at stage 10 around the prospective blastopore. This expression domain perstists as gastrulation proceeds and is restricted to the posterior end of the embryo at early neural plate stages. Additionally, at early neural plate stages expression of *FGF8* appears at the anterior end of the embryo in form of three stripes. The most anterior of these stripes lies in a domain around the anterior neural plate similar to that of *Six1* (Christen and Slack, 1997 and Fig. 9 E–H). In order to determine the exact spatial relationship between *FGF8* expression and the neural plate, in situ hybridisation with a probe for *FGF8* followed by immunohistochemical detection of Sox3 protein as a marker for the neural plate were performed. In sagittal sections, the border of the neural plate is clearly recognizable by greenly fluorescing Sox3 positive cells (Fig. 9 N). As mentioned above, anterior expression of *FGF8* forms three distinct stripes (Fig. 9 G, M). The posterior two of these stripes lie within the neural plate as clearly evident when compared with Sox3 positive cells (Fig. 9 O). In contrast, the most anterior stripe of *FGF8* expression is situated around the anterior neural plate, presumably slightly ventral to the placodal *Six1* expression domain in its anterior part but partly overlapping with it laterally (Fig. 9 D, H, O). Because of its expression pattern in the anterior neural plate, *FGF8* was further investigated concerning its possible role in the induction of placodal *Six1* expression. Anterior neural plate grafts continue to express *FGF8* when transplanted into belly ectoderm, which was verified in 6 out of 6 cases (Fig. 10 G–I, Tab. 2). Thus, *FGF8* could indeed be responsible for the inductive activity of the neural plate observed in the transplantation experiments reported here.

However, in grafts of the lateral panplacodal domain to the belly, *FGF8* is expressed only weakly, which is in accordance with the observation that no *Six1* expression is induced in host ectoderm after such grafts (Fig. 10 J, Tab. 2).

Overexpression of *FGF8* turned out to be unsuitable for investigating direct effects on the placodal *Six1* expression. After injection of a relatively large amount of *FGF8* mRNA (250pg) into one or two blastomeres of an embryo at the two or four cell stage, respectively, all embryos (n=17) show severe gastrulation defects. 11 embryos were labelled for *Six1* and in these embryos normal *Six1* expression was severely disturbed (Tab. 6). Because of the severe side effects, clearly seen morphologically but also confirmed by labelling with *NeuroD* (n=6), with no normal domains of *NeuroD* left and ectopic expression in belly ectoderm (data not shown), it is not possible to distinguish between direct effects of *FGF8* on placodal *Six1* expression and secondary effects resulting from the disturbed gastrulation in these cases. In a next step, a lower amount of *FGF8* mRNA (31,25 pg) was injected into a more restricted part of the embryo, viz. in 1 cell at the 8-cell stage (n=42). Additionally, *FGF8* DNA (25pg) was injected the same way (n=25) to delay translation of *FGF8* and thus minimize such gastrulation defects. These restricted injections of lower amounts of mRNA or DNA had similar results and will be considered together. 35 embryos were analyzed for the expression of *Six1*. All but one of these embryos managed to gastrulate properly but show a neural tube/neural plate which is enlarged on the expense of the placodal domain. This was additionally confirmed by labelling with *Sox3* (n=32), revealing a broadening of the neural plate domain of *Sox3* expression on the expense of the placodal domain in 28 embryos. In some cases (n=3), also small secondary axes can be detected (Tab. 6). Additional embryos that were labelled with *NeuroD* (n=10) show again ectopic expression in the belly ectoderm in eight cases (data not shown). These experiments show that injection of *FGF8* has an effect on the development of the neural plate making it again impossible to distinguish between direct effects of *FGF8* on the placodal *Six1* expression and secondary effects resulting from the broadening of the neural plate.

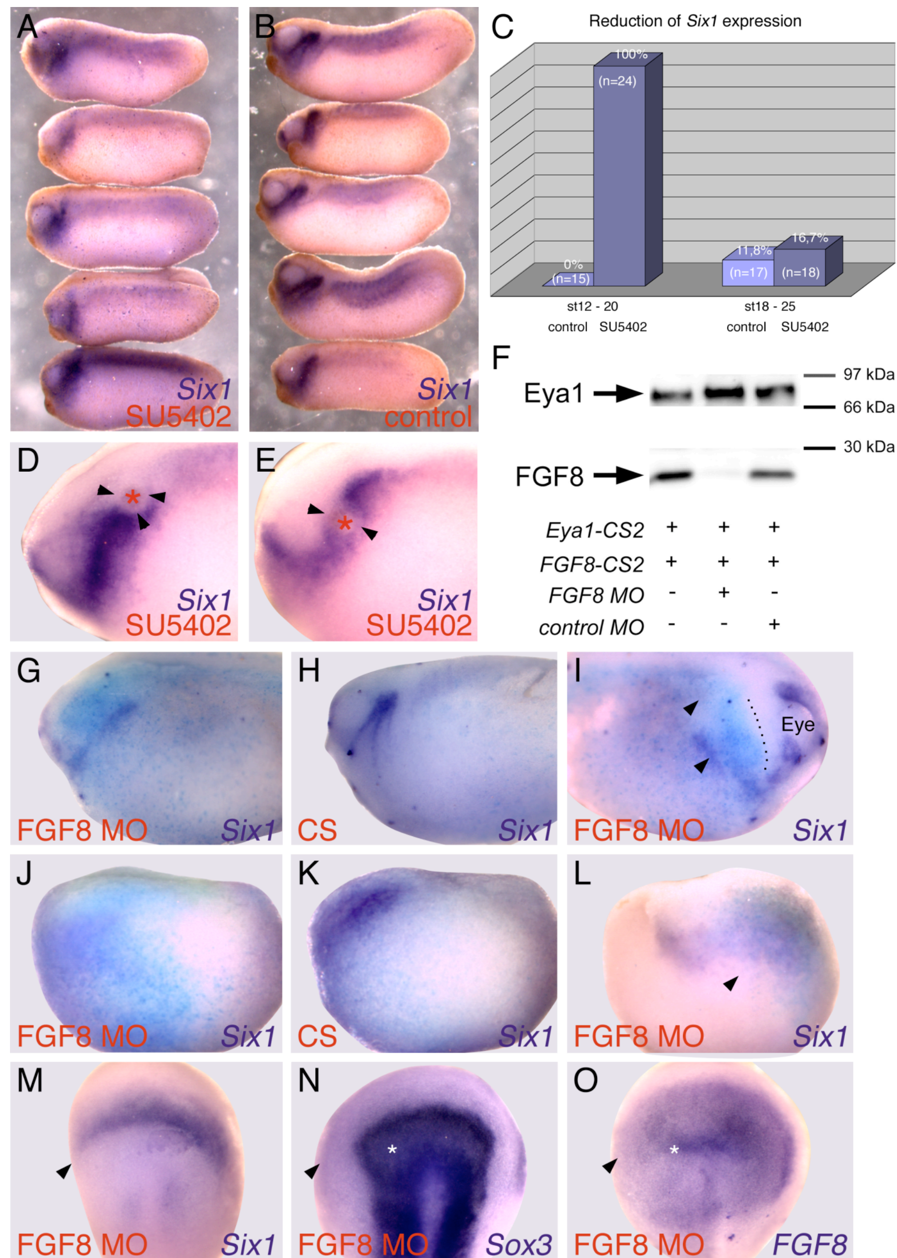

Fig. 8 FGF signalling is necessary for placodal *Six1* induction. **A** Placodal *Six1* expression is reduced after incubation in the FGF inhibitor SU5402 from stage 12-20 compared to controls (**B**). **C** Percentage of embryos showing reduction of *Six1* expression after treatment with SU5402 at different stages. **D, E** Beads soaked with SU5402 locally inhibit *Six1* expression (arrowheads) after implantation into the region of the prospective ear placode (**D**) or of prospective trigeminal, lateral line and epibranchial placodes (**E**). **F** Western blot after in vitro transcription and translation reveals that the FGF8 MO but not an unspecific control MO specifically blocks translation of *FGF8*, whereas *Eya1* remains unaffected (kindly provided by Dr. Schloßer). **G-O** Embryos injected with FGF8 MO show strong reductions (arrowheads) of placodal *Six1* (**G-L**), *Sox3* (**N**) and *FGF8* (**O**) expression, while neural plate domains of *Sox3* as well as *FGF8* expression are not reduced (asterisks). The injected side is to the left in panels **G-K** and **M-O**. Placodal domains are more strongly reduced posteriorly but analysis of tailbud stage embryos (**G**, **H**, lateral view of injected and control sides, respectively; **I** (midline indicated by dotted line) reveals loss or reductions of *Six1* expression not only in the ear (upper arrowhead) but also in more anterior placodes, e.g. the olfactory placode (lower arrowhead). Moreover, when injected into both blastomeres (**L**), the FGF8 morpholino also strongly reduces *Six1* expression in anterior portions (arrowhead), visible at neural plate stages.

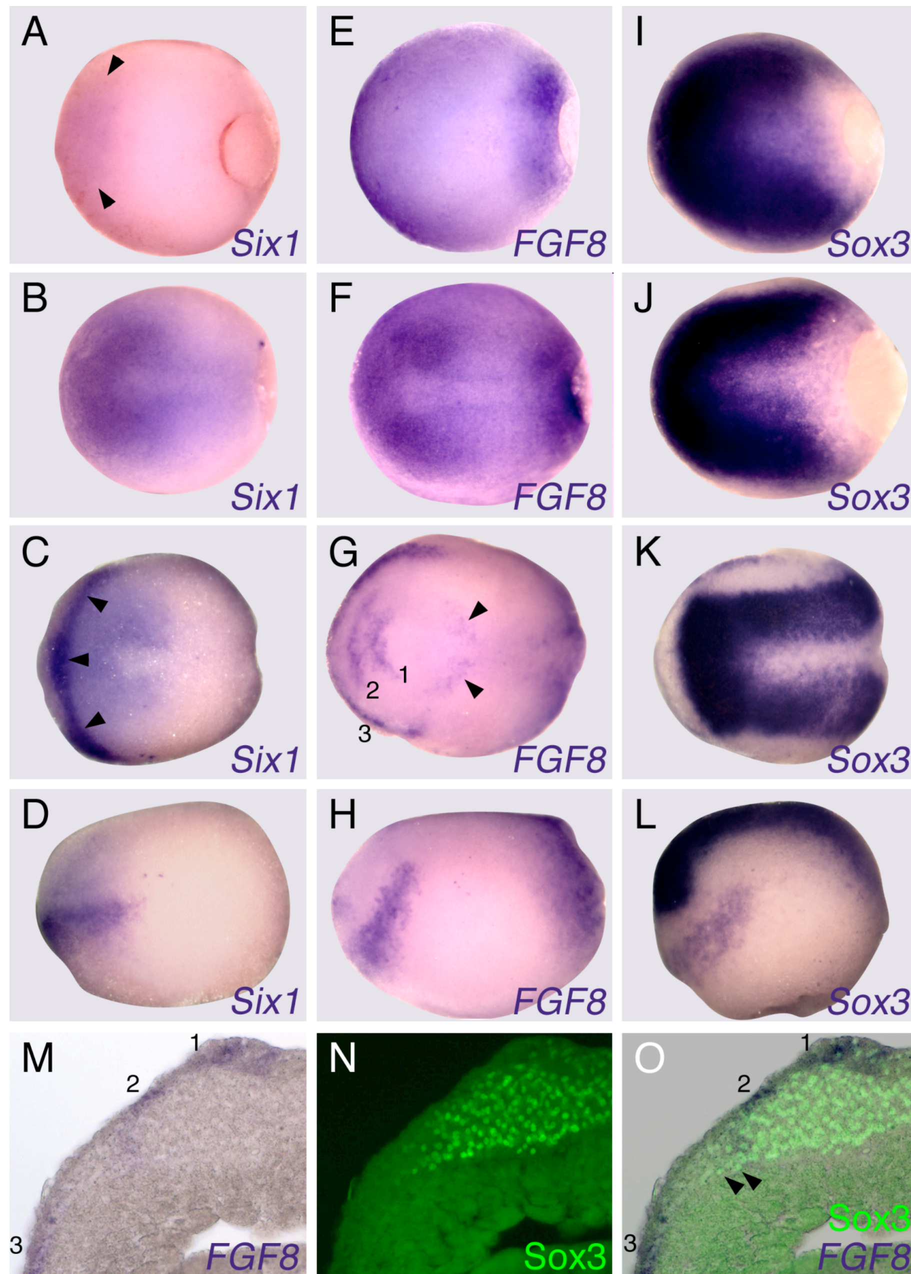

Fig. 9 Comparison of *Six1*, *FGF8*, and *Sox3* expression at stages 11.5 to 12.5. Embryos are shown in dorsal (**A**-**C**, **E**-**G**, **I**-**K**) or lateral (**D**,**H**,**L**) views with anterior to the left. **A-D** Weak *Six1* expression first appears in dorsoanterior ectoderm (arrowheads) at stage 11,5 (**A**), intensifies at stage 12 (**B**), and becomes restricted to a crescent around the anterior neural plate while being downregulated in the anterior neural plate at stage 12.5 (**C**,**D**). **E-H** In addition to circumblastoporal expression, a new domain of *FGF8* expression in dorsoanterior ectoderm appears around stage 11,5 (**E**) and becomes stronger by stage 12 (**F**). By stage 12.5, expression of *FGF8* is downregulated in dorsoanterior ectoderm except for the isthmic region (arrowheads) and three arcs (1-3) in and around the anterior neural plate (**G**,**H**). **I-L** *Sox3* expression, for comparison, is broadly expressed in dorsal and dorsoanterior ectoderm at stage 11.5 (**I**) becoming restricted to the developing neural plate at stage 12 (**J**). At stage 12.5 an additional crescent shaped expression domain of *Sox3* expression around the anterior neural plate appears (**K**,**L**). **M-O** Sagittal sections through embryo depicted in (**G**,**H**). Immunostaining for Sox3 (**N**,**O**) reveals that the two posterior arcs (1,2) of *FGF8* expression (**M**,**O**) are situated within the anterior neural plate, whereas the anteriormost arc (3) is located outside of the neural plate. Arrowheads indicate Sox3 immunopositive nuclei immediately rostral to the second arc of *FGF8* expression.

Thus, in order to test whether FGF8 is able to induce ectopic *Six1* expression a different strategy was pursued. Embryos were injected with *FGF8* mRNA in all blastomeres at the two or four cell stage. Animal caps of these injected embryos were then transplanted into the belly of host embryos. *Six1* expression of 29 embryos was analyzed. 19 of these embryos did not show any ectopic *Six1* expression around the graft, and the placodal expression domain of the host looked more or less normal. However, ten cases showed some effects (Tab. 4). Six of these cases have a broadened placodal *Six1* expression domain that runs towards the anterior edge of the graft (Fig. 10 B). The other four cases show a weak induction of ectopic *Six1* expression at the anterior border of the graft. Induction of *Six1* expression by FGF8 injected animal caps shows a similar pattern to that caused by *noggin* injected animal caps (Fig. 10 A). The most obvious effect after grafting of either *noggin* injected or *FGF8* injected animal caps is a broadening of the normal placodal expression domain of *Six1*. But this effect is much more pronounced after grafting noggin animal caps than after grafting FGF8 animal caps, although animal caps of *FGF8* injected embryos are also neuralized as confirmed by labelling with *Sox3* in 4 out of 6 cases (Tab. 4).

The inductive activity of FGF8 was then tested in the absence of other molecules. Heparin beads that had been soaked with FGF8 were implanted in the belly of host embryos. Ectopic expression of *Six1* is visible in none of the investigated nine cases (Fig. 10 E, Tab. 5) where the bead ended up in an appropriate position. However, one bead is situated just ventral and posterior to the placodal *Six1* expression domain and in this case the placodal expression domain of the host is slightly extended toward the bead. These results suggest that FGF8 alone is not sufficient to induce placodal *Six1* expression.

In order to check for synergistic effects of noggin and FGF8, embryos were coinjected with *noggin* mRNA and *FGF8* mRNA. Animal caps of these coinjected embryos were then transplanted into the belly of host embryos. Similar to the results after transplantations of animal caps that were injected with either *noggin* or *FGF8* alone, the normal placodal expression domain of *Six1* is broadened in 17 of these manipulated embryos (n=19). But in contrast to the grafts of animal caps injected with either *noggin* or *FGF8* alone, these *noggin* and *FGF8* coinjected caps additionally induced a strong ectopic expression of *Six1* localized at the border of the transplant in 14 cases. This ectopic expression lies in a crescent around the anterior part of the graft (Fig. 10 C).

noggin and *FGF8* coinjected caps are also neuralized as visualized by hybridisation with *Sox3* (n=5). Additionally, the placodal *Sox3* expression domain slightly runs to the anterior border of the graft.

noggin and FGF8 together were also tested directly for their ability to induce ectopic *Six1* expression in the absence of other molecules. Beads that had been soaked with noggin and FGF8 protein were implanted in the belly of host embryos. Eight beads ended up in an appropriate position. After in situ hybridisation, there is a clear ectopic *Six1* expression visible in the ectoderm adjacent and superficial to the bead in 6 cases (Fig. 10 F, Tab. 5). Thus, noggin and FGF8 together are sufficient to induce ectopic expression of *Six1* in anterior belly ectoderm.

In order to test whether FGF signalling is necessary for the induction of placodal *Six1* expression, mRNA of a dominant negative form of the receptor FGFR4 was injected into one or two blastomeres at the two or four cell stage, respectively. Based on its expression pattern (Riou et al., 1996; Golub et al., 2000) and functional studies (Ornitz et al., 1996), FGFR4 is the most likely candidate for the receptor mediating effects of FGF8 in placode induction. The same problems as mentioned above concerning overexpression of *FGF8* were observed. On high amounts of injected *dnFGFR4* (1ng) all embryos (n=27) show severe gastrulation effects (Tab. 6). After injection of a smaller amount of *dnFGFR4* mRNA (125 pg) or injection of DNA (50 pg) into one of eight blastomeres, embryos that were analyzed for the expression of *Six1* (n=33) show a reduced expression on the injected side in 15 cases (Tab. 6). In at least five cases the neural tube is reduced and the head is smaller on the injected side; additionally these embryos show spina bifida. Embryos analyzed for the expression of *Sox3* (n=31) show spina bifida in five cases. In four cases the placodal expression of *Sox3* is disturbed on the injected side. At least in one embryo, *Sox3* expression was also reduced in the neural tube (Tab. 6). The severe defects seen in some of these embryos, like spina bifida and reduced heads may be due to the fact that the FGF receptor 4 is not only able to mediate signalling by binding FGF8 but also other members of the FGF family, involved in several developmental processes. Because of these many side effects precluding the investigation of direct effects of *FGF* inhibition on placodal *Six1* expression, injections of *dnFGFR4* were not pursued further.

Whether FGF8 is necessary for the induction of placodal *Six1* expression was instead tested by injections of an FGF8 morpholino, thus specifically blocking translation of *FGF8* in the embryo. After in situ hybridisation with a probe for *Six1* (n=66), 65% of the em-

bryos show a dramatic reduction of the placodal *Six1* expression domain (Fig. 8 G–M). In three cases the placodal *Six1* expression is more or less completely lost (Tab. 7). Effects on the development of placodes were additionally investigated by hybridisation with a probe for *Eya1*. In nine out of 14 cases the injected side showed a slightly reduced expression of *Eya1* (Tab. 7). Analysis of *Eya1* labelled embryos turned out to be more difficult because the label is much weaker than *Six1* labels. Placodal *Sox3* expression is reduced also, as detected in 33 out of 60 embryos analyzed, in one case placodal *Sox3* expression is lost on the experimental side (Fig. 8 N, Tab. 7). Five additional cases show a disturbed expression pattern. 14 embryos were analyzed with a probe for *FGF8*, eight embryos show a reduction in the placodal expression domain of *FGF8* (Fig. 8 O). However, injection of the FGF8 morpholino did not reduce neural plate expression domains of *Sox3* or *FGF8* in embryos that were fixed at neural plate stages, there was even a slight broadening of the neural plate on the injected side.

I finally tested the ability of *FGF8* injected animal caps to rescue placodal *Six1* expression after unilateral removal of the neural plate. Embryos were co-injected with *FGF8* and *BMP4* or *FGF8* and *dnFGFR4* to avoid neuralization of the graft and transplanted unilaterally into the neural plate. Indeed, *FGF8* and *BMP4* co-injected animal caps were able to rescue *Six1* expression in 3 out of 5 cases (Tab. 4) and *FGF8* and *dnFGFR4* co-injected animal caps in 7 out of 11 cases (Tab. 4). However, co-injection of *BMP4* or *dnFGFR4* with *FGF8* was not able to prevent neuralization, as *FGF8* and *dnFGFR4* co-injected animal caps grafted into the neural plate express *Sox3* in 2 out of 2 cases (Fig. 10 L, Tab. 4) and also the embryo shown in Fig. 10 M with an animal cap graft co-injected with *FGF8* and *BMP4* shows Sox3 expression in cross sections within the graft, as revealed by immunohistochemistry (Fig. 10 O,P). Thus, it cannot be ruled out that rescue of *Six1* expression in these experiments may simply be due to neuralization of the graft.

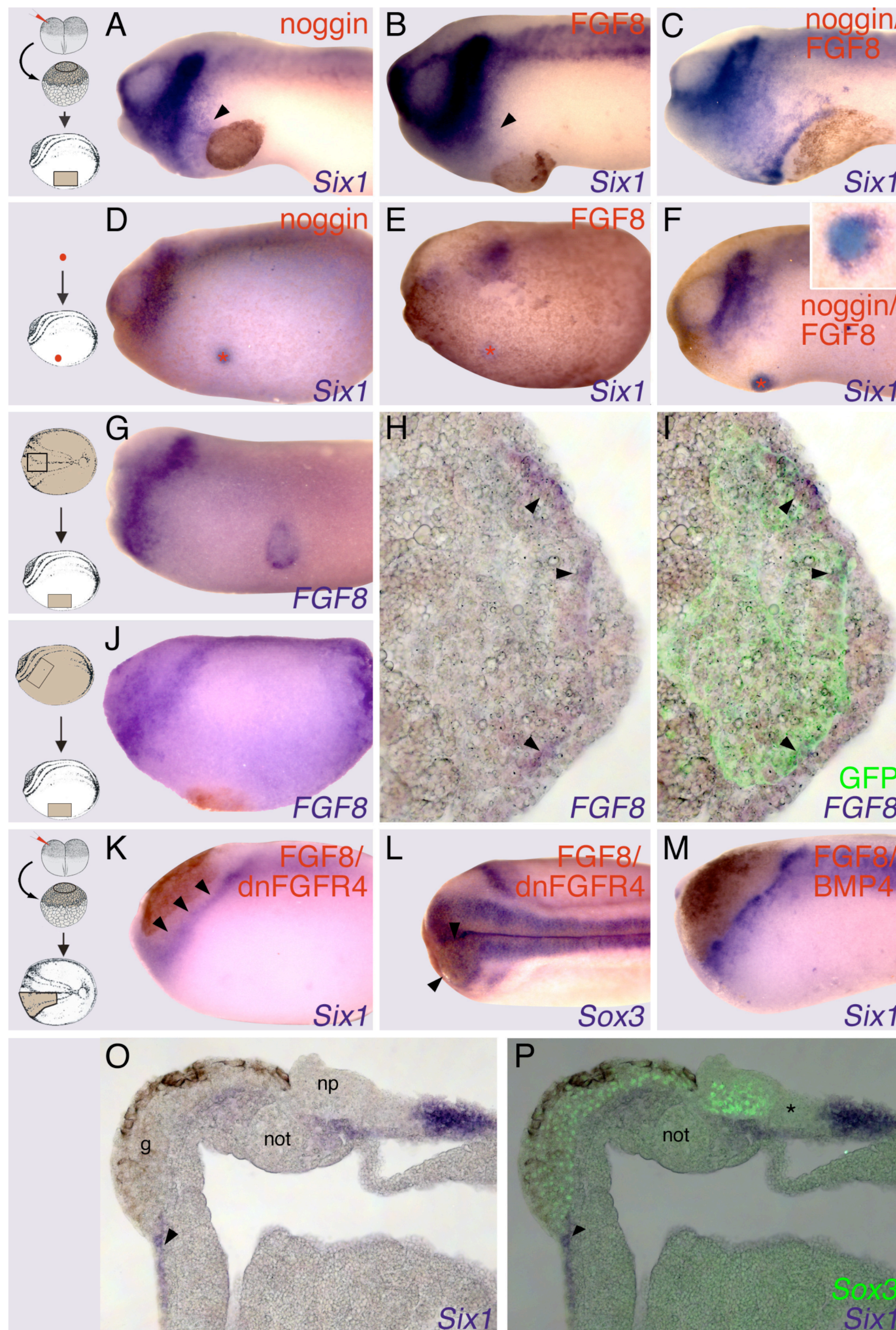

Fig. 10 *FGF8* can induce placodal *Six1* expression. Embryos are shown in lateral (**A-G**,**J**,**K**,**M**) or dorsal (**L**) views with anterior to the left. **A-C** Placodal *Six1* expression is drastically broadened towards anterior border of the graft after transplanting an animal cap from *noggin* injected embryos into belly ectoderm at stage 13 (**A**), whereas animal caps from *FGF8* injected embryos have little effect (**B**). **C** In addition to broadening of the normal placodal expression domain of *Six1*, strong ectopic *Six1* expression is induced at anterior border of the graft after transplantion of animal caps from *noggin* and *FGF8* co-injected embryos into belly ectoderm at stage 13. **D-F** Beads (red asterisks) soaked with noggin (**D**) or FGF8 (**E**) alone do not, but beads soaked with both noggin and FGF8 (**F**) do induce ectopic *Six1* expression in adjacent belly ectoderm (shown at higher magnification in insert). **G-I** *FGF8* continues to be expressed in the anterior neural plate after transplantation into belly ectoderm at stage 13. Transverse sections (**H**,**I**) through the embryo shown in (**G**) clearly show *FGF8* expression (**H**) confined to

the GFP-labeled graft (**I**). **J** In contrast, *FGF8* expression is not maintained in prospective placodal ectoderm after transplanting into belly ectoderm at stage 13. **K, L** Placodal *Six1* expression (**K**) is induced at the border of the graft (arrowheads) after unilateral replacement of the anterior neural plate at stage 13 by an animal cap from a *FGF8* and *dnFGFR4* co-injected embryo. Grafts are neuralized as indicated by *Sox3* expression (arrowhead) (**L**). **M-P** Placodal *Six1* expression is also induced at the border of the graft after unilateral replacement of the anterior neural plate at stage 13 by an animal cap from a *FGF8* and *BMP4* co-injected embryo. A transverse section through the embryo shown in (**M)** shows that *Six1* is induced just adjacent to the pigmented graft (g) in host ectoderm (arrowhead) (**O**). Immunostaining for Sox3 (**P**) reveals that the graft is neuralized. Note that only on the unoperated side the Sox3 positive neural plate is separated from placodal *Six1* expression by a zone free of label, occupied by the neural crest (asterisk).

3.3.3. Wnt proteins

In order to test whether posteriorizing factors like Wnt proteins may play a role in restricting placodal *Six1* expression to anterior portions of the neural plate border, a dominant negative form of *Wnt8*, *dnWnt8*, was coinjected either with *noggin* or *FGF8* alone, or with *noggin* and *FGF8*.

Transplanting animal caps from embryos that had been coinjected with *dnWnt8* and *noggin* led in eight out of ten cases to a broadening of the host´s placodal *Six1* expression domain (Tab. 4). This broadening is very similar to that observed after transplantion of animal caps injected with *noggin* alone. There was no ectopic expression domain found at the posterior end of the graft. When *dnWnt8* was co-injected with *FGF8* (n=23), no effect on placodal *Six1* expression was visible (Tab. 4).

After transplantation of animal caps coinjected with *dnWnt8*, *noggin* and *FGF8*, which are neuralized as verified by hybridisation with a probe for *Sox3* in seven out of seven cases, 35 of 41 embryos showed induction of *Six1* expression (Tab. 4). Eight of these cases possess a broadened placodal expression domain of *Six1* toward the anterior border of the graft. 16 additional cases show an ectopic expression domain at the anterior border of the graft. In 12 cases, however, ectopic expression is not restricted to the anterior border of the graft, but the ectopic expression domain lies ringshaped around the graft. Thus, *Wnt8* might indeed play a role in restricting the panplacodal *Six1* expression to the anterior part of the embryo.

Table 4

Expression of *Six1*, *Sox3* and *FGF8* after grafting animal caps of mRNA and/or DNA injected embryos

molecule tested	place of implan-tation (st13)	probe	number of experiments	number of individuals	induction of expression in host	reduction or loss of expression in host	expression within the graft
noggin	belly	*Six1*	4	29	23	0	0
		Sox3	3	17	12	0	17
dnBMPR	belly	*Six1*	6	34	8	0	0
		Sox3	2	12	6	0	12
FGF8	belly	*Six1*	3	29	10	0	0
		Sox3	1	6	4	0	4
noggin +FGF8	belly	*Six1*	2	19	17	0	0
		Sox3	1	5	5	0	5
dnwnt8 +noggin	belly	*Six1*	1	10	8	0	0
dnwnt8 +FGF8	belly	*Six1*	3	23	0	0	0
dnwnt8 +noggin +FGF8	belly	*Six1*	3	41	35	0	0
		Sox3	1	7	7	0	7
BMP	lateral placodal domain	*Six1*	1	7	0	7	0
FGF8 +BMP4	neural plate	*Six1*	1	5	3	2	0
FGF8+dn FGFR4	neural plate	*Six1*	4	11	7	4	0
		Sox3	1	2	2	0	2
uninjected control	belly	*Six1*	2	19	5	0	0
		Sox3	1	4	0	0	4
uninjected control	lateral placodal domain	*Six1*	1	7	0	0	1
		Sox3	1	7	0	0	7

Tabelle 5

Expression of *Six1* after implantation of beads soaked with different proteins or FGF inhibitor

molecule tested	place of implantation (st13)	probe	number of experiments	number of individuals	induction of expression in host	loss of expression in host
noggin	belly	*Six1*	4	14	0	0
FGF8	belly	*Six1*	3	9	(1) (broadening of host domain)	0
noggin+FGF8	belly	*Six1*	3	8	6	0
SU5402	lateral panplacodal domain	*Six1*	1	9	0	8
BMP4	lateral placodal domain	*Six1*	1	2	0	2
control heparin beads	belly	*Six1*	1	4	0	0
control affi-blue beads	lateral placodal domain	*Six1*	1	2	0	0
control resin beads	lateral placodal domain	*Six1*	1	2	0	0

Table 6

Effects of FGF8 and dnFGFR4 mRNA and DNA injections

molecule tested	conc. (pg)	probe	number of experi-ments	number of indivi-duals	induction of expression in placodal domain	reduction or loss of expression in placodal domain	broadening of the neural plate	gastru-lation defects
FGF8 mRNA	250	*Six1*	1	11	0	11	-	11 (strong)
	31,25	*Six1*	1	24	0	24	24	0
		Sox3	1	18	0	14	14	0
FGF8 DNA	25	*Six1*	1	11	0	4	10	1 (slight)
		Sox3	1	14	0	14	14	0
dnFGFR4 mRNA	1000	*Six1*	1	10	-	-	-	10 (strong)
		Sox3	1	17	-	-	-	17 (strong)
	125	*Six1*	1	13	0	7	0	5 (slight)
		Sox3	1	10	0	3	0	4 (slight)
dnFGFR4 DNA	50	*Six1*	1	20	0	8	0	0
		Sox3	1	21	0	1	1	1 (slight)

Table 7

Effects of FGF8 morpholino injections

probe	number of experiments	number of individuals	induction of expression in placodal domain	reduction or loss of expression in placodal domain	reduction of neural plate expression
Six1	7	66	0	43	not determined
both blasto-meres injected	1	2	0	2	not determined
Eya1	2	14	0	9	not determined
Sox3	5	60	0	33	0
FGF8	3	14	0	8	0

4. Discussion

4.1. *Six1* as a panplacodal marker gene

In this study, the transcription factor *Six1* was used as a marker for placodal tissue, because there is strong evidence that the early expression domain of *Six1* demarcates a panplacodal primordium from which all neurogenic placodes arise.

Fate mapping studies in chick embryos indicate that all placodes develop from a region that is located in a horseshoe-shaped domain around the anterior neural plate (Couly and Douarin, 1987; Couly and Douarin, 1990; Baker and Bronner-Fraser, 2001). Although there is no detailed fate map for the respective region in *Xenopus laevis*, existing fate mapping studies of amphibians suggest that all placodes arise from a common placodal primordium situated around the anterior neural plate also in amphibians (Röhlich, 1931; Carpenter, 1937; Keller, 1975; Eagleson and Harris, 1989; Eagleson et al., 1995).

In early neural plate stages of *Xenopus laevis*, the expression of *Six1* is situated in a horseshoe-shaped domain around the anterior neural plate and thus matches this domain (Fig. 9 C and D). Furthermore, during subsequent embryonic development, *Six1* continues to be expressed in all neurogenic placodes (Pandur and Moody, 2000; Ghanbari et al., 2001). This pattern of ectodermal expression confined to placodes is identical with the one described for *Six4* (Ghanbari et al., 2001) and *Eya1* (David et al., 2001).

Additionally, Six and Eya proteins have been shown to play a functional role in the development of placodes. *Six1* deficient mice show malformations of the inner ear and nasal cavity, structures that are formed from neurogenic placodes. The external and middle ears of such mice develop completely normal, but the inner ear, which is derived from the otic placode is disorganized (Laclef et al., 2003). Zheng et al. (2003) report that *Six1* deficient mice fail to form any structure, i.e. chambers and canals, of the inner ear (confirmed by Li et al., 2003), because its development is arrested at the otic vesicle stage. Additionally, the ganglia of the VIII. and the IX. cranial nerve are absent (confirmed by Ozaki et al., 2004). Similarly, in *Eya1* deficient mice, no inner ear structures form, because development of the inner ear arrests at the otic vesicle stage. Furthermore, the development of several cranial ganglia with placodal contributions is disturbed (Xu et al., 1999). Likewise, zebrafish mutants that lack functional Eya1 show defects in the development of the ear (Whitfield et al., 2002). Double *Six1*/*Eya1* null mice mutants show effects additional to

those observed in single mutants, among them a 5-10 fold reduced size of the pituitary (Li et al., 2003). The fact that not all placodes are affected by mutations of *Six1* and *Eya1*, respectively, may be due to other Six and Eya proteins assuming redundant functions in the development of placodes. Recently, it has indeed been shown in our lab that Eya1 in *Xenopus laevis* embryos influences neurogenesis in all neurogenic placodes. Overexpression of *Eya1* leads to an increase of the expression of *NeuroD*, a marker for neuronal differentiation. Conversely, inhibition of *Eya1* by morpholino injections results in a disturbance of neuronal differentiation indicated by the disturbed expression pattern of *NeuroD* in all neurogenic placodes (Völker, 2003). These results suggest that Six and Eya proteins are essential for developmental processes and properties that are common to all placodes, such as morphogenetic movements and neurogenesis.

Six and Eya proteins, as transcription factors and coactivators, respectively, are known to form complexes with each other and act together in order to initiate transcription of their target genes (Heanue et al., 1999; Relaix and Buckingham, 1999; Ohto et al., 1999; Kawakami et al., 2000; Epstein and Neel, 2003; Li et al., 2003). Moreover, *Six* and *Eya* together with *Pax* and *Dach* genes belong to a common regulatory network that was first discovered in *Drosophila* compound eye development. In the meantime, it has become clear that this Pax-Six-Eya-Dach regulatory network is reused in other developmental processes and the formation of various organs and tissues. In vertebrates, this network, for example, plays an important role during myogenesis by driving common processes underlying the formation of muscle cells, like proliferation of myogenetic progenitor cells or activation of the myogenic program through Myf5 and/or MyoD (Relaix and Buckingham, 1999; Kawakami et al., 2000).

The fact that Six and Eya are members of a regulatory network that is reused in different developmental contexts, being essential for the development of common properties of certain cell types, together with the ectodermal expression of *Six1*/*Six4* and *Eya1*, which is confined to placodes, and with the defects seen in mutants, led to the suggestion that *Six1*/*Six4* and *Eya1* are involved in developmental processes common to all placodes and thus demarcate a panplacodal primordium in the early neural plate embryo.

In this study, generally *Six1* was used as a panplacodal marker gene, because it is a strong reliable probe. Nevertheless, some individuals were also hybridised with a probe for *Eya1* as another panplacodal marker gene and *Sox3* which labels a subset of placodes (for details see below).

4.2. Time window for the induction of placodes

4.2.1. Placodal *Six1* expression is specified and committed at neural fold stages

As a basis for further investigations, first, the time window for the induction of placodes was determined. At the beginning of its expression during gastrula stages, *Six1* is visible in anterior dorsal ectoderm of the embryo (Fig. 9 A and B). As development proceeds *Six1* is partly downregulated in the neural plate and becomes restricted at early neural plate stages to the horseshoe-shaped domain around the anterior neural plate, designated here as the panplacodal primordium (Fig. 9 C and D). However, this restriction to the presumed precursor region of all neurogenic placodes does not reveal when this portion of ectoderm is specified or even committed to express *Six1*. In order to investigate the time of specification of placodes, ectoderm adjacent to the lateral neural folds encompassing a major part of the placodal expression domain of *Six1* was explanted at different developmental stages and held in culture until at least neural fold stages and afterwards investigated for the expression of *Six1*. The stage at which the placodal *Six1* expression is specified, was considered as that stage at which more than 50% of the investigated explants show *Six1* expression. According to this criterion, the findings of the present study indicated that placodal *Six1* expression is specified at early neural fold stages (Fig. 2 B).

Commitment of the placodal *Six1* expression occurs slightly later as revealed by transplanting ectoderm from the putative placodal region into the belly of a stage host embryo, which represents a foreign environment for this part of ectoderm. At late neural fold stages more than 50% of the investigated transplants showed *Six1* expression, indicating the onset of commitment of placodal *Six1* expression (Fig. 2 D).

Specification and commitment for placodal *Six1* expression occurs rather late compared with other ectodermal cell types, viz. the neural plate and the neural crest. In *Xenopus*, neural tissue is known to become specified during gastrulation (Knecht and Bronner-Fraser, 2002). Explants from the prospective neural folds of *Xenopus laevis* embryos showed expression of the neural crest marker *Xsnail* even when dissected at stage 12 (Mayor et al., 1995) and of the neural crest marker *Xslug* when dissected at stage 13 (Mancilla and Mayor, 1996). Thus, the neural crest forming domain is specified at early

neural plate stages in contrast to early neural fold stages when the panplacodal domain is specified. Unfortunately, nothing is known about when the neural crest is committed.

4.2.2. Signals for the induction of placodal *Six1* decline after neural plate stages

The time during which signals for the induction of placodal *Six1* expression are present, was elucidated by orthotopically transplanting unspecified stage 13 ectoderm of the putative placodal region into two different stages, viz. stage 13 and stage 16. As expected when transplanted into a stage 13 donor, the *Six1* expression pattern looks normal, because, as demonstrated above, the placodal *Six1* expression is not yet specified at that early neural plate stage and, thus, placodal *Six1* inducing signals must still be present. Importantly, the normal pattern after orthotopic transplantations performed at that early stage also shows, that the transplantation procedure itself does not disturb the placodal expression of *Six1* (Fig. 3 A).

In contrast, in stage 16 hosts the expression pattern of placodal *Six1* is disturbed, indicating that they do not possess an adequate amount of *Six1* inducing signals (Fig. 3 B). These experiments suggest, that the availability of signals for the induction of placodal *Six1* declines after stage 13. Nevertheless, as discussed above, commitment for the placodal expression of *Six1* does not occur before late neural fold stages. Thus, the amount of signals present at stage 16 (early neural fold stage) may only be enough to maintain *Six1* expression in ectoderm that has already been exposed to inducing signals for a protracted time period. Alternatively, it is possible that signals that are necessary for the induction of *Six1* decline after stage 13, whereas additional signals that account for the maintenance of placodal *Six1* expression persist longer.

4.2.3. Competence for the induction of *Six1* persists at least until neural tube closure

Ectodermal competence to respond to the *Six1* inducing signals was tested by grafting belly ectoderm from donor embryos of different stages into ectoderm adjacent to the lateral neural folds of a stage 13 host embryo. As shown by the preceding experiments, signals to induce *Six1* expression are present at this stage of the host. *Six1* expression was visible within grafts from all stages investigated. It is important to mention, that the expression pattern within the graft sometimes was not normal, indicating that competence to respond to *Six1* inducing signals is compromised although not lost. But even in the oldest embryos investigated, viz. stages 18 to 22, only 40% of the embryos showed severe abnormalities in the expression of *Six1* within the graft. Thus, ectodermal competence for the induction of panplacodal *Six1* expression persists much longer than inducing signals, viz. at least until neural tube closure (Fig. 3 E and F). This observation is in agreement with findings of Schlosser and Northcutt, 2001 who demonstrated that competence for lateral line placode induction in the axolotl also persists until early tailbud stages.

Importantly, competence of the ectoderm to respond to *Six1* inducing signals persists much longer than ectodermal competence to respond to inducing signals for neural plate and neural crest. Neural competence of the ectoderm is lost at the end of gastrulation (Kintner and Dodd, 1991; Servetnick and Grainger, 1991). Similarly, Mancilla and Mayor (1996) showed via conjugation experiments that ectodermal competence to respond to neural crest inducing signals derived from mesoderm is lost at the end of gastrulation. However, competence to form neural crest in response to mesodermal signals is not equivalent to general competence of the ectoderm to form neural crest, because in vivo additional signals from other tissues may play a role in the induction of neural crest. Signals from the mesoderm are required for the induction of the neural crest, but additionally it has been shown that an interaction between the neural plate and epidermis also plays a role in neural crest induction (Moury and Jacobson, 1989; Mancilla and Mayor, 1996; Bastidas et al., 2004). In order to address this concern, Mancilla and Mayor (1996) transplanted animal caps into the prospective neural crest region and showed that ectodermal competence to respond to inducing signals is lost at stage 12 also in this experimental context. Surprisingly however, belly ectoderm was able to respond to neural crest inducing signals from

anterior neural plate grafts until after the end of gastrulation at stage 13 (Mancilla and Mayor, 1996).

The fact that the induction of placodes takes place much later in development (viz. during neural fold stages) than the induction of neural plate (during gastrulation) and neural crest (during early neural plate stages) contradicts certain models concerning the induction of neural plate, neural crest, placodes and epidermis which suggest a common mechanism for the induction of all these different ectodermal cell types. This will be discussed later in a separate paragraph.

4.3. Tissues involved in placode induction

4.3.1. Neural plate ectoderm is sufficient to ectopically induce *Six1* and necessary for its induction in vivo

In amniotes as well as anamniotes it is possible to induce neural crest at experimentally created neural plate boundaries (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Moury and Jacobson, 1989, 1990; Mancilla and Mayor, 1996). In the axolotl, it has been shown that neural folds formed at experimentally created boundaries between neural plate and epidermis (Moury and Jacobson, 1989, 1990). The appearance of melanocytes indicated that these ectopic neural folds form neural crest cells. Similar experiments performed in *Xenopus laevis* confirmed these results. When Mancilla and Mayor (1996) grafted the anterior neural plate into lateral epidermis at early neural plate stages and looked for the induction of the neural crest marker *slug*, ectopic expression of *slug* was visible in a ring at the boundaries of the graft, both within the graft and in the surrounding ectoderm.

As the placodal *Six1* expressing domain borders the neural crest laterally and is situated just adjacent to the neural plate anteriorly, in this study, the neural plate was investigated concerning its ability to induce placodal gene expression in competent ectoderm. Indeed, a piece of anterior neural plate that was transplanted into epidermal ectoderm, induced expression of placodal *Six1* (Fig. 5 B) and placodal *Eya1* (data not shown) in a ring around the graft. Similar experiments performed by Woda et al. (2003) and Glavic et al. (2004) confirm the ability of neural plate ectoderm to induce *Six1*. Additionally, Glavic et al. (2004) showed induction of *Xiro1* by grafting the anterior neural plate into belly ecto-

derm. *Xiro1* is expressed in a subset of placodes, viz. profundal, trigeminal, lateral line, otic and epibranchial placodes from early neural plate stages until midtailbud stages when it starts to get downregulated in the epibranchial placodes (Schlosser and Ahrens, 2004). Thus, *Xiro1* may be considered a marker for the posterior part of a panplacodal primordium. Together with the result reported here, these observations strongly indicate that anterior neural plate is sufficient to induce a panplacodal domain in belly ectoderm. Nevertheless, in contrast to what has been observed for the induction of the neural crest marker *slug* (Mancilla and Mayor, 1996) in the experiments reported here, the ectopically induced *Six1* expression was always confined to host ectoderm. These results suggest that signals emanating from the neural plate induce placodal *Six1*/*Eya1* expression in the epidermis and thus an interaction between neural plate ectoderm and epidermis is involved in the induction of placodal *Six1* as it is in the induction of the neural crest (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; reviewed in Knecht and Bronner-Fraser, 2002).

However, Mayor and Aybar (2001) suggested that neural plate ectoderm may induce ectopic *slug* expression because it merely mimicks the functions of signals from the earlier acting organizer. Indeed, the present study shows, that it is also possible to induce ectopic expression of placodal *Six1* by grafting the organizer into belly ectoderm of a host embryo (Fig. 4 A). However, the pattern of ectopic expression of *Six1* induced by the organizer does not look exactly the same as ectopic expression of *Six1* induced by the neural plate. The latter forms a ring around the transplant, whereas ectopic *Six1* expression induced by the organizer is typically observed solely at the anterior border of the graft.

The results obtained by grafting the organizer into belly ectoderm were confirmed in vitro by additionally performing conjugation experiments. The dorsal marginal zone (DMZ), which is part of the organizer, induces the expression of *Six1* in unspecified animal cap ectoderm (Fig. 4 B). Similar results have been reported by Mayor et al. (1995) for the neural crest marker *slug*, whereas Bonstein et al. (1998) showed that in comparison with the dorsal lateral marginal zone (DLMZ), the DMZ was only rarely able to induce the expression of *slug* or neural crest derived melanocytes in conjugated animal caps.

The results reported here can be interpreted in two different ways: either the organizer directly induces the expression of *Six1*, or it operates indirectly via the induction of a neural plate.

Although it cannot be ruled out that the organizer plays a direct role in very early steps of placode induction, it cannot be the only source of *Six1* inducing signals in vivo, because

as shown above, specification and commitment of the placodal *Six1* expressing domain takes place later in development, viz. from early neural fold stages on, thus, after disappearance of the organizer.

However, it is possible that axial mesoderm derived from the organizer retains some organizer characteristics and thus, may be the source of *Six1* inducing signals in vivo. In contradiction to that hypothesis, the expression of placodal *Six1* looks normal after removal of the organizer derived chordamesoderm (Fig. 6 C), indicating that this tissue is not necessary for the induction of placodal *Six1*.

Thus, in contrast to what has been proposed by Marchant et al., 1998, the neural plate is unlikely to mimick the effects of organizer derived signals, but rather vice versa the organizer and the DMZ may induce *Six1* indirectly via inducing a neural plate, which is a source for *Six1* inducing signals. A transplantation series performed by Bastidas et al. (2004) suggests that direct interactions between neural plate and epidermis are similarly involved in neural crest induction. When grafting animal caps into different regions of the embryo, they observed that *slug* induction was always accompanied by the induction of the neural plate marker *Sox2*, independently of where the graft was placed, indicating that also in these grafts neural crest is induced via an interaction of neural plate ectoderm and epidermis.

Here I show in addition, that the neural plate, in contrast to the organizer derived chordamesoderm, is necessary for the induction of placodal *Six1* expression, because placodal *Six1* expression was almost completely lost after removal of the anterior neural plate on one side of the embryo (Fig. 5 J). Additionally, when the removed neural plate was replaced by belly ectoderm, *Six1* expression was induced not at the normal location within the host ectoderm as one would expect if the induction was independent of neural plate signals in vivo, but more dorsally, within the graft, indicating that induction of *Six1* takes place at the newly created boundary of neural plate and epidermis. Nevertheless, the induced expression was not confined to the edges of the graft but was distributed rather homogenously (Fig. 5 K).

The ability of the neural plate to induce placodal expression of *Six1* is not distributed ubiquitarily along its anterior posterior axis. Conforming to the location of placodal *Six1* expression in the embryo around the anterior neural plate, only anterior neural plate was able to induce ectopic expression of *Six1* (Fig. 5 B and G). Mancilla and Mayor (1996) report the same result for the expression of the neural crest marker *slug*, whereas they were

able to induce ectopic expression of *slug* only in 2 out of 28 cases after grafting posterior neural plate into lateral epidermis of host embryos.

The results reported here additionally show, that the most anterior part of the neural plate, the anterior neural ridge (ANR) is also able to induce *Six1* in competent ectoderm. In some of the ANR grafts, *Six1* expression was not only visible outside the graft in host belly ectoderm, but also inside the graft (Fig. 5 F). This can be easily explained, because the anterior boundary of the ANR could not be determined exactly and, thus, grafts often must have contained parts of the normal placodal *Six1* expression domain situated just in front of the ANR. The ANR is not exclusively responsible for the induction of the placodal *Six1* domain in vivo, because placodal *Six1* expression was reduced but never lost after removal of the ANR leaving the rest of the anterior neural plate intact (Fig. 5 I). These results may indicate that the ANR is responsible for induction of a part of the placodal *Six1* expression, but not for the induction of the whole placodal *Six1* domain.

It would be interesting to analyze whether the anterior neural ridge is also able to induce neural crest markers, because neural crest is not found in this most anterior part of the neural folds, but can be induced there by posteriorizing signals (Villanueva et al., 2002), which either may compensate for missing neural crest inducing signals, or may lift repression of neural crest induction by antagonizing factors.

Besides the spatial differences in the ability of the neural plate to induce *Six1*, the results reported here also reveal temporal differences in the inductive ability of the neural plate. Whereas stage 13 neural plate was able to induce ectopic expression of placodal *Six1*, younger neural plate grafts from stage 12 embryos were only rarely able to do so (Fig. 5 H). These results indicate that the neural plate is not able to induce placodal *Six1* expression before the end of gastrulation, matching the timing of initial upregulation of *Six1* expression in the placodal region.

4.3.2. Lateral endomesoderm is not sufficient to ectopically induce *Six1* but necessary for its induction in vivo

As discussed in the preceding paragraph, axial mesoderm is not necessary for the induction of placodal expression of *Six1*. I additionally investigated the role of non-axial mesoderm in the induction of placodal expression of *Six1*, because this type of tissue has been shown

to be involved in the development of the neural plate as well as the neural crest. Whereas the neural plate in *Xenopus* is induced by axial mesoderm, non-axial mesoderm is also involved in patterning the central nervous system (Bang et al., 1997, 1999; Muhr et al., 1997). During neural crest development, the paraxial mesoderm seems to be essential for its induction (Bonstein et al., 1998; Marchant et al., 1998) as well as for proper emigration of neural crest cells (Sela-Donenfeld and Kalcheim, 2000).

The experiments reported here show that endomesoderm that directly underlies the lateral expression domain of placodal *Six1*, encompassing dorsal lateral plate mesoderm and the subjacent endoderm, is likewise necessary for its induction, because in embryos where the lateral endomesoderm has been removed, placodal *Six1* expression is strongly reduced or even lost (Fig. 6 B). This is similar to what has been reported for neural crest, because the expression of *slug* is strongly reduced and embryos lack melanocytes when the paraxial mesoderm underlying the neural crest forming region has been removed at gastrula stages (Bonstein et al., 1998; Marchant et al., 1998). Furthermore, *slug* expression is reduced in embryos dorsalized by LiCl treatment, which possess a reduced DLMZ (Bonstein et al., 1998). These results indicate that lateral endomesoderm is necessary for the formation of placodes, whereas paraxial mesoderm is necessary for the neural crest induction.

Paraxial mesoderm is not only necessary but also sufficient to induce neural crest in experimental contexts as reported by several authors. Bonstein et al. (1998) report that DLMZs combined with animal caps induce the expression of *slug* and *twist*, a cranial neural crest marker, and the formation of melanocytes within the animal cap. Furthermore, in these experiments the DLMZ was shown to be a more potent neural crest inducer than the DMZ. Additionally, ventral marginal zones that had been dorsalized either by *noggin* or by a dominant negative receptor for BMP4 were able to induce melanocytes in competent ectoderm (animal caps). These results are confirmed by Marchant et al. (1998) and Monsoro-Burq et al. (2003), because these authors also show that conjugates of DLMZ with animal caps showed a strong expression of *slug* in the ectodermal part (Marchant et al., 1998). In older stages, viz, stage 12,5, notochord, mesoderm proximal to the notochord, and the region proximal to the lateral plate including some cells of the lateral plate were able to induce *slug* (Marchant et al., 1998).

In contrast, as shown here, the lateral endomesoderm is not sufficient to induce *Six1* expression in competent ectoderm, because after transplanting this tissue into belly ectoderm of host embryos, ectopic expression of *Six1* was not induced in any case (Fig. 6 A).

In conjugation experiments, the DLMZ was also tested for its ability to induce *Six1*. However in contrast to *slug* induction, the DLMZ was able to induce *Six1* expression only in 50% of the cases and rather weakly, whereas the DMZ induced a stronger *Six1* expression in all cases (Fig. 4 B,C). As discussed above, the DMZ is proposed to induce *Six1* expression indirectly via induction of a neural plate. The residual inductive activity of the DLMZ reported here may be attributed to the fact that DMZ and DLMZ are not two well seperated entities so there may be some overlap in inducing activities, as it was also proposed for the low *slug* inducing activity of the DMZ by other authors (Monsoro-Burq et al., 2003). Thus, those cases in which the DLMZ induced *Six1* expression in animal caps may have contained some of the inducing activity of the DMZ, indirectly inducing expression of *Six1* suggesting that neither DLMZ nor older lateral mesoderm is able to induce *Six1*.

The results described in this section, identified the anterior neural plate as well as the lateral endomesoderm as tissues involved in the induction of placodes. The anterior neural plate is sufficient to induce *Six1* expression in experimental contexts and is necessary for its induction in vivo, the lateral endomesoderm is additionally necessary but not sufficient for placodal *Six1* indcution. In the following section the nature of the inducing molecules involved shall be discussed.

4.4. Molecules involved in placode induction

4.4.1. BMP-inhibition is necessary but not sufficient for the induction of placodal *Six1*

As discussed above, I showed that it is possible to induce ectopic *Six1* expression by grafting the anterior neural plate into belly ectoderm, indicating that placodes are induced by an interaction between neural plate and the adjacent epidermis. As already mentioned, several authors suggested that such interactions are also essential for the establishment of the neural crest region. However, Marchant et al., 1998 postulated that grafts of the neural plate could be a source of BMP inhibitors like chordin (Sasai et al., 1994) or noggin (Lamb et al., 1993) coming from co-grafted underlying dorsal mesoderm (Smith et al., 1993; Sasai et al., 1994) or the neural plate itself (Knecht and Harland, 1997), because inhibition of BMP

is believed to be essential for neural crest induction (Morgan and Sargent, 1997; LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998).

More specifically, Marchant et al. (1998) suggested that not the interaction between neural plate and epidermal ectoderm may be essential for the induction of the neural crest, but a certain value of BMP, which could be established by BMP inhibitors diffusing from an anterior neural plate graft. This gradient model (see below) is based on the observation, that it is possible to induce the neural crest marker *slug* in animal caps by injecting a dominant negative form of a BMP receptor, thereby lowering the level of BMP activity in this tissue. Moreover, *noggin* injected animal caps were able to induce *slug* in conjugated uninjected animal caps (Marchant et al., 1998). Additionally, when a piece of ectoderm of *noggin* injected embryos was grafted into lateral epidermis of uninjected host embryos, *slug* was induced either within the graft or in host tissue depending on the concentration of *noggin*, suggesting that a certain level of BMP inhibitors is sufficient to induce neural crest cells.

In the present study, I show that not only the anterior neural plate can induce placodal *Six1* expression when grafted into belly ectoderm, but that it is also possible to induce *Six1* by transplanting animal caps injected with *noggin* (Fig. 7 B). Thus, lowering BMP concentration in the ectoderm may be indeed important for the induction of placodal *Six1* expression.

However, an alternative explanation of these experimental data is also possible, because *noggin* is known to be capable of dorsalizing not only mesoderm (Jones et al., 1996) but also ectoderm and thus acts as a neural inducer. The induced neural tissue is of an anterior character (Smith and Harland, 1992; Lamb et al., 1993; Knecht et al., 1995). Neuralization of *noggin* injected animal caps in the present study was demonstrated by labelling with the neural plate marker *Sox3*. The whole graft was labelled intensely (Fig. 7 A). Thus, contrary to what has been proposed by Marchant et al. (1998), it is equally possible that ectopic expression of *slug* as well as ectopic *Six1* expression around *noggin* injected animal caps, is induced secondarily via induction of an anterior neural plate within the graft. The observation that *slug* is induced either within the grafted animal cap or in host tissue depending on the concentration of *noggin* may also be attributed to induction of the neural plates of different sizes, depending on the availability of *noggin* to inhibit BMPs.

If neuralisation of the transplanted animal cap were exclusively responsible for the induction of ectopic *Six1* expression, the same inductive activity would emanate from an

animal cap graft that had been injected with a dominant negative receptor for *BMP4*. Injection of *dnBMPR* also neuralises the graft, shown here via labelling with *Sox3* (Fig. 7 C), but in contrast to noggin, dnBMPR is not diffusible. The results reported here show, that *dnBMPR* injected animal caps induced *Six1* expression only weakly and rarely (Fig. 7 D), indicating that neuralisation of the graft alone is not sufficient for the induction of ectopic *Six1*.

Together, these finding indicate that BMP inhibition seems to play a role in the induction of *Six1*. However, several results of the present study indicate that BMP inhibition alone is not sufficient to induce *Six1* expression. *Six1* expression that is observed after grafting *noggin* injected animal caps, is always confined to the anterior border of the graft (Fig. 7 B). Additionally, the induced expression domain appears always as a broadening of the normal placodal *Six1* expression domain, indicating that the anterior portion of the embryo demarcates a permissive region for the inducing activity of *noggin* injected caps, whereas posterior portions of the embryo are not permissive. In contrast, ectopic *Six1* expression induced by transplanting the anterior neural plate into an equivalent region of host embryos occurs in a ring around the entire graft (Fig. 5 B). This suggests that neural plate ectoderm is the source of an additional factor that permits induction of ectopic *Six1* expression also at posterior portions of the embryo. This assumption is also supported by the bead experiments reported here. Beads soaked with *noggin* alone were not able to induce expression of *Six1* in neighbouring ectoderm (Fig. 7 E).

However, even though BMP inhibition alone is not sufficient, it seems to be necessary for the induction of placodal *Six1* expression. When the amount of BMP in the prospective placodal region is too high, expression of *Six1* is disturbed, as shown by grafting *BMP4* injected animal caps or implanting beads soaked with BMP4 into the respective position. BMP4 diffusing from the graft or the bead, respectively, inhibits expression of *Six1*, leading to a white ring around the *BMP4* injected animal cap or a white spot above the implanted bead (Fig. 7 F and G). Moreover, FGF8, which was tested as a second candidate molecule for its inducing activity (see below), is not able to induce ectopic *Six1* expression in belly ectoderm by itself, which was shown by implanting beads soaked with FGF8 (Fig. 10 E). But when the BMP level is lowered in the surrounding ectoderm by soaking beads with noggin in addition to FGF8, ectopic *Six1* expression is induced (Fig. 10 F).

The experiments reported here do not support the model that a certain BMP threshold has to be reached to induce *Six1*, as proposed by several authors (see below). As shown

here, belly ectoderm that was grafted to the neural plate unilaterally, expressed *Six1* (Fig. 5 K), indicating that the BMP level, present in the neural plate is compatible with the induction of placodal *Six1* expression. Conversely, animal caps that were transplanted into the placodal region expressed *Sox3* but not *Six1* (Fig. 7 H and I), suggesting that the BMP level existing in the placodal domain allows neural induction if grafted ectoderm is still neurally competent. Thus, the experiments reported here suggest that it is not necessary to establish intermediary concentrations of BMP to induce *Six1*, but that inhibition of BMP leads to the establishment of a permissive environment for the induction of placodes.

4.4.1.1. ***Cerberus*, a candidate molecule mediating BMP-inhibition during placodal *Six1* induction in vivo**

In the present study, *noggin* was used as a BMP inhibiting protein, but *noggin* is just one member of a set of different BMP antagonists present in the embryo. The results reported here do not necessarily indicate that *noggin* is the factor, which is responsible in vivo for lowering the BMP level in the future placodal region and, thus, allowing the induction of *Six1* expression. In fact, *noggin* is expressed only in axial mesoderm, which was shown here to be unimportant for the induction of placodal *Six1* expression, and so seems to be an unprobable candidate. A more likely candidate molecule is *cerberus* (Bouwmeester et al., 1996), which is also capable of binding BMPs (Piccolo et al., 1999) thereby inhibiting its function in vivo (Silva et al., 2003).

cerberus is strongly expressed in gastrula and neurula stages in *Xenopus* embryos. During early neural plate stages it is expressed in a broad anterior domain in the dorsolateral endomesoderm (Bouwmeester et al., 1996), which was shown here to be necessary for *Six1* induction. Thus, *cerberus* may be one molecule responsible for the induction of placodal *Six1* expression, being expressed in the right place at the right time.

Also, functionally *cerberus* seems to be related to the formation of anterior structures. Embryos, which overexpress *cerberus* are anteriorized with large cement glands and missing trunk-tail mesoderm. Additionally, *cerberus* overexpressing embryos possess cyclopic eyes, indicating that the eye field failed to split because of missing signals from the prechordal plate. Importantly overexpression of *cerberus* is able to induce anterior neuroectodermal structures like brain and olfactory placodes (Bouwmeester et al., 1996). Converse-

ly, morpholino-mediated knock down of *cerberus* results in an inhibition of head formation (Kuroda et al., 2004). In contrast to *noggin*, *cerberus* does not only inhibit BMP signalling but is also an antagonist of the nodal and Wnt signalling pathways (Glinka et al., 1997; Piccolo et al., 1999; Piccolo et al., 1999), which may account for its head inducing ability. Whereas nodal and Wnts are required for trunk formation, in head formation these pathways need to be inhibited. It is possible that the Wnt inhibiting function of *cerberus* also plays a role in the induction of placodal *Six1* expression, restricting the placodal domain to anterior parts of the embryo (see below), but this has to be further investigated.

To summarize, it is proposed here, that *cerberus* may be the molecule accounting for the necessity of the lateral endomesoderm for placodal *Six1* expression in vivo, but this needs to be tested in further studies.

4.4.2. Fibroblast Growth Factors

The experiments presented thus far indicate that at least one other inducing factor in addition to a BMP inhibiting protein is involved in the induction of the placodal *Six1* expression domain. Another group of molecules, necessary for a diverse array of developmental processes and discussed to be involved in the induction of other ectodermal cell types, viz. the neural plate and the neural crest, are fibroblast growth factors (FGFs) (Kengaku and Okamoto, 1993; Launay et al., 1996; reviewed in Dono, 2003; Lamb and Harland, 1995).

4.4.2.1. FGFs in embryonic development

In early embryonic development, FGFs seem to play a role in the process of gastrulation, during which the three different germ layers are formed Nutt et al., 2001 Frazzetto et al., 2002. Moreover, FGFs have been shown to directly induce mesoderm (Kimelman and Kirschner, 1987; Slack et al., 1987; Isaacs, 1997). Later on during organogenesis, FGFs are involved in several developmental processes, e.g. in limb bud formation, where they mediate its induction as well as the proliferation inducing activity of the apical ectodermal ridge (Crossley et al., 1996; reviewed in Powers, 2000; Ornitz and Itoh, 2001).

Several members of the FGF family appear to be involved in neural crest formation because they are able to induce neural crest when combined with BMP inhibitors or transcription factors. Animal caps injected with *bFGF* and *Sox2*, which is downstream of the BMP inhibitor chordin, developed neural crest derived melanophores, whereas neither of the molecules alone induced neural crest derived cells (Mizuseki et al., 1998). In agreement with these findings, it was possible to isolate the neural crest marker FoxD3 from animal caps treated with a combination of chordin and bFGF (Sasai et al., 2001). Additionally, animal caps cultured in noggin and bFGF expressed *slug*, whereas animal caps that had been treated with either protein alone did not (Mayor et al., 1995). Interestingly, FGF8 injected animal caps grown in isolation showed expression of a subset of neural crest markers, as reported by Monsoro-Burq et al. (2003), although it may be possible that neural crest markers were induced indirectly by neural tissue, because FGF8 is also a potent neural inducer (Fürthauer et al., 1997; Koshida et al., 2002; Pera et al., 2003; Phillips et al., 2004). In vivo injection of *FGF8* led to an expansion of the expression domain of *slug*. Additionally, in conjugation with animal caps, paraxial mesoderm as a source of FGF8 induced the expression of *slug* (Monsoro-Burq et al., 2003).

Moreover, *FGF8* has been shown to play a role in the development of the telencephalon (Eagleson and Dempewolf, 2002; Lupo et al., 2002) and the isthmic organizer. Animal caps, co-injected with *chordin* and *cer-S* to inhibit mesoderm formation, which were conjugated with beads soaked with FGF8, showed a strong induction of the ventral forebrain marker *Xnkx2.1* compared to *chordin* and *cer-S* co-injected animal caps not conjugated with beads. Additionally, the dorsal marker *eomes* was activated, whereas the dorsal marker *Xemx* was not or only slightly induced. Control animal caps did not show any expression of either of these dorsal markers (Lupo et al., 2002). These results indicate that FGF8 may play a role in patterning the telencephalon of *Xenopus laevis* by promoting ventral forebrain fates and additionally function within the dorsal telencephalon. These observations were confirmed by Eagleson and Dempewolf (2002), who implanted beads soaked with FGF8 into the anterior neural ridge (ANR). The ANR is proposed to be the source of FGF8 signalling responsible for the patterning of the telencephalon. When FGF8 was locally overexpressed in this region, expression of *XBf-1* as a marker for the telencephalon was shifted posteriorly. This suggests that a certain level of FGF8 implies positional information, in that it is optimal for the induction of *Bf1*. Moreover, embryos possessed fused telencephali (Eagleson and Dempewolf, 2002). An involvement of FGF8 in the deve-

lopment of the telencephalon of zebrafish embryos was also shown by Shinya et al., 2001, who report a reduced expression of the subpallial marker *Nk2.1b* after injection of a FGF8 morpholino. Loss of function of the *FGF8* gene in zebrafish can also be investigated in the ace mutant which lacks functional FGF8 (Reifers et al., 1998). In agreement to what has been observed after FGF8 morpholino injection into zebrafish embryos, ace mutants show a reduction in the expression of *Nk2.1b* in the telencephalon. Additionally, *Lim6* and *Lim1*, markers for neuronal differentiation were also reduced in the telencephalon (Shanmugalingam et al., 2000).

Besides its role in patterning of the telencephalon, FGF8 was shown to be necessary for the formation and function of the midbrain-hindbrain boundary (MHB). In chick embryos, misexpression of FGF8 induces ectopic expression of markers for the midbrain-hindbrain boundary (reviewed in Dono, 2003). In zebrafish ace mutants which lack functional FGF8, the MHB fold and the primordium of the cerebellum are missing (Reifers et al., 1998). Moreover, FGF8 morpholino injections into zebrafish embryos result in a loss of *Pax2* expression in the isthmic region (Maroon et al., 2002). In *Xenopus*, *FGF8* is also expressed at the level of the isthmus (Christen and Slack, 1997, Pera et al., 2002, present study) and was shown to be ectopically induced by Otx2, a protein proposed to be involved in the establishment of the midbrain-hindbrain boundary (Tour et al., 2002). Moreover, the induction of *En2* expression, as a marker for the MHB, observed after conjugation of animal caps injected with *Otx2* and *Gbx2*, respectively, fails when *Gbx2* is co-injected with a dominant negative receptor for FGF signalling (XFD) (Glavic et al., 2002). Similar results were obtained in Keller explants, containing dorsal ectoderm and the organizer, which show an anteroposterior pattern of the neural plate similar to that of whole embryos. When FGF signalling is blocked via XFD in such transplants, the expression of *En* is lost (Holowacz and Sokol, 1999). These results suggest, that also in *Xenopus*, FGF signalling, most likely mediated by FGF8, is necessary for the establishment of the midbrain-hindbrain boundary.

Additionally, FGF8 is involved in the development of the otic placode (Adamska et al., 2001; Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002; Liu et al., 2003; Phillips et al., 2004), as will be discussed in a separate section.

In the present study, *FGF8* was investigated concerning its potential role in the induction of placodal *Six1* expression, because besides its expression within the prospective placodal region itself, *FGF8* is expressed in two stripes in the anterior neural plate (Christen

and Slack, 1997; Eagleson and Dempewolf, 2002; present study), which was shown here to be necessary for the induction of placodal *Six1* (see above).

4.4.2.2. FGF8 is sufficient to induce *Six1* when BMP is inhibited

In order to investigate the potential of FGF8 to induce placodal *Six1*, animal caps of *FGF8* injected embryos were transplanted into belly ectoderm of host embryos. In one third of the embryos that received *FGF8* injected animal cap grafts, an effect on the placodal expression of *Six1* was visible. Similar to what has been observed after grafting of *noggin* animal caps, the main effect of *FGF8* grafts was a broadening of the normal placodal *Six1* expressing domain (Fig. 10 B), although this effect was less intense and occurred in a much smaller percentage of embryos than after grafting *noggin* injected animal caps. These results indicated that FGF8 alone is not sufficient to induce placodal *Six1* expression, and this was further confirmed in the bead assay. Beads soaked with FGF8 alone were not able to induce ectopic *Six1* expression (Fig. 10 E), similar to what was observed after implantation of beads soaked with noggin alone (see above). These results are in agreement with observations that FGF8 soaked beads implanted in zebrafish embryos were only able to expand ear vesicles, whereas ectopic ears were not induced (Léger and Brand, 2002). However, Phillips et al. (2004) report ectopic expression of otic markers, e.g. *Pax8* and formation of ectopic otic vesicles in a few cases after spatially restricted overexpression of *FGF8* in zebrafish. In the chick embryo, beads soaked with FGF8 that had been implanted posterior to the otic vesicle induced an increased size of the otocyst and enlarged the otic expression domains of *cNkx5-1*, *SOHo1* and *Pax2*, but did not lead to the formation of ectopic ears (Adamska et al., 2001). When FGF8 soaked beads were implanted into the lateral anterior neural ridge of *Xenopus laevis* embryos, an increase in the expression of *XBf1* was observed in the epibranchial placodes, but no ectopic epibranchial placodes were induced (Eagleson and Dempewolf, 2002). Together with the observations in the present study, these results indicate that FGF8 may be involved in the induction of placodal tissue, but that it might act in combination with other signals.

As discussed in the preceding section, BMP inhibition is necessary but not sufficient for the induction of placodal *Six1*. Thus, in a following series of experiments the ability of FGF8 to induce placodal *Six1* expression in combination with BMP inhibition was investi-

gated. Indeed, *noggin* and *FGF8* co-injected animal caps induced expression of placodal *Six1* when transplanted into belly ectoderm of host embryos (Fig. 10 C). Importantly, in contrast to animal caps injected with *noggin* or *FGF8* alone, co-injected animal caps not only broadened the normal placodal *Six1* expression domain towards the anterior border of the graft, but induced a strong ectopic expression domain immediately adjacent to the graft in nearly all cases investigated. Thus, *noggin* and *FGF8* co-injected animal caps were able to partly mimick the inductive activity coming from neural plate transplants, because they induced an ectopic expression domain of *Six1* adjacent to the graft, but this induction was restricted to the anterior border of the graft. Moreover, in contrast to beads soaked with either FGF8 or noggin alone, beads soaked with both noggin and FGF8 were able to induce *Six1* in belly ectoderm (Fig. 10 F). This suggests that the effect of noggin and FGF8 directly induces *Six1* and that the expression of *Six1* observed after grafting *noggin* and *FGF8* co-injected animal caps is not the indirect result of activity of other signals emanating from the graft. Together, these experiments show that FGF8 in combination with noggin is sufficient to induce placodal *Six1* expression in competent belly ectoderm.

4.4.2.3. *FGF8* overexpression perturbs neural development and cannot be analyzed for placodal defects

Although in vivo effects of *FGF8* overexpression on the neural crest had been reported by Monsoro-Burq et al. (2003), who describe a strong increase in *slug* expression after injection of *FGF8* mRNA, in the present study overexpression of *FGF8* was found to be unsuitable for the investigation of direct effects on the placodal *Six1* expression domain. Injections of high amounts of *FGF8* mRNA led to severe gastrulation defects, due to perturbation of posterior mesoderm induction (Hardcastle et al., 2000) making it impossible to investigate its effects on the placodal *Six1* expression domain. Hardcastle et al. (2000) also report spina bifida in embryos injected with *FGF8* at higher doses (270pg). Although the concentration is comparable to the one used here, the defects reported by Hardcastle et al. (2000) are not as strong as those observed in the present study, but also suggest an interference of FGF8 with the induction of posterior mesoderm. In contrast to other members of the FGF family (Isaacs, 1997), FGF8 is a poor mesoderm inducer (Christen and Slack, 1997), but perturbs mesoderm induction (Isaacs, 1997; Hardcastle et al., 2000).

Although embryos injected specifically into the dorsal animal blastomere of an eight cell stage with a lower amount of *FGF8* mRNA or with *FGF8* DNA gastrulated properly, these embryos also did not allow to investigate the direct effects of FGF8 on placodal *Six1* expression, because the neural plate was often expanded at the expense of the placodal domain. In agreement with this observation, other authors also report a dorsalisation effect after injection of *FGF8* or *FGF3* mRNA or DNA in zebrafish embryos, leading to an expansion of the neural plate at the expense of placodal ectoderm (Phillips et al., 2004; Koshida et al., 2002; Fürthauer et al., 1997).

The observed enlargement of the neural plate at the expense of placodal and epidermal ectoderm most likely reflects the ability of FGF8 to potently induce (Chalmers et al., 2002; Pera et al., 2003) and/ or posteriorize neural tissue (Christen and Slack, 1997). Due to these collateral effects after *FGF8* injection, it was not possible to distinguish between direct and indirect effects of *FGF8* overexpression on the placodal *Six1* expression.

4.4.2.4. Requirement of FGF8 for placodal *Six1* induction

In order to investigate whether FGF8 is necessary for placodal *Six1* expression, FGF signalling was blocked in the embryo via injecting a dominant negative form of FGFR4. This receptor has been shown to possess a high affinity to FGF8 (Ornitz et al., 1996) and is expressed at neural plate stages in a domain located at the anterior border of the neural plate (Riou et al., 1996; Golub et al., 2000), likely encompassing the prospective placodal *Six1* expression domain, making it the most likely candidate receptor for mediating FGF8 signalling in the prospective placodal domain. However, *FGFR4* expression is not restricted to this placodal domain and furthermore is most likely to transduce additional signals from other members of the FGF family (Ornitz et al., 1996). Thus, one could predict that interference with FGFR4 will lead to more than merely placodal defects. In embryos injected with *dnFGFR4* in the present study, defects like spina bifida could be detected, even when *dnFGFR4* was injected spatially restricted into dorsal animal blastomeres at the eight cell stage. Severe gastrulation defects after injection of *dnFGFR4* are also confirmed by others (Hongo et al., 1999; Monsoro-Burq et al., 2003), and were also reported for a dominant negative form of FGFR1 (Xu, 1997). Moreover, the development of the nervous system seems to be disturbed in *dnFGFR4* injected embryos, as in some injected embryos the neu-

ral tube looks reduced in size. Injection of *dnFGFR4a* was also shown to strongly suppress the expression of the panneural marker *Nrp-1* in addition to a reduction of the anterior neural markers *Bf-1*, *Rx-1* and *En-2* (Hongo et al., 1999). When signalling via FGFR4 is more broadly inhibited, embryos lack part of the telencephalon and show reduced heads with fused eyes (present study and Hongo et al., 1999). Thus, due to its many side effects, over–expression of *dnFGFR4* was not suitable to investigate whether signalling by FGF8 is necessary for the induction of placodal *Six1* expression.

A different method to block FGF signalling in the embryo is incubation in the FGF inhibitor SU5402. An advantage of this approach is the possibility to perform inhibition of FGF signalling through defined time periods and to observe different effects of FGF inhibition at different developmental stages. Embryos that had been treated during gastrulation in the present study did not properly complete gastrulation and failed to neurulate, in accordance with what was observed after injections of high amounts of *dnFGFR4*. Disturbing FGF signalling in these early stages of development leads to severe defects because FGFs promote a variety of developmental processes including mesoderm formation and gastrulation movements (reviewed in Slack et al., 1996). In contrast, embryos incubated at stages from the end of neurulation to early tailbud did not develop obvious defects, because most basic developmental processes, in which FGF signalling is involved, were already completed. In order to test whether FGF signalling is necessary for placodal *Six1* induction, embryos were incubated in SU5402 from stage 12 to 20, the time window during which induction of placodal *Six1* expression is completed (see above). SU5402 treated embryos showed reductions in the expression pattern of placodal *Six1* (Fig. 8 A), indicating that FGF signalling is necessary for the induction of placodes. However, results observed after incubation with SU5402 were rather variable, most likely due to insufficient penetration (see also Lombardo and Slack, 1998). Moreover, incubation during stage 12-20 also led to side effects outside of the placodal region. Embryos seemed to be shortened in their anterioposterior axis, which is in agreement with the observation of Amaya et al. (Amaya et al., 1991, 1993). In zebrafish embryos, incubation in SU5402 from 60% epiboly onwards led to smaller eyes and brains with absent commissures (Shanmugalingam et al., 2000). Implantation of beads soaked with SU5402 into the placodal domain allowed to avoid such side effects, because of their locally restricted action. Moreover, this experimental procedure ensures that SU5402 inhibits FGF signalling directly in the desired tis-

sues. *Six1* expression was strongly reduced in ectoderm just overlying the bead while the surrounding expression pattern looked normal (Fig. 8 D and E).

These observations indicate that FGF signalling is necessary for the induction of placodal *Six1*, but they do not show whether FGF8 is the member of the FGF family, which is necessary for placodal *Six1* induction. In order to specifically test the latter hypothesis, a morpholino against *FGF8* was injected unilaterally into embryos at the two cell stage. Expression of *Six1* was strongly reduced or lost after injection of the morpholino (Fig. 8 G–M). Placodal expression of another panplacodal marker, *Eya1* was also reduced as was placodal expression of *Sox3* (Fig. 8 N) an additional marker gene labelling a subset of placodes and placodal expression of *FGF8* (Fig. 8 O) itself. In contrast, injections of a non-specific control morpholino showed typically no defects. Thus, the defects observed after FGF8 morpholino injections can be really attributed to the specific loss of functional FGF8 signalling. In contradiction to what was observed after inhibition of FGF8 in *Xenopus* in the present study, the expression of markers for the early panplacodal domain, such as *Dlx3*, *Eya1* and *Six4.1* is not affected in zebrafish ace mutants, which lack functional FGF8 (Léger and Brand, 2002; Shanmugalingam et al., 2000). The unaffected expression pattern of *Dlx3*, *Eya1* and *Six4.1* in ace mutants may be due to other members of the FGF family exerting redundant functions. That different members of the FGF family can have redundant functions has been shown for example for FGF8 and FGF3 during the development of the otic placode (Phillips et al., 2001; Maroon et al., 2002). Unfortunately, mouse mutants of *FGF8* die early due to gastrulation defects and could not be analyzed for later placodal defects (Sun et al., 1999)

Results obtained in the present study strongly indicate that in *Xenopus laevis* FGF8 is necessary for the induction of placodal *Six1* expression. Nevertheless, one has to keep in mind that FGF8 also seems to be involved in the development of the central nervous system as discussed above and some observed effects on placodal *Six1* expression may thus be due to secondary effects resulting from a disturbed development of the neural plate. However, when FGF8 morpholino injected embryos where analyzed with a probe for *FGF8*, they showed a reduction of *FGF8* expression in the placodal domain, whereas the expression pattern of *FGF8* in the neural plate was not disturbed (Fig. 8 O). Similarly, only placodal *Sox3* expression was deficient in FGF8 morpholino injected embryos, while *Sox3* expression in the neural plate was largely normal (Fig. 8 N). This indicates that inhibiting FGF8 activity directly suppresses the induction of placodal marker genes whereas pattern-

ing of the neural plate is not influenced except for a slight broadening of the neural plate after morpholino injections.

4.4.2.5. Source of FGF8 activity

Because of the expression pattern of *FGF8* described above with two domains within the anterior neural plate (Fig. 9 G, M–O) and the observed expression of *FGF8* in neural plate grafts transplanted into belly ectoderm (Fig. 10 G–I), it is most likely that the anterior neural plate, which was shown here to be necessary for the induction of placodal *Six1*, is the source of FGF8 for placodal *Six1* induction. Although Monsoro-Burq (2003) suggested non-axial mesoderm to be the source of FGF8 for neural crest induction, and lateral endomesoderm was also shown to be necessary for the induction of placodal *Six1* in the present study, it is not likely to be a major source of FGF8 involved in placodal *Six1* induction, because expression of *FGF8* in mesodermal tissue is restricted to a very posterior part of the embryo.

Unfortunately, rescue experiments performed in this study could not be analyzed unequivocally. Animal caps of *FGF8* and *dnFGFR4* or *FGF8* and *BMP4* co-injected embryos that replaced the neural plate unilaterally rescued *Six1* expression in the panplacodal domain (Fig. 10 K and M), but in spite of injection of *dnFGFR4* or *BMP4*, respectively, these caps were neuralized (Fig. 10 L and P). Thus, rescue of placodal *Six1* induction may be due to the neuralization of the grafted animal caps rather than to direct effects of the injected *FGF8*.

4.4.3. Wnts

As discussed above, *noggin* and *FGF8* together were sufficient to induce *Six1* when *noggin* and *FGF8* co-injected animal caps were grafted into belly ectoderm as well as in the bead assay. However, *noggin* and *FGF8* co-injected animal caps could just partly mimick the induction of *Six1* observed after transplantation of the anterior neural plate. Whereas anterior neural plate grafts were able to induce *Six1* in adjacent host ectoderm at the anterior as well as at the posterior border of the graft, *noggin* and *FGF8* co-injected animal caps were

only able to do so at their anterior border. Thus, it seems that the embryo is permissive for the induction mediated by *noggin* and *FGF8* only in its anterior part, suggesting that there is a factor emanating from the anterior neural plate that establishes this permissiveness also in more posterior parts of the embryo after transplantation into belly ectoderm.

For the induction of the neural crest it has been postulated that the restriction of this cell type to more posterior parts of the embryo with exclusion from anterior parts may be due to an anteroposterior gradient of posteriorizing molecules, e.g. *Wnt8* (Villanueva et al., 2002). Indeed it has been shown that a gradient of the Wnt/β-catenin pathway seems to be established in the presumptive neural plate of *Xenopus* embryos at gastrula stages (Kiecker and Niehrs, 2001). Additionally, there are several indications that Wnts may play a functional role in the induction and/or maintenance of neural crest cells (reviewed in Yanfeng et al., 2003; Wu et al., 2003). Overexpression of different members of the Wnt family leads to an expansion of the neural crest domain. After injection of *Wnt3a*, *Wnt8* or *Wnt1* mRNA, an increased expression of *slug* was detected in embryos at neurula stages (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Deardorff et al., 2001). Similarly, injection of *Wnt7b* expands the expression domain of *twist*, another marker for the neural crest (Chang and Hemmati-Brivanlou, 1998). Moreover, other members of the canonical Wnt signalling pathway are also able to expand the neural crest forming domain. Overexpression of the Wnt receptor frizzled3 increased the expression of *slug* (Deardorff et al., 2001). Similar results were obtained after injection of *Wnt1* (Deardorff et al., 2001), or of the co-receptor LRP6 (Tamai et al., 2000). In the canonical pathway of Wnt signalling, binding of Wnt to its receptor leads via dishevelled to the inhibition of degradation of β-catenin catalysed by GSK-3β (Jones and Jomary, 2002; Yanfeng et al., 2003). In agreement with that, an increase of β-catenin leads to a dramatic increase in *slug* expression (LaBonne and Bronner-Fraser, 1998). Conversely, a surplus supply of GSK-3β resulted in a decrease or even absence of the expression of *Krox20* in migrating neural crest cells (Saint-Jeannet et al., 1997).

Wnts were not only shown to increase the expression of several neural crest markers in vivo, but also to induce these markers in competent ectoderm in vitro. In animal caps neuralized by the injection of *noggin* or *chordin*, co-injection of *Wnt1* or *Wnt3a* reduced the induction of the pan-neural marker *nrp1* as well as of the forebrain marker *Otx1*, which are induced by *noggin* alone, but induced the expression of the neural crest markers *Krox20*, *AP2*, *slug* and *FoxD3* (Saint-Jeannet et al., 1997; Sasai et al., 2001) as well as neural crest

derivatives like melanocytes (Deardorff et al., 2001). Similarly, *Wnt7b* was able to induce *slug* expression when co-injected with *noggin* (Chang and Hemmati-Brivanlou, 1998) and *Wnt8* was shown to induce *Pax3* in neuralized animal caps (Bang et al., 1999).

Besides their potential to expand the neural crest domain when overexpressed or to induce neural crest in neuralized ectoderm, Wnts were also shown to be necessary for the proper induction and maintenance of the neural crest. Injections of dominant negative forms of *Wnt8*, *frizzled3* and *LRP6* or injections of a morpholino against frizzled3 reduced the expression of *slug* on the experimental side (LaBonne and Bronner-Fraser, 1998; Tamai et al., 2000; Deardorff et al., 2001). Additionally, mouse mutants which lack both Wnt1 and Wnt3a show reduced expression of *AP2* and an altered formation of the neural crest derived parts of the trigeminal and vagal nerves and the glossopharyngeal ganglion (Ikeya et al., 1997).

Whereas Wnt signalling is necessary for the development of the neural crest, it has to be suppressed for the formation of the head including anterior parts of the central nervous system (reviewed by Lake and Kao, 2003). In zebrafish embryos, overexpression of *Wnt1* or *Wnt8* in the margin of the neural plate (ANB) leads to an expansion of the isthmic *Pax2* domain, whereas expression of telencephalic markers *FGF8* and *Emx1* was inhibited (Houart et al., 2002). Conversely, misexpression of *dickkopf1* (*dkk1*), a Wnt antagonist, in *Xenopus* embryos leads to enlarged head domains, with larger eyes and telencephalon, demonstrated by the expanded expression domains of the telencephalic marker *Bf1* and the anterior neural plate markers *Xanf1* and *Otx2*. In contrast, expression of more posterior markers, like *En2*, and *Krox20* is reduced in these embryos (Kazanskaya et al., 2000; Kiecker and Niehrs, 2001). These results are similar to those observed after injection of the Wnt antagonist *Frzb1*, that cause embryos with shortened anteroposterior axes and enlarged heads, eyes and cement gland (Leyns et al., 1997; Pera and Robertis, 2000). Likewise in zebrafish, injection of a morpholino against *wnt8* reduced expression of posterior neural markers such as *huC*, *isl1*, *krx20* and *ephA4/rtk1* (Erter et al., 2001), whereas anterior markers viz. *otx2* and *opl* have expanded expression domains (Lekven et al., 2001).

Comparable to their possible role in restricting the neural crest to posterior domains of the neural folds and allowing the expression of anterior neural markers only at anterior positions within the neural plate, Wnts could also play an opposite role in restricting the panplacodal domain to the anterior part of an embryo. In the present study, *dnWnt8* was used to investigate a possible role of Wnts in spatial restriction of placodal tissue. When

animal caps of embryos co-injected with *dnWnt8* and *noggin* were grafted into belly ectoderm, the observed induction of *Six1* was not distinguishable from that observed after grafting animal caps injected with *noggin* alone. In contrast when *dnWnt8* was co-injected with *noggin* and *FGF8*, indeed some embryos showed a ringshaped expression of *Six1* around the graft, indicating that *Six1* was also induced at the posterior border. However, only 29% of all the embryos, in which an ectopic expression domain of *Six1* was induced, showed expression also at the posterior border of the graft. Additionally, grafts were not localized within the embryo at exactly the same position. Ectopic *Six1* expression induced by *noggin* and *FGF8* co-injected animal caps was detected in ectoderm positioned anteriorly to the fourth somite. This is nearly the same posterior limit of *Six1* expression as was observed after grafting *noggin*, *FGF8* and *dnWnt8* co-injected caps. Only three out of 35 cases investigated showed ectopic expression of *Six1* behind the fourth somite, i.e. up to the fifth somite. Therefore, it is possible that the observed induction of *Six1* at the posterior border of *noggin*, *FGF8* and *dnWnt8* co-injected animal caps may rather be due to a more anterior position of the graft than to the activity of dnWnt8. Thus, the present results do not clearly establish a role of Wnt signalling in anterior restriction of the panplacodal domain. Further experiments are necessary to elucidate the importance of Wnt signalling in placodal *Six1* induction.

4.5. Models for the induction of placodes

4.5.1. BMP gradient model

Because different ectodermal cell types, viz. neural plate, neural crest and placodes are localized precisely immediately adjacent to each other, several models have been proposed that connect the induction of these different ectodermal derivatives. One of these models is the BMP gradient model.

At early developmental stages, *BMP4* is expressed ubiquitously in the embryo but during gastrulation *BMP4* expression becomes restricted to the ventral part of the embryo (Fainsod et al., 1994; Wilson and Hemmati-Brivanlou, 1995). During gastrulation, BMP inhibitors - among them noggin, chordin and follistatin - diffuse from the organizer and its derivative, the chordamesoderm. These diffusible molecules directly bind BMPs and in-

hibit BMP signalling in the overlying ectoderm, thus allowing the formation of the neural plate. The BMP inhibitors may spread throughout the entire embryo and establish a gradient of BMP, with high levels in ventral parts and low levels in dorsal parts of the embryo (Mayor et al., 1995; Knecht and Harland, 1997; Neave et al., 1997; Jones and Smith, 1998; Marchant et al., 1998; Dale and Jones, 1999), which according to the gradient model carries positional information, so that certain thresholds of BMP lead to the development of different ectodermal fates (Sasai and Robertis, 1997; Wilson et al., 1997; Chang and Hemmati-Brivanlou, 1998; Barth et al., 1999; Dale and Wardle, 1999; Aybar and Mayor, 2002; Tribulo et al., 2003). However, there is no unequivocal evidence supporting this simple model of induction of different ectodermal cell types.

The BMP gradient model for dorsal-ventral patterning of the ectoderm implies that the development of different ectodermal cell fates takes place simultaneously. However, as I show here, the induction of placodes takes place significantly later in development than the induction of the neural plate and the neural crest (see above), arguing against this simple model for the induction of different ectodermal cell fates.

Furthermore, the gradient model apparently cannot account for neural crest induction. A certain threshold of BMP does not seem to be sufficient to induce neural crest fate in competent ectoderm (LaBonne and Bronner-Fraser, 1998; Streit and Stern, 1999). Animal caps of embryos injected with *chordin*, *noggin* or *dnBMPR* that were cultured until stage 17 when normally neural crest markers such as *slug* and *snail* are strongly expressed in the embryo, never expressed neural crest markers in the absence of epidermal or neural markers. However, as discussed above, neural crest is known to be induced by the juxtaposition of neural plate and epidermis. Thus, the expression of neural crest markers observed at low doses of chordin may be due to the result of interactions between neural tissue induced by *chordin* and cells of the animal cap with an epidermal character. Moreover, these embryos were analyzed with RT-PCR and the observed expression of the neural crest markers was below a level detectable by in situ hybridisation and thus, below levels of the endogenous *slug*/*snail* expression. Higher doses of chordin never led to the expression of neural crest markers, but induced the expression of NCAM in the absence of *epidermal keratin*, suggesting that the whole animal cap had been neuralized by chordin injection and not enough epidermal cells were left to interact with the induced neural plate to further induce neural crest (LaBonne and Bronner-Fraser, 1998).

In apparent contrast to these observations, Marchant et al. (1998) report, that they were able to induce either neural plate or neural crest markers in animal caps depending on the injected concentration of a dominant negative receptor for BMP (dnBMPR). At high doses of *dnBMPR* only the neural plate marker *Sox2* was induced, whereas at lower concentration the neural crest marker *slug* was induced. Nevertheless, neural plate markers were also induced, though only weakly, at that specific BMP concentration. Marchant et al. (1998) propose that these results reflect the ability of certain threshold values of BMP to specifically induce different ectodermal fates, but these results can also be interpreted in a different way. As the lowest concentration of *dnBMPR* used in their experiments induced *slug* only in a very few cases and *Sox2* was also induced rarely at that concentration (Marchant et al., 1998), the probability for expression of the neural crest marker may be linked to the probability of neural plate induction and consequently to the opportunities for interactions between induced neural plate and epidermal tissue. This is supported by Bastidas et al. (2004), who report that induction of *slug* in animal cap transplants, grafted into ectoderm of host embryos was always accompanied by the induction of the neural plate marker *Sox2*.

Additional experiments reported by Marchant et al. (1998), which suggested neural crest induction by a certain BMP threshold can also be explained differently. In conjugates of different parts of mesoderm with animal cap ectoderm, expression of *slug* was induced at varying distances from the mesodermal part of the conjugate. It could be observed that axial mesoderm induced expression of the neural crest marker *slug* at a distance from the mesodermal part of the conjugate, whereas lateral mesoderm induced *slug* just adjacent to it. Marchant et al. (1998) reasoned that axial as well as lateral mesoderm are sources of BMP antagonists that diffuse into the animal cap part of the conjugate, inducing neural crest when reaching the precise threshold value of BMP. However, it has been reported that in contrast to paraxial mesoderm axial mesoderm is a very poor neural crest inducer but a strong inducer of neural plate tissue (Marchant et al., 1998; Bonstein et al., 1998). Thus, the results reported by Marchant et al. (1998) could also be due to direct induction of neural plate tissue by the axial mesoderm. In a second step neural crest is induced at the newly created border between the induced neural plate and cells of the animal cap with an epidermal character. In contrast, paraxial mesoderm may induce neural crest directly via a different inducing molecule, resulting in *slug* expression just adjacent to it. For that reason, experiments presented thus far, which at a first glance support the role of a BMP gradient for the development of the neural crest can also be interpreted in an alternative fashion and

do not provide unambiguous support for this proposed mechanism of ectodermal patterning.

Glavic et al. (2004) favour the model of a BMP gradient also for the induction of placodes, but results of the present study strongly argue against applicability of the BMP gradient model to placode induction. As mentioned above, belly ectoderm, grafted to replace the neural plate unilaterally, strongly expressed *Six1* (Fig. 5 K). Thus, the low level of BMP present in the neural plate region of an embryo seems to be compatible with the induction of placodal tissue. Conversely, animal caps that had been transplanted to the placodal region expressed *Sox3* but not *Six1* (Fig. 7 H and J), confirming preceding observations that competent ectoderm grafted to the lateral epidermis of a host embryo express *Sox2* (Mancilla and Mayor, 1996). Moreover, Bastidas et al. (2004) showed that animal caps expressed *Sox2* when grafted to anterior, posterior as well as ventral regions of the embryo. These findings indicate that the BMP level in the placodal region and even in more ventral parts of the embryo seems to be low enough to be compatible even with neural induction. These observations strongly indicate that differences in competence rather than in the BMP level are crucial for establishing different ectodermal cell fates (Servetnick and Grainger, 1991).

Together these results suggest that a gradient of BMP is not sufficient to establish different ectodermal cell fates in a dorsoventral order. Moreover, additional molecular signals would also be necessary to provide positional information along the anterior-posterior axis of the embryo, because the BMP gradient model cannot explain why placodes only develop in the head region and why neural crest do not form in the most anterior part of the embryo. To solve this problem for the induction of the neural crest, Villanueva et al. (2002) postulated a model for neural crest induction, which combines the dorsoventral gradient of BMP with an anteroposterior gradient of other morphogens, such as eFGF and Wnt8, which are expressed in the ventrolateral mesoderm. During gastrulation these signals are confined to posterior parts of the embryo and from there could spread through the embryo establishing an anteroposterior gradient of Wnt8 and/or eFGF. According to the model proposed by Villanueva et al. (2002), a certain threshold of BMP induces a neural plate border region with an anterior character and afterwards a gradient of posteriorizing molecules specifies posterior parts of this neural plate border to become neural crest (Aybar and Mayor, 2002). As discussed above, Wnts could also play a role in restricting the placodal *Six1* expression to anterior parts of the embryo, which needs to be further in-

vestigated. However, at present, there is no evidence that these posteriorizing molecules act in a graded fashion to allow induction of placodal *Six1* expression and an alternative model will be proposed in the following paragraph.

4.5.2. A new model for the induction of placodes

On the basis of the new insights gained in the present study combined with previous publications, a new model for the induction of placodes shall be proposed here. Any model for the induction of placodes should also account for other ectodermal cell types, because placodes are induced at a position immediately adjacent to the anterior neural plate and the neural crest, which are also precisely located relative to each other. While the BMP gradient model allows to account for this precise positioning of different ectodermal fates, it could not be confirmed by the results obtained in this study regarding the induction of placodes. In particular, the idea of an intermediary level of BMP being responsible for the establishment of a placodal bias is not supported (see above).

Moreover, there a several indications that placodes and neural crest, which are generally considered as neural plate border fates, do not develop from a distinct neural plate border region situated between the neural plate and the epidermis as it has been suggested by several authors (Marchant et al., 1998; Morgan and Sargent, 1997; Nguyen et al., 1998; Wilson et al., 1997; Streit, 2002; Streit and Stern, 1999; Woda et al., 2003; McLarren et al., 2003; Tribulo et al., 2003). In contrast, several studies using different experimental approaches indicate that neural crest can be induced predominantly in neural plate tissue (LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002; Chang and Hemmati-Brivanlou, 1998; Luo, 2003; Moury and Jacobson, 1989; Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995), whereas placodes are only induced in epidermal ectoderm (Woda et al., 2003; Glavic et al., 2004; present study). Although it has been reported elsewhere that neural crest could also be induced in epidermal tissue (Mancilla and Mayor, 1996; Selleck and Bronner-Fraser, 1995), this may only be possible at early stages of development. In preliminary experiments where the expression of the neural crest marker *FoxD3* was analyzed after grafting the anterior neural plate into belly ectoderm at early neural plate stages in *Xenopus*, expression of *FoxD3* was only detectable within the graft, i.e. in neural plate ectoderm. This has also been verified in

cross sections (Ahrens and Schlosser, unpublished observation). This observation suggests that either published results suggesting *slug* induction in the epidermis after grafting the neural plate into belly ectoderm in *Xenopus* were misinterpretations because embryos were only analyzed as wholemounts, or, more likely, that epidermis looses its ability to form neural crest cells at some early developmental stage.

Here, it is proposed, that before gastrulation prospective ectoderm has the competence to form all ectodermal cell types with a preference to form neural plate, which is inhibited, however, by high BMP levels. During the process of gastrulation, differential suppression of BMP levels plays a major role in defining a border between ectoderm biased towards a neural fate and ectoderm biased to form epidermis, whereupon two differentially biased ectodermal regions are generated that are suggested to possess different competences and inducing capacities. That part of the ectoderm which is biased towards neural fates is proposed to be competent to form neural plate as well as neural crest cells (as argued above), whereas the epidermally biased region is competent to form either epidermis or placodes. Evidence for the latter assumption is given here by the observed expression of placodal *Six1* after transplanting the neural plate into belly ectoderm. The induced expression of *Six1* was always confined to the epidermal host tissue, indicating that epidermal tissue was competent to form placodes in response to inducing signals emanating from the neural plate. Moreover, belly ectoderm grafted to the anterior neural plate expressed *Six1*, indicating that the epidermal tissue was competent to form placodes, whereas the neural plate that is exposed to the same inducing signals in that environment is not. Conversely, neural plate grafts transplanted lateral to the neural plate where normally *Six1* is placodally expressed do not express *Six1* (Ahrens and Schlosser, personal observations). Moreover, animal caps, i.e. undifferentiated ectoderm which is not epidermally biased, did not express placodal *Six1* but *Sox3* when transplanted into the panplacodal domain (Fig. 7 H and I), indicating that animal cap ectoderm was still competent to establish neural fates. Similarly, even animal caps transplanted into belly ectoderm expressed *Sox3* (Bastidas et al., 2004). Together, all these observations favour the idea that the distribution of competence rather than the distribution of inductive signals is crucial for the development of placodes at a precise position relative to other ectodermal cell fates. The central importance of competence for the formation of different ectodermal cell types has already been proposed by Albers (1987) but in contrast to the model proposed here, which is summarized in Figure 11, Albers

postulated that different ectodermal cell types are induced by a common inductor spreading from the dorsal midline.

Competence of the epidermally biased territory to form placodes may depend on Dlx3 in *Xenopus* embryos. The expression of *Dlx3* in the inner ectodermal cell layer at neural plate stages precisely matches the dorsal border of the panplacodal expression domain of *Six1* (Schlosser and Ahrens, 2004). Moreover, *Dlx3* is expressed widely in the epidermis until tailbud stages (Schlosser and Ahrens, 2004), which is in agreement with the observation of the present study that epidermal competence for placodal *Six1* induction persists at least until neural tube closure. Functionally, it has been shown that neural plate grafts fail to induce expression of *Six1* in belly ectoderm at experimentally created neural plate boundaries, when Dlx3 activity was downregulated in the responding host tissue (Woda et al., 2003).

Besides the differences in competence, the two differentially biased ectodermal regions, epidermis and neural plate, may emit different inductive activities that induce neural crest and placodes in the adjacent part of the neural plate and the epidermis, respectively. The epidermally biased ectoderm is known to be a source of Wnt signals involved in neural crest induction (see above). The neurally biased ectoderm, on the other hand, was shown here to be one source of signals necessary for the induction of placodal *Six1*, because after removal of the neural plate on one side of the embryo, placodal *Six1* expression was lost on the experimental side. In the present study two signalling molecules were elucidated to be involved in the induction of placodal tissue. Because of its localized expression in the anterior neural plate, it is proposed here that FGF8 constitutes the inductive activity emanating from the anterior neural plate necessary for placodal *Six1* expression in competent, i.e. epidermally biased ectoderm. Additionally, the present study demonstrated that the endomesoderm underlying the prospective placodal domain is necessary for its induction and that BMP must be inhibited to allow the induction of *Six1* in competent ectoderm. The expression of the BMP inhibitor *cerberus* in the lateral endomesoderm suggest that the endomesoderm is a major source for BMP inhibitors involved in placode formation. The molecular activities and their likely sources proposed here to be involved in the induction of placodes are shown in Figure 12.

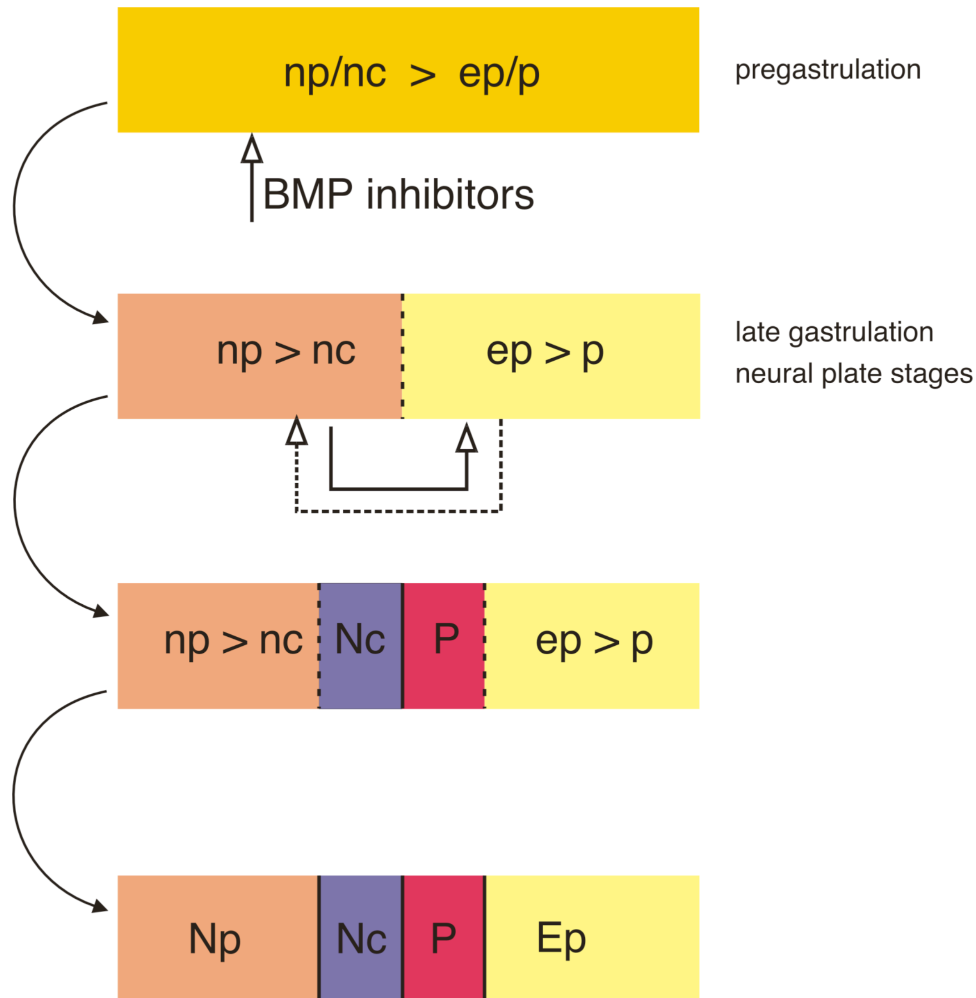

Fig. 11 Model for generic placode induction relative to other ectodermal cell fates. Before gastrulation, ectoderm is competent to form all ectodermal cell fates and has a neural default fate (indicated by np/nc > ep/p). During gastrulation a border is established between a dorsal (orange) and a ventral (yellow) ectodermal domain with low BMP levels being ultimately required for stabilizing the dorsal ectodermal domain (reviewed in Weinstein and Hemmati-Brivanlou, 1999; Harland 2000; Wilson and Edlund, 2001). The two established domains differ in competence and have neural (np > nc) and epidermal (ep > p) default fate, respectively. Besides inducing activity from the mesoderm (not shown) the two differentially biased ectodermal domains emit different inductive activities resulting in placode (p) induction at the border of the epidermal biased domain, whereas neural crest (nc) is induced at the border of the neurally biased ectodermal domain. During further development, boundaries between the different ectodermal domains are sharpened and four differentially committed domains are created (Np, Nc, P and Ep).

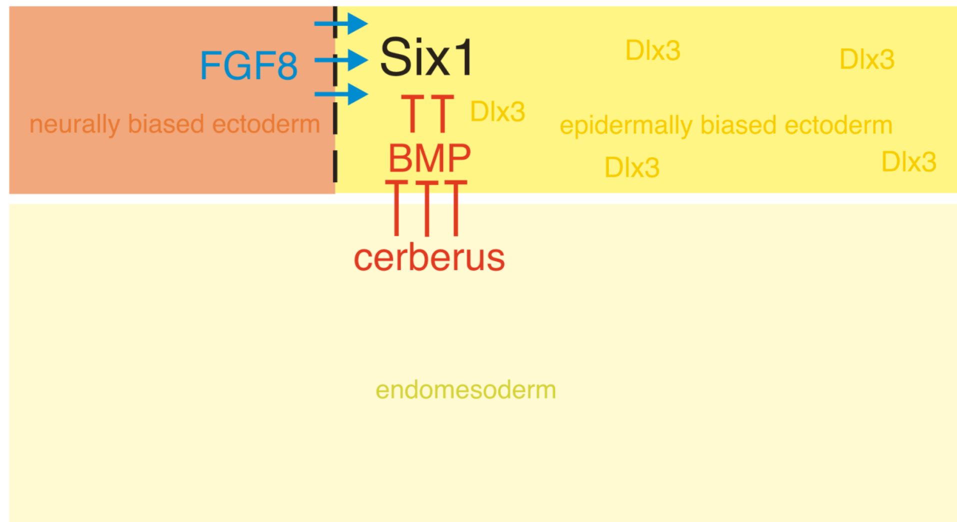

Fig. 12 Molecules involved in the induction of placodal *Six1*. FGF8 signals from the neural plate (blue arrows) induce expression of generic placodal markers such as *Six1* at the border of the epidermal/placodal competence region at neural plate/fold stages. The dorsoventral extent of generic placode induction is probably restricted to a region receiving permissive signals from the underlying anterior endomesoderm, possibly cerberus (red bars), which inhibit BMPs and probably Wnts. Dlx3 may be involved in mediating placodal competence in the epidermally biased ectoderm.

4.5.2.1. Induction of distinct types of placodes

The present study elucidated the time window during which placodal tissue expressing *Six1*, is induced. Moreover, the necessity of different types of tissue for this induction was investigated and additionally, the involvement of two different types of molecules in this process was demonstrated. In the preceding section, a new model for the induction of placodes was postulated mainly based on the new insights in placode induction obtained in this study. It is important to point out that the results presented here as well as the proposed model only address the induction of a common primordium biased to form placodal tissue. This panplacodal primordium is demarcated by the expression of members of the *Six* and *Eya* gene families, which might promote generic placodal properties, like the ability to perform morphogenetic movements or neurogenesis (see above and Schlosser and Ahrens, 2004). In order to develop a specific type of placode, such as the otic or epibranchial placodes, however, additional molecular signals are needed.

There is evidence that the generic step of placode induction, investigated in the present study, and the induction of specific types of placodes act at least partly in parallel rather than in series. Gene expression patterns in the panplacodal primordium indicate that already during neural plate stages, some individual placodes are molecularly demarcated from neighbouring tissue (Schlosser and Ahrens, 2004). Moreover, it has been shown that, for example, the olfactory and the otic placodes are already specified during neural plate stages in anurans (Bando, 1930; Choi, 1931; Zwilling, 1940; Zwilling, 1941; Ginsburg, 1995; Gallagher et al., 1996). However, for the complete differentiation of the inner ear, additional signals are necessary during later stages of development.

From early neural plate stages up to tailbud stages, the transcription factors *Pax2* and *Pax8* are expressed in a posterior part of the panplacodal domain in *Xenopus laevis*. These expression patterns most likely demarcate a posterior placodal area that is able to form otic, lateral line and epibranchial placodes (Schlosser and Ahrens, 2004). The decision towards an otic placodal fate is depending on additional molecular signals, most likely emanating from the neural tube as well as the mesendoderm (Harrison, 1938; Harrison, 1945; Kogan, 1939; Jacobson, 1963a;Jacobson, 1963b; Mendonsa and Riley, 1999). In contrast, the epibranchial placodes, which also develop from the posterior placodal area have been shown to be induced by the pharyngeal pouches, most likely via signalling of BMP7 (Begbie et al., 1999). Lateral line placodes, as the third group of descendants of the

posterior placodal area may also be induced by signal derived from the neural plate (Mangold, 1929) and the mesoderm, but no inducing signals are known so far. Possibly, lateral line placodes form from the posterior placodal area, when neither otic nor epibranchial placodes are induced, thus lateral line placodes would constitute the default state of the posterior placodal area (Fritzsch et al., 1998; Vendrell et al., 2000; Schlosser and Ahrens, 2004).

In the following section the induction of the inner ear will be discussed in more detail, because there seem to exist many similarities between the induction of generic placodal fate and the induction of this specific type of placode. However, differences between the results presented in this study and the findings concerning the induction of the otic placode will be pointed out.

4.5.2.1.1. Induction of the otic placode

Members of the FGF family have also been proposed to be specifically involved in the induction of the otic placode (reviewed in Noramly and Grainger, 2002; Riley and Phillips, 2003). Beads soaked with FGF2 or FGF3, respectively were able to induce ectopic ear vesicles in posterior dorsal ectoderm of *Xenopus* embryos (Lombardo and Slack, 1998; Lombardo et al., 1998). The capacity of FGF3 to induce ectopic otic placodes was also observed in chick embryos (Vendrell et al., 2000). The induced ectopic otic vesicles show at least partly normal patterning as revealed by the expression of *Wnt3a* or *Pax2* and *Nkx5.1*, respectively (Lombardo and Slack, 1998; Vendrell et al., 2000). In zebrafish and chick embryos, beads soaked with FGF8 led to an enlargement of the ear vesicles, though they were not able to induce ectopic otic placodes (Adamska et al., 2001; Léger and Brand, 2002). Similarly, injections of mRNA of DNA for either *FGF8* or *FGF3* led to an expansion of the preotic domain and ectopic expression domains of *Pax8* and *Foxi1*, *Pax2a* and *Dlx3b*, as later preotic markers (Phillips et al., 2004; Solomon et al., 2004). Only a very small fraction of the embryos investigated developed ectopic ear vesicles (Phillips et al., 2004). Likewise, Léger and Brand (2002) report an expanded expression domain of *Pax2.1* in the placodal domain after injection of *FGF8* mRNA. Upregulation of *Pax2.1* expression by overexpression of *FGF8* was limited to the normal preotic region and could not be observed elsewhere in the embryo (Léger and Brand, 2002).

FGF signalling was also shown to be necessary for the complete induction of otic placodes. Zebrafish embryos incubated in SU5402 between 30-70% epiboly, and tailbud or 2-somite stage have reduced or even no ears with a strong reduction or complete loss of the expression of *Pax8*, *Pax2.1* and *Dlx3* (Maroon et al., 2002; Léger and Brand, 2002). Incubation of the embryos during later phases of development, viz. tailbud and 18-somite stage revealed that FGF signalling is not only involved in the induction of the otic placode but is also required for the maintenance of the expression of the otic placode markers *Pax8*, *Pax2.1* and *Dlx3* (Léger and Brand, 2002).

Effects on otic placodal development are also visible in zebrafish ace mutants, which lack functional FGF8. Development of the otic placode is disturbed as revealed morphologically and by the expression of several markers. The otic placode and the otic vesicle are reduced in size compared to wildtype embryos and there is only one otolith, or two misplaced ones. Moreover protrusions of the inner ear fail to form correctly. The expression of several genes, viz. *Pax2.1*, *Pax8*, *Pax5*, *MshD*, *Otx1*, *Zdk1* is reduced in the otic placode of ace mutants (Léger and Brand, 2002). Partly, also the development of other placodally derived structures seems to be affected in ace mutants, because these embryos develop only a strongly reduced number of neuromasts, suggesting perturbation of lateral line placodes. However, the expression of *Nkx5.1* used as a marker for the lateral line showed no defects in its expression pattern (Léger and Brand, 2002). Thus, loss of FGF8 may possibly also have an effect on the induction of the lateral line placodes, which also develop from the posterior placodal domain.

FGF8 morpholino injection into zebrafish embryos also led to defects in placodal development. Injected embryos possess small otocysts, with a weaker and less extensive expression of *Dlx3* and *Pax2.1*, whereas the earliest marker for the otic placode *Pax8* was still present (Maroon et al., 2002).

Embryos injected with a morpholino against FGF3 show similar effects than those injected with FGF8 morpholino or ace mutants (Phillips et al., 2001; Maroon et al., 2002; Léger and Brand, 2002). These effects are aggravated when the function of both genes is interrupted by coinjection of morpholinos against FGF3 and FGF8 or by FGF3 morpholino injection into ace mutants, which in the most affected cases lead to a complete loss of otic vesicles and the lack of Dlx3, Pax2.1 and Pax8 expression (Maroon et al., 2002; Phillips et al., 2001; Léger and Brand, 2002; Solomon et al., 2004). These results indicate that FGF3 and FGF8 promote redundant functions during otic placode induction. Similarly, in mouse

embryos FGF3 and FGF10 were proposed to perform redundant function during the induction of the otic placode (Alvarez et al., 2003; Wright and Mansour, 2003), because double mutants show a severe reduction of the otic vesicles, whereas single mutants for either FGF3 or FGF10 only show mild effects.

This paragraph gave a short overview on the possible role of FGFs among them FGF8 in the induction of the otic placode. It is possible that FGF8 plays a role in the induction of a panplacodal domain as well as in the induction of a specific type of placode, because of a specific interaction with other signalling molecules or transcription factors. Because of the observed redundant functions of FGF3 and FGF8, it is also possible that FGF8 promotes panplacodal induction, whereas FGF3 is involved in the induction of the otic placode. However, many of the above mentioned studies were only concerned with the induction of the otic placode and did not look for the expression of other placodal marker genes, but Léger and Brand (2002) report that in ace mutants the expression of *Six4.1*, *Eya1* and *Dlx3* around the anterior neural plate is unaffected, whereas there are clear otic defects (see above). Moreover, FGF3 and FGF8 zebrafish double morphants exhibit *Dlx3* expression that is induced normally at the edge of the neural plate but fails to be up-regulated in the preotic primordium. It is possible that for the induction of the panplacodal primordium another member of the FGF family promotes a redundant function in zebrafish embryos, which could account for these obvious differences between the results observed in the zebrafish and the results reported here for *Xenopus laevis*. Unfortunately, experiments where FGF8 was overexpressed in zebrafish were analyzed just with markers for the otic placode and not with panplacodal marker genes (Léger and Brand, 2002). Thus, it is unknown if such an overexpression has an effect on other placodes too, as the altered number of neuromast in ace mutants may indicate. However, it is also possible, that some of the different results in zebrafish and *Xenopus* reflect real species differences.

Importantly, one has to exclude that the results reported in the present study may only concern otic placode induction. There are several indications that this is not so. *Six1* expression clearly extends the otic placode domain. Although after FGF8 morpholino injections anterior parts of this expression domain are partly still present, this is most likely due to inductive activities from the uninjected control side of the embryo, because two cases which had been injected on both sides miss placodal *Six1* expression in anterior parts (Fig. 8 L). Moreover, after removal of the anterior neural ridge, the most anterior source of FGF8 activity, *Six1* was reduced in the placodal domain anterior to the otic domain. Addi-

tionally, beads soaked with SU5402 do not only inhibit the expression of *Six1* in the otic placode but also in more anterior placodal areas depending on their location in the embryo. Furthermore, FGF8 morpholino injected embryos have a reduced or even absent expression of *Pax2* and *Pax8* not only in the otic part of their expression domain but in the whole placodal expression domain encompassing also the prospective lateral line and epibranchial placodes. *Pax3*, which is expressed in the profundal placode is also reduced in *Xenopus* FGF8 morphants (unpublished observations).

Most importantly, *FGF8* in *Xenopus* is not expressed in the hindbrain, in contrast to zebrafish (Phillips et al., 2001), suggesting that a different member of the FGF family act as an otic inducer in *Xenopus* as has also been shown in mammals (Wright and Mansour, 2003, Alvarez, 2003).

These observations clearly show that the observed influence of FGF8 on the *Six1* expression is not restricted to the otic placodes. Thus, it is proposed here, that all placodes develop from a common panplacodal primordium demarcated by the expression of *Six* and *Eya* genes, which is induced by BMP inhibition coming from the underlying lateral endomesoderm and FGF8 signalling emanating from the anterior neural plate (see above) and that distinct types of placodes form because a unique combination of transcription factors is established due to the influence of a diverse array of additional inducing molecules.

Zusammenfassung

Plakoden sind spezialisierte ektodermale Strukturen, die essentiell sind für die Bildung der meisten Sinnesorgane des Wirbeltierkopfes und deren Innervation. Genexpressionsmuster, Untersuchungen von Mutanten und Schicksalsstudien weisen darauf hin, dass alle Plakoden aus einer gemeinsamen Vorläuferregion entstehen. Bisher war über die Entstehung eines solchen panplakodalen Primordiums nichts bekannt. Ziel dieser Dissertation war es die Induktion des panplakodalen Primordiums zu untersuchen und dabei sowohl Gewebetypen, die hierbei eine Rolle spielen, zu identifizieren, als auch die molekulare Identität der von diesen Geweben ausgehenden induktiven Aktivität zu klären.

Im ersten Teil der vorliegenden Arbeit werden Daten zum räumlich–zeitlichen Expressionsmuster unterschiedlicher plakodaler Markergene zusammengefasst, die bereits publiziert sind (David et al., 2001; Schlosser and Ahrens, 2004). Zunächst wird *Eya1* als panplakodales Markergen identifiziert, das im Neuralplattenstadium ein panplakodales Primordium markiert, das um die anteriore Neuralplatte herum liegt. Dabei überlappt der anteriore Bereich dieser Expression mit der dorsalen Expressiondomäne von *Sox3*, während lateral zwischen diesen beiden Expressiondomänen eine Lücke klafft, die von *FoxD3* exprimierenden Neuralleistenzellen besetzt wird. Im weiteren Verlauf der Entwicklung ist *Eya1* in allen Plakoden mit Ausnahme der Linsenplakode exprimiert und sein Expressionsmuster entspricht dem von *Six1*. Beide Gene eignen sich somit gleichermaßen als panplakodaler Marker für weiterführende Studien.

Die Analyse der Expression weiterer plakodaler Marker zeigt, dass bereits im Neuralplattenstadium bzw. frühen Neuralfaltenstadium das panplakodale Primordium in molekular distinkte Bereiche unterteilt ist. Diese Expressionsmuster ermöglichen die Vorhersage, dass die olfaktorische und Adenohypophysenplakode aus einer anterioren Domäne hervorgehen – im Neuralplattenstadium durch die Expression von *Sox2*, *Sox3* und *Pax6* charakterisiert –, und dass sich Trigeminus- und Profundusplakode bereits in diesem frühen Stadium durch die distinkte Expression von *Pax6* respektive *Pax3* voneinander abgrenzen. Die otische, Seitenlinien- und Epibranchialplakoden scheinen aus einer gemeinsamen Vorläuferregion zu entstehen, die durch die Expression von *Pax2* und *Pax8* identifiziert werden kann.

Im zweiten Teil der Arbeit werden die Ergebnisse aus diversen Explantations-, Transplantations- und Injektionsversuchen dargestellt und diskutiert. Diese beziehen sich

auf die Identifizierung des Zeitfensters der Plakodeninduktion, die Rolle unterschiedlicher Gewebetypen bei dieser Induktion sowie die Moleküle, die hierbei involviert sind. Eine erste Versuchsreihe zeigt, dass plakodales Gewebe in späten Neuralfaltenstadien determiniert wird, *Six1* zu exprimieren, während die Kompetenz hierfür wesentlich länger besteht. Weiterführende Experimente wurden an Stadien durchgeführt, in denen Signale für die Induktion von plakodalem *Six1* vorhanden sind, das Ektoderm aber noch nicht determiniert ist.

Explantations- und Transplantationsversuche zeigen, dass sowohl die anteriore Neuralplatte als auch das dem panplakodalen Primordium unterliegende Endomesoderm in vivo notwendig sind für die Induktion von *Six1*, wohingegen nur die Neuralplatte in der Lage ist *Six1* ektopisch zu induzieren. Die Neuralplatte erlangt ihre Fähigkeit *Six1* zu induzieren mit Beginn der Neuralplattenstadien. Die induktive Fähigkeit ist auf die anteriore Neuralplatte beschränkt. Chordamesoderm hingegen spielt in vivo keine Rolle bei der Induktion von *Six1*.

Mittels Transplantationen von unspezifiziertem animal cap Ektoderm aus mit verschiedenen mRNAs oder DNAs injizierten Spenderembryonen in uninjizierte Empfängerembryonen und bead-Implantationen werden BMP-Inhibitoren und Fibroblastenwachstumsfaktor 8 als Moleküle identifiziert, die die Induktion von plakodalem *Six1* vermitteln. Eine zu hohe Konzentration von BMP verhindert die Induktion von *Six1*, während FGF8 *Six1* induzieren kann, wenn BMP inhibiert wird. Wird hingegen die Funktion von *FGF8*, durch die Injektion eines spezifischen Morpholino oder durch Implantation eines mit einem Inhibitor getränkten beads blockiert, so ist die Expression von *Six1* in der plakodalen Domäne stark reduziert. Daraus kann abgeleitet werden, dass *FGF8* für die normale Induktion plakodaler *Six1* Expression notwendig ist.

Auf der Basis dieser Daten zusammen mit der relevanten Literatur wird ein neues Modell zur ektodermalen Musterbildung vorgeschlagen. Hiernach werden Plakoden in einem Teil des Ektoderms, das die Tendenz hat, Epidermis zu bilden und die Kompetenz zur Plakodenbildung besitzt, induziert. Diese Annahme wird von der Beobachtung gestützt, dass Neuralplattentransplantate *Six1* nur im angrenzenden epidermalen Wirtsgewebe induzieren können. Aufgrund der Ergebnisse der vorliegenden Arbeit wird vorgeschlagen, dass die plakodale Domäne von FGF8 induziert wird, das aus der Neuralplatte stammt. Es wird gezeigt, dass *FGF8* in der Neuralplatte und auch in Neuralplattentransplantaten exprimiert

wird. Zusätzlich wird BMP durch Inhibitoren inhibiert, die vermutlich aus dem unterliegenden Endomesoderm stammen.

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