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Clonal analyses in the anterior pre-placodal region: Implications for the early lineage bias of placodal progenitors

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Abstract

Cranial ectodermal placodes, a vertebrate innovation, contribute to the adenohypophysis and peripheral nervous system of the head, including the paired sense organs (eyes, nose, ears) and sensory ganglia of the Vth, VIIth, IXth and Xth cranial nerves. Fate-maps of groups of cells in amphibians, teleosts and amniotes have demonstrated that all placodes have a common origin in a horseshoe shaped territory, known as the preplacodal region (PPR), which surrounds the presumptive neural plate of the late gastrula/early neurula stage embryo (reviewed in McCabe and Bronner-Fraser, 2009; Schlosser, 2010; 2006). Given the extensive regional overlap of progenitors for different placodes in the chick embryo (Bhattacharyya et al., 2004; Streit, 2002; Xu et al., 2008), it has been a matter of debate as to whether individual cells in the PPR are truly multipotent progenitors, with regard to placodal identity, or rather are lineage-biased or restricted to a specific placodal type prior to overt differentiation. Utilizing clonal analyses *in vivo*, we demonstrate that the anterior PPR comprises some precursors that contribute either to the olfactory or lens placode well before they are spatially segregated or committed to either of these placodal fates. This suggests that lineage bias towards a specific placodal fate may coincide with induction of the PPR.

Keywords

placode; olfactory; lens; pre-placodal region; lineage

Introduction

Cranial placodes are transient ectodermal thickenings that are induced in localized regions of the embryonic head. The derivatives of the cranial sensory placodes include the lens of the eye, the olfactory epithelium of the nose and the inner ear. Given the seminal contributions that placodes make to the peripheral nervous system, their developmental origin has been examined in much detail. In particular, dye labeling small populations of cells in chick and *Xenopus* embryos has revealed that all placodes originate within a horseshoe-shaped domain that surrounds the anterior neural plate in early neurula stage embryos, termed the pre-placodal region (PPR) (Bhattacharyya et al., 2004; Pieper et al., 2011; Streit, 2002; Xu et al., 2008). However, within this domain, anterior-posterior regionalization relegates adenohypophyseal, olfactory and lens precursors to the anterior third of the PPR (known as the extended anterior placodal area: Toro and Varga, 2007) while the

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middle third is occupied mainly by trigeminal with a few otic precursors and finally the posterior third contains mostly precursors to the otic placode and all precursors of the epibranchial placodes. Despite this regionalization, focal dye injections often label adjacent placodes. While placode cells are multipotent, capable of giving rise to various cell types including neurons, hair cells, secretory cells and supporting cells amongst others, it has been unclear whether individual cells in the PPR can contribute to more than one type of placode. To address this question, we dye injected single cells in the anterior PPR and found that individual clones contributed to either the olfactory or lens placode (at early stages they also were able to contribute to either the epidermis or neural epithelium) but we were unable to detect any clones that spanned both the olfactory and lens placodes. While we cannot rule out the possibility that there are indeed precursors in the chick PPR that contribute to more than one type of placode, our results raise the intriguing possibility that lineage bias towards a particular placodal fate occurs concomitantly with demarcation of the PPR.

Results

Our population fate-map analyses in the early chick embryo established that olfactory and lens placode precursors show extensive overlap in the anterior PPR at neurula stages (Hamburger-Hamilton stage 6 (HH6)), with domains that contain either olfactory or lens precursors beginning to segregate away from each other at slightly later stages (HH8) (Bhattacharyya et al., 2004). In order to determine the fate of single cells at each of these time-points, individual precursors within the anterior PPR were iontophoretically injected with rhodamine dextran at HH6 and HH8 (Fig. 1A). Two criteria were used to verify single cell labeling. First, voltage measurements were carried out during penetration of the cell membrane, revealing a characteristic drop in membrane potential. Second, visualization under a fluorescence microscope was performed to confirm that only a single cell was labeled. Embryos were then allowed to develop for 12 to 48 hours. Typically, this resulted in clone sizes ranging from 50–100 labeled cells. The results show that, at HH8 (n=65 surviving embryos), clones derived from a single cell contribute exclusively to the olfactory epithelium (Fig. 2C-C''), the lens (Fig. 2F-F''), the forebrain or the ectoderm (data not shown). In sections through the olfactory epithelium, rhodamine dextran can be seen in olfactory sensory neurons, marked by their expression of neurofilament and β III-tubulin (Fig. 2D, D', E). These results suggest that olfactory and lens precursors are already segregated in terms of prospective placodal fate by HH8.

This raises the intriguing prospect of a lineage decision having occurred at a prior stage. To address this possibility, we performed an analogous investigation in HH6 (neurula stage) embryos (n=31 surviving embryos), the earliest stage accessible to *in ovo* lineage analysis. The results show that even single cells injected at HH6 are restricted in their ability to contribute to one or the other placode. Interestingly, however, more medial injections (performed closer to the midline of the embryo) give rise to cells in the forming olfactory placode (Fig. 2A-A'') and in the forebrain. Slightly more lateral injections produce cells in the lens and neighboring ectoderm (Fig. 2B-B''). These results show that cells in the anterior ectoderm of HH6 embryos can contribute to more than one embryonic cell type. In spite of this bipotency, we did not find clones that contribute to multiple manners of placode. It should be noted that the region closest to the tip of the prechordal plate predominantly

contains nasal precursors while the posterior-most fourth of the shared lens/olfactory domain is populated almost exclusively by lens precursors (Bhattacharyya et al., 2004). Therefore, it is possible that some of our injections targeted these already partitioned domains of ectoderm. Furthermore, we saw a 2:1 ratio of clones that contributed to the olfactory as opposed to the lens placode across stages; however, this may simply reflect a tendency to inject closer to the midline rather than any biological difference in the numbers of olfactory versus lens progenitors. Nonetheless, our most parsimonious conclusion is that some individual placode progenitors within the anterior PPR are fated to contribute to a particular placodal type as early as the neurula stage. Interestingly, clones of cells derived from a single cell at either stage do not disperse throughout the area but rather form compact streams of cells even after the several rounds of cell division and fairly vigorous morphogenetic movements that occur (Fig. 1B).

Discussion

Given the extensive co-labeling of both the lens and olfactory placodes, when small groups of cells were labeled in the anterior chick PPR (Bhattacharyya et al., 2004), we expected this propensity for dual contribution to be mirrored at the level of individual cells. However, a recent study in *Xenopus*, mathematically deduced that the co-labeling of adjacent placodes by a single dye injection marking 5–15 cells is in fact “compatible with the possibility that adjacent placodes originate from non-overlapping regions of the PPR at neural plate stages” (Pieper et al., 2011). Our single cell lineage analyses in the chick embryo support the idea that focal injections could indeed label a heterogeneous population of differentially lineage biased placode precursors at the neurula stage albeit we lack sufficient statistical power in our data to rule out the possibility that there may be PPR cells whose progeny contribute to more than one placode. Interestingly, single cell lineage analyses in gastrula stage zebrafish embryos also reveals early fate restriction – progeny of anterior placodal progenitors mostly contribute either to the olfactory epithelium, lens or the pituitary (Dutta et al., 2005; Whitlock and Westerfield, 2000).

A key question that remains unresolved is whether individual cells within the PPR are in fact lineage-restricted and show targeted movements to their final destination or rather randomly change neighbors and acquire their fate based on local gradients of signaling cues. One striking observation in this study is that all clones derived from cells injected at either stage are found in a reasonably tight cluster as opposed to dispersing throughout the ectoderm. This is also compatible with the *Xenopus* study wherein no evidence was found for large-scale cell sorting when tracking the movement of single cells in a field of labeled placodal precursors; however, cells appeared to move as a coherent group within the ectoderm with local rearrangements contributing to limited cell mixing (Pieper et al., 2011). While the present study has not exhaustively labeled every cell in the anterior PPR, when taken together with our population fate-map, it suggests that there is likely to be an intermingling of precursors, which would argue for a need for small-scale cell sorting perhaps guided by signals in the microenvironment. Notably, local differences in the availability of Fgf4 and in the responsiveness of individual cells within the inner cell mass of the early mouse blastocyst results in a “salt and pepper distribution” of lineage-biased precursors for the pluripotent epiblast and primitive endoderm (Kang et al., 2012).

At first glance, our data suggesting the presence of lineage-biased placodal progenitors appear to contradict several lines of evidence for a shared “ground state” of the PPR. These include 1) a unique transcriptional profile – placodal cells co-express and their derivatives show a functional requirement for the transcription factors *Eya1*, *Six1* and *Six4* (Schlosser, 2010; 2006; Streit, 2007), 2) only cells that have acquired generic placodal properties can be induced to differentiate into specific placodal cell types (Martin and Groves, 2006) and 3) placodal precursors at all axial levels are specified as lens in an *in vitro* culture system (Bailey et al., 2006). However, increasing evidence points to cell-to-cell variation in gene expression, epigenetic and signaling responses in groups of cells previously thought to be homogenous. For example, both hematopoietic stem cell and embryonic stem cell populations are often heterogeneous in that individual cells express pluripotency genes unevenly and also show differential lineage-specific gene expression (reviewed in (Enver et al., 2009)). One possibility is that pluripotency in these systems is a meta-stable condition that might allow easier transition to a new cell state resulting in a more robust system supportive of highly regulative development (Silva and Smith, 2008). Consistent with this idea, developmental bias to contribute to either the inner cell mass or the trophectoderm has been observed as early as the 4-cell mouse embryo (Tabansky et al., 2013). While oriented cell divisions might be causal to this bias (reviewed in (Zernicka-Goetz, 2005)), it is currently thought that epigenetic factors (Torres-Padilla et al., 2007) and differing kinetics of Oct4 DNA binding (Plachta et al., 2011) may in fact perpetuate this bias.

Thinking of the PPR in a similar context, it is possible that stochastic fluctuations in gene expression perhaps in response to different signaling cues could result in an early compartmentalization of the ectoderm. We have shown previously that *Dlx5* and *Pax6* are expressed at varying levels in the anterior ectoderm with some cells expressing higher levels of *Dlx5* and lower levels of *Pax6* and vice versa (Bhattacharyya et al., 2004). Since *Dlx5* expression is ultimately strengthened in the olfactory placode but diminishes in the lens, it is possible that cells expressing initially higher levels of *Dlx5* may be predisposed to an olfactory fate. In line with this hypothesis, *Dlx5* over-expressing cells are excluded from the lens (Bhattacharyya et al., 2004). Furthermore, *Dlx5* overexpression appears to alter the adhesive properties of cells such that all *Dlx5* expressing cells cluster together (Bhattacharyya et al., 2004). Thus, there could be internal and spatial constraints on multipotency resulting in lineage bias early in development. When these constraints are removed, either by placing the progenitors in an *in vitro* culture system (Bailey et al., 2006; Bhattacharyya and Bronner-Fraser, 2008) or by transplantation to a different environment in the embryo, the lack of commitment to a particular lineage is exposed (Bhattacharyya and Bronner-Fraser, 2008). Taken together, this suggests that the PPR may be a region of progenitors uniquely biased towards *specific* placodal fates despite sharing certain general properties. This premise deserves to be further explored by more extensive analyses of the fate of individual cells within the PPR coupled with live imaging to follow the cell movements of the clonal descendants. Technical advances in imaging and improved molecular and computational tools that allow single cells, their progeny and their interactions with their neighbors to be followed over time and space will facilitate such analyses (Dempsey et al., 2012; Sellmyer et al., 2013; Tabansky et al., 2013). Highlighting the significance of such an approach, a recent study utilizing *in toto* imaging of the zebrafish

ventral neural tube, discovered that differentially specified progenitors intermingle with each other as a consequence of heterogeneous responses to a signaling cue prior to being sorted into distinctly defined progenitor domains (Xiong et al., 2013). The PPR might thus provide an excellent developmental context within which to interrogate the cellular and molecular mechanisms that result in the underlying variability that precedes the achievement of a stereotypic pattern of tissue growth and differentiation.

Materials and methods

Single cell injections

Injecting electrodes were made from heat pulled aluminosilicate glass microcapillaries (A-M Systems) using a horizontal Sutter electrode puller. Electrodes were back-filled with 1 μ l of high molecular weight (10kDa) rhodamine dextran. *This* does not pass through gap junctions and leak into neighboring cells; hence, it is required for unambiguously labeling a single cell. Just prior to injecting, the rhodamine dextran in the electrode was overlaid with 1.2M LiCl. The electrode is then placed in the electrode holder reservoir and the reservoir is similarly filled with 1.2M LiCl. Once the electrode is prepared, the egg is windowed and India ink diluted 1:20 is injected into the sub-blastodermal cavity to visualize the embryo. A ground electrode is placed in the egg white via a small hole in the shell. At this point, the injecting electrode is lowered into Ringer's solution bathing the embryo and an intact electrical circuit is evidenced trace is observed on the oscilloscope. The resistance of the electrode is measured. A tip resistance on the order of 20–30 M Ω is indicative of a sharp electrode and can be used to penetrate the cell membrane of a single cell. An increase in noise is noted as the electrode approaches the embryo. The electrode is “rung” by very quickly turning the capacitance knob. If the electrode enters a cell, a drop in voltage, ranging from 10–40 mV is noted. The dye is then released into the cell by injecting 4nA of current for 5 seconds. The electrode is then quickly removed from the cell and the oscilloscope trace should return to its ground value. Signals were amplified using an intracellular amplifier (Axon Instruments) and electrical activity was observed on an oscilloscope (Hitachi). The success of the injection was verified using epifluorescence. Embryos were harvested 24–48 hours later and fixed in 4% paraformaldehyde overnight at 4°C. Approximately 90% of the embryos survived 24 hours later (96 out of 131 embryos injected).

Histology and immunofluorescence

Fixed embryos were washed several times in PBS and then scored for labeled cells in the head region using an upright Zeiss Axiophot fluorescence microscope. Labeled embryos were photographed at 10X magnification using an Axiocam digital camera attached to the fluorescence microscope. Some of these embryos were then processed for cryosectioning. The embryos were equilibrated in PBS containing 5% and 15% sucrose before being embedded in 7.5% gelatin (300 bloom, Sigma) and 15% sucrose. The embryos were sectioned at 10 μ m and the slides stored at 4°C. For purposes of immunostaining, sections through embryos were degelatinised by soaking in PBS warmed to 42°C for 5–10 minutes. Neurofilament (1:250) and β III-tubulin were diluted in blocking buffer as the primary antibodies with a goat anti-mouse IgG Alexa 488 used as the secondary antibody to detect both primary antibodies. Several washes with PBS containing 0.1% Tween were carried out

after each application of antibody. Slides were finally rinsed in distilled water before being mounted in Permount containing 10µg/ml DAPI, a nuclear stain.

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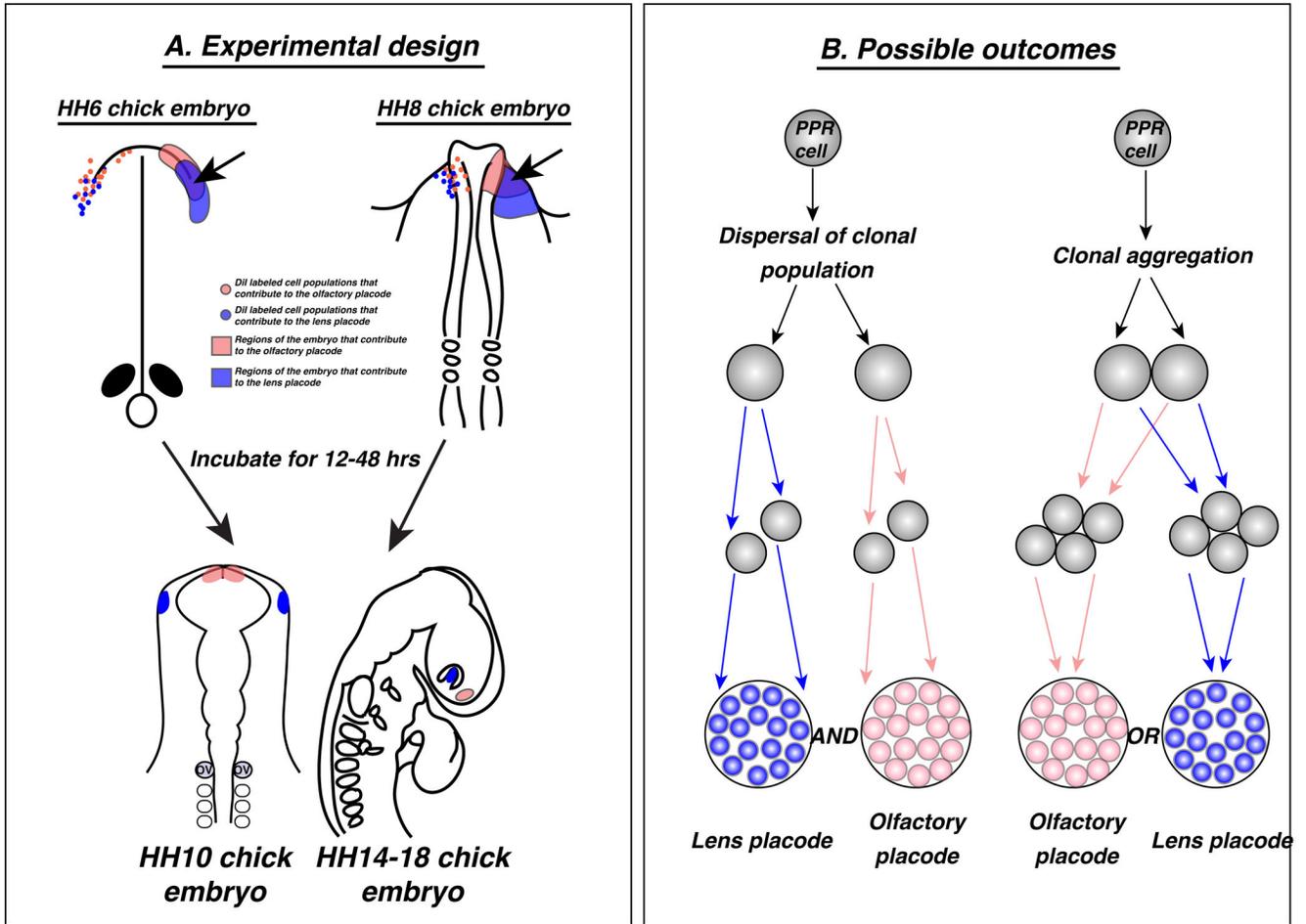


Figure 1. Experimental design and hypothesis

A: Experimental design. Black arrows point to the area targeted for injections in HH6 and HH8 chick embryos respectively. Pink dots/regions demarcate areas of the embryos where injecting small populations of cells results in labeling in the olfactory placode. Similarly, blue dots/regions correspond to labeled areas that contribute to the lens placode. Significant overlap is seen between these regions (3). Embryos were collected 12–48 hours later at which time olfactory and lens placode territories had completely separated (HH10) and were morphologically identifiable as placodes (HH14–18). **B: Possible outcomes.** Individual progenitor cells in the PPR (PPR cell) can either divide and disperse to contribute to two different placodes, or can be lineage-biased and undergo minimal cell mixing to contribute to a single placode. Our data suggest that the latter is the case.

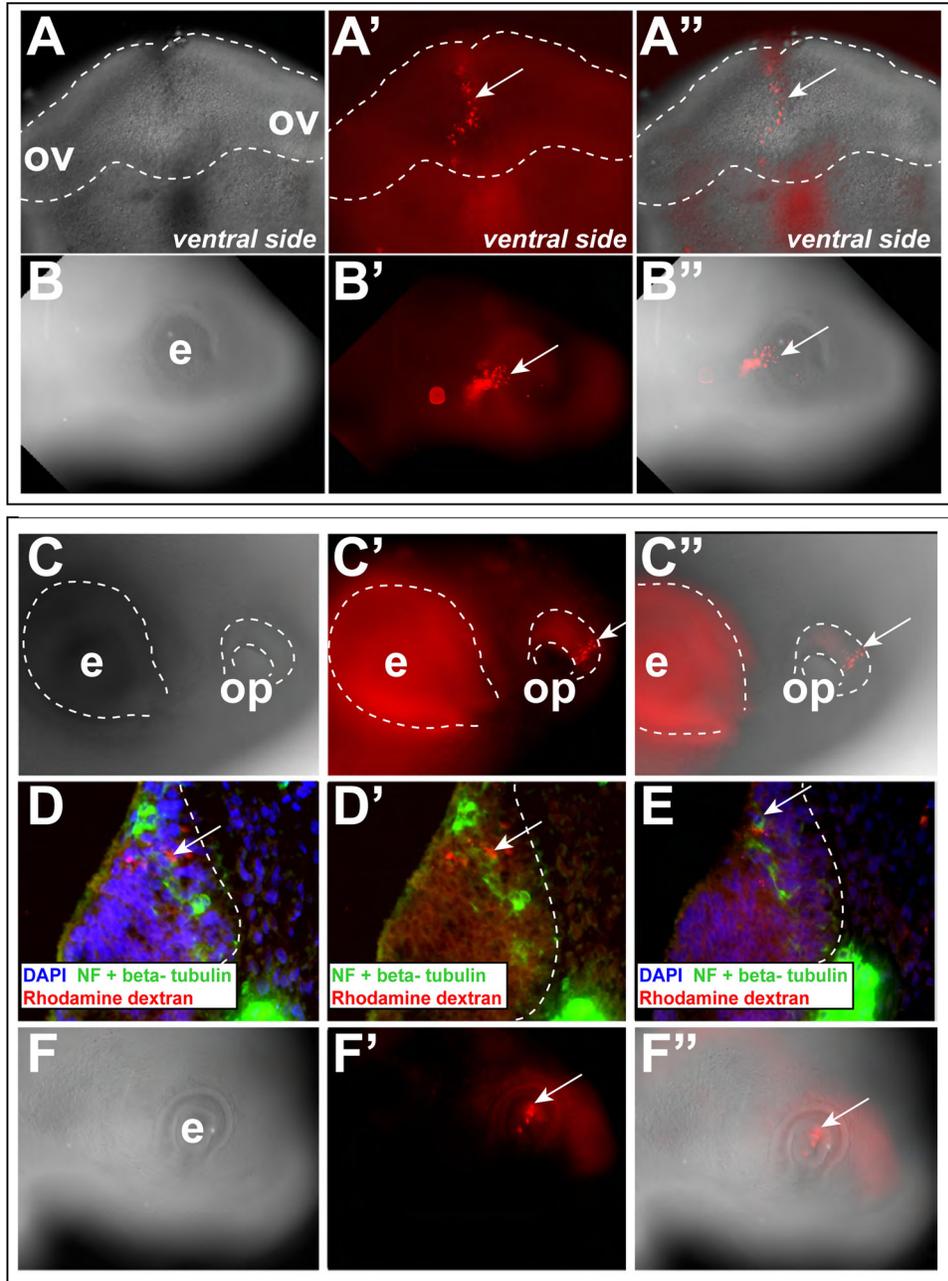


Figure 2. Single cells in the pre-placodal region (PPR) are lineage-biased to an olfactory or lens placode fate

A-A'': Example of an embryo in which a single cell was labeled at HH6 and whose progeny contributes to the olfactory territory. A: Bright field image of embryo collected at HH10. The rhodamine dextran labeled stream of cells is clearly visible in A' (arrow) and an overlay of the two images is presented in A''. Dotted lines demarcate the presumptive forebrain of the embryo at this stage. B-B'': Example of an embryo collected at HH14 in which a single cell was labeled at HH6 and whose progeny contributes to the lens and adjacent ectoderm. B: Bright field image; B': Fluorescent image; B'': Overlap of the bright field and fluorescent

images. Arrows in B' and B'' point to labeled cells in the lens. C-C'': Example of an embryo injected at HH8 and collected at HH18. A cluster of cells is clearly labeled within the olfactory epithelium. C: Bright field image; C': Fluorescent image; C'': Overlay. Dotted lines demarcate the eye and the olfactory placode in the head of the embryo. Arrows indicate labeled cells in the olfactory placode. D-D'': Example of an embryo collected at HH15/16 in which a single cell was labeled at HH8. Rhodamine dextran is clearly visible in the lens. D: Bright field image. D, E: Adjacent sections through embryo depicted in C-C''. Blue: DAPI; Green: Neurofilament and beta-tubulin staining; Red: rhodamine dextran. D': Same section as shown in D without DAPI staining. Arrows in D, D' and E point to a neuron in the olfactory epithelium born from the rhodamine labeled single cell. Dotted lines denote the basement membrane of the olfactory epithelium. F-F'': Example of an embryo injected at HH8 and collected at HH16 in which the clone contributes to the lens of the eye (arrows). F: Bright field image; F': Fluorescent image; F'': Overlay. ov: optic vesicle; e: eye; op: olfactory placode.