Development 139, 3741-3751 (2012) doi:10.1242/dev.081091

Redefining the structure of the hair follicle by 3D clonal analysis

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SUMMARY

The hair follicle (HF) is a multi-tissue mini-organ that self-renews periodically. However, the cellular organisation of this much-studied model is not fully understood. The structures of the outer layer and of the bulb, which ensures HF growth, have not been completely established. To clarify these points, we have conducted in vivo clonal analyses with 3D imaging in mice. The upper two-thirds of the HF outer layer consists of two clonally unrelated groups of cells that exhibit different modes of growth. They correspond to the basal outer root sheath (ORS) and the companion layer (Cp). The basal ORS has an unusual anisotropic mode of growth from a suprabulbar zone, which we named the privileged proliferation zone. The Cp has a stem/transient-amplifying mode of growth and is shown to be an HF internal structure. Furthermore, we describe an additional element, the bulb outer layer, which is contiguous and shares markers (e.g. Lgr5) with the basal ORS but is formed by a separate lineage that belongs neither to the ORS nor Cp lineage. It represents a novel element with proximal cells that are contiguous with the germinative layer in the bulb. In reference to its shape and position we named it the lower proximal cup (LPC). These clonal hierarchies reveal a novel model of HF organisation and growth based on two major entities: the basal ORS and the LPC plus the seven internal layers.

KEY WORDS: Hair follicle, Outer root sheath, Companion layer, Germinative layer, Lower proximal cup, Lgr5, Mouse

INTRODUCTION

The organisation of cells into organs is the starting point for analysis of their cellular homeostasis, formation, collective behaviour and evolution. The hair follicle (HF), a much-studied mini-organ, provides an ideal model with which to elucidate these processes (Fuchs et al., 2001; Legué et al., 2012). The HF is among the few structures of the adult mammal that renews periodically through neomorphogenesis (Fuchs and Horsley, 2008; Legué et al., 2010). Each cycle begins with a growth phase (anagen), during which groups of cells actively divide to maintain the structure, followed by a programmed regression (catagen) and then a resting phase (telogen) (Millar, 2002; Müller-Röver et al., 2001; Stenn and Paus, 2001).

The HF consists of concentric layers of different keratinocyte types that account for its extraordinary physical properties, all organised into three compartments: first, the hairshaft, which is composed of medulla, cortex and cuticle; second, more radially, the inner root sheath (IRS) composed of the IRS cuticle, the Huxley and Henle layers; and finally the outer layer of the HF (outer root sheath, ORS), which is contiguous with the interfollicular epidermis (Hardy, 1992). Cellular homeostasis of the internal layers during anagen is achieved by a stem/transient-amplifying mode of growth from a germinative layer in the proximal HF (the bulb). Further, in the ORS, cellular homeostasis is controlled by a regional proliferative mode of growth coupled with apoptosis that coordinates the development of outer and inner structures (Legué and Nicolas, 2005). At each new cycle, the HF undergoes neomorphogenesis, which is achieved by a group of HF stem cells that survive catagen and give rise to ORS progenitors and the germinative layer. The germinative layer cells are clonally related to each other (Kopan et al., 2002; Legué et al., 2010). This widely accepted scheme is based on clonal analysis and morphological approaches (Blanpain and Fuchs, 2006; Legué et al., 2012).

Major uncertainties still exist as to the cellular definition of the ORS and of the bulb. For some, the outer layer consists of two compartments: the basal ORS, which expresses keratin 14 (K14), and the companion layer (Cp), an innermost layer that expresses keratin 75 (K75) (Ito, 1986; Ito, 1988; Ito, 1989; Pinkus et al., 1981). For others, it consists only of the $K14^+$ ORS cell layer. In this model, the $K75^+$ Cp cells either belong to the IRS compartment (Gu and Coulombe, 2007; Zhang et al., 2009) or constitute a separate HF structure (Orwin, 1971; Rothnagel and Roop, 1995; Wang et al., 2003; Winter et al., 1998). Related to these issues, there are several possibilities as to the origin of the Cp cells during anagen. They may either share a common progenitor with the $K14^+$ basal ORS cells or derive directly from the $K14^+$ cells themselves. The lower limit of the $K14^+$ ORS is also subject to debate. Many consider that it extends to the bottom and even inside the bulb (Langbein and Schweizer, 2005), whereas others restrict it to the suprabulbar region (Coulombe et al., 1989). Recently, these questions have come to light again with the analysis of a new molecular marker, Lgr5. Lgr5 is expressed by the outer layer of the anagen HF including the bulb and cells that abut the dermal papilla proximally (Jaks et al., 2008). This suggests, but does not prove, a cellular continuity of the HF outer layer from the bulb to its most distal part. A definitive resolution of these issues is a prerequisite for an understanding of HF homeostasis and morphogenesis and its cyclic regeneration from stem cells.

To clarify these issues, the clonal relationships of the HF outer layer cells and its radial and longitudinal compartmentation were studied. We conducted clonal analysis using several reporter mice, including one that allows confocal 3D imaging (mT/mG). This permits unambiguous resolution of the cellular structure of the HF outer layer and determination of the 3D structure of the clones. We also used various Cre driver lines. We establish that the two types of

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Accepted 26 July 2012

cells of the HF outer layer, i.e. the basal ORS and the Cp, originate from separate cell lineages with distinct modes of growth. The Cp is part of the HF internal lineage and follows a stem/transient-amplifying mode of growth. We reveal a novel proliferative growth mode for the $K14^+$ basal ORS from a privileged proliferation zone (PPZ) at its proximal end that incorporates an anisotropic growth activity. We also reveal that the outer layer of the HF bulb is a previously unidentified HF structure that is contiguous with, but distinct from, the $K14^+$ basal ORS. We call it, in reference to its position and shape, the lower proximal cup (LPC). Surprisingly, the LPC that covers the precursors of the Cp in the bulb is associated with the internal lineages and not with the basal ORS.

MATERIALS AND METHODS

Mouse strains

The $CMVCreER^T$ strain was provided by D. Metzger (Feil et al., 1996), $RosaCreER^{T2}$ by L. Grotewold and A. Smith (Legué et al., 2010), Rosa26R *lacZ* reporter by P. Soriano (Soriano, 1999), and $Lgr5EGFPIresCreER^{T2}$ mice (Barker et al., 2007) and mT/mG reporter mice (Muzumdar et al., 2007) were obtained from Jackson Laboratory. The CMV promoter confers widespread expression in all HF cells (Metzger and Chambon, 2001) and the Rosa26 promoter confers ubiquitous expression on *lacZ*, mT/mG and $CreER^{T2}$. mT/mG is a double-fluorescent Cre reporter mouse that expresses membrane-targeted Tomato protein (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision (Muzumdar et al., 2007). The localisation of fluorescent proteins in the membrane provides an outline of cell morphology.

Synchronisation of the HF cycle, 4-hydroxytamoxifen preparation and injection

Experimental designs are described in Fig. 1A and Fig. 5A. We start the experiment in the last synchronous telogen [postnatal day (P) 55] and stimulate anagen by depilation. To have two different stages of the HF cycle in the same animal at the induction day, two zones were delineated in the back skin (Fig. 1A). To induce clones in telogen, zone A was depilated 2 or 5 days after induction (D-5 or D-2), and to induce clones in anagen zone B was depilated 3 or 8 days before induction (D3 or D8) (Fig. 1A). We verified the HF stage at induction in a control biopsy. 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich) was suspended as described (Petit and Nicolas, 2009). Biopsies and HF collection were performed as described (Legué et al., 2010).

Histology, immunostaining and electron microscopy

X-gal staining in toto and in frozen skin sections was performed as described (Legué et al., 2010). For electron microscopy, individual HFs were microdissected, embedded in Epon resin as described (Hsu et al., 2011). Semi-thin sections (1 μm) of Epon-embedded samples were stained with Toluidine Blue dye. Immunofluorescence of skin cryosections was performed as described (Legué et al., 2010) with antibodies against K5 or K14 (both Covance, 1:1000), K75 (previously named K6hf; Progen, 1:300) and Ki67 (Novocastra, 1:1000). Sections were incubated with the corresponding secondary fluorescent antibodies (Alexa Fluor 488 or 633, Invitrogen, 1:150). For whole-mount immunostaining, HFs were fixed in 4% paraformaldehyde (20 minutes, 4°C) followed by 3 days incubation with primary antibodies and 2 days for secondary antibodies (4°C). Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Skin sections (mounted in Vectashield, Vector Labs) and whole-mount HFs were observed using a Zeiss LSM700 confocal microscope. *z*-stacks were reconstructed in 3D using ImageJ (NIH) and Zen (Zeiss) software.

RESULTS

Two clonal categories for the HF outer layer

Bulge HF stem cells exhibit heterogeneous contributions to the renewed HF structure (Claudinot et al., 2005; Ghazizadeh and Taichman, 2001; Legué et al., 2010; Zhang et al., 2009), possibly dependent on their position relative to the dermal papilla (Legué et al., 2010). Yet, among the restricted clonal contributions, that which is restricted to the distal ORS (rORS clones) is not consistent with the current view of the HF structure, as the ORS is believed to cover the bulb. To resolve this puzzling observation, we labelled single cells in vivo by injecting low doses of 4-OHT (supplementary material Table S1) into mice that differ in the inducer construct ($CMVCreER^T$ or $RosaCreER^{T2}$) or in the reporter construct (Rosa26R or mT/mG), at different times before (zone A experiments) or during (zone B experiments) anagen (Fig. 1A). For zone A experiments, clones were induced at P55 when all HFs are in the second telogen (the last synchronous telogen stage, which was confirmed by skin biopsy). As a result, clones labelled in zone A were not derived from cells at different phases of the cycle, but exclusively from stem cells in telogen bulge or hair germ. For zone B experiments, HFs are in anagen. As a result, clones labelled in zone B and observed one complete cycle later were derived exclusively from stem cells in anagen.

First, we tested for $CreER^T$ and $CreER^{T2}$ activity in telogen cells with 4-OHT injections at P55 in $CMVCreE^{RT}$ and $RosaCreER^{T2}$ inducer lines. The patterns of labelling observed 3 to 5 days after induction show $CMVCreER^T$ and $RosaCreER^{T2}$ expression in all bulge and hair germ cells (Fig. 1B-G). Then, clones were observed during the following anagen at D14 (zone A experiment, telogen induction at D-5 or D-2; see Materials and methods) or one complete cycle later (zone B experiment, anagen induction at D3 or D8, observation at D14+1 cycle) (Fig. 1A). In all conditions tested, rORS clones were observed (Fig. 1H), representing 31-55% of the labelling (Fig. 1I). Analysis of their contribution to the HF proximal-distal (PD) axis confirmed the absence of labelled cells at the proximal HF levels (0-30 units of the PD axis; Fig. 1H,I, arrowheads). We also observed additional clones that supply descendant cells to the outermost layer of the HF proximal end and also contribute to internal structures (Fig. 1J,J′). Strikingly, these clones also participate in the outer layer of the HF at its distal part (30-100 units along the PD axis; Fig. 1K). We called these ‘complex clones’ (30-56% of the labelling; Fig. 1K).

Together, rORS clones and complex clones labelled all HF lineages (Fig. 1H,J,J′; see also Fig. 2G-J and Fig. 4C,E; supplementary material Movie 1). This is a strong internal control for general expression of the two inducers and the two reporter lines in the F1 crosses, consistent with the previous findings for *lacZ* under the Rosa26 promoter (Legué and Nicolas, 2005; Legué et al., 2010). However, it remains possible that other types of clone might have escaped the analysis due to potential non-uniform induction of Cre function.

Thus, these analyses clearly demonstrate that the HF outer layer originates from two clonal contributions corresponding to complex clones and to the rORS clones that contribute to only the distal two-thirds.

Cells of the two clonal categories are of different shape and position

To determine whether these different clonal contributions correspond to cell type heterogeneities, we analysed transverse HF sections of representative clones. Complex clones contribute internal flattened cells that juxtapose the HF Henle layer and are covered by more external cells (*n*=20 clones; supplementary material Table S1, CMV#1; Fig. 2A,B, arrowheads). By contrast, rORS clones contribute cuboid external cells (*n*=15 clones; supplementary material Table S1, CMV#1; Fig. 2C,D, arrowheads). The two clonal categories contributed to cells of distinct shapes occupying different radial positions. Electron

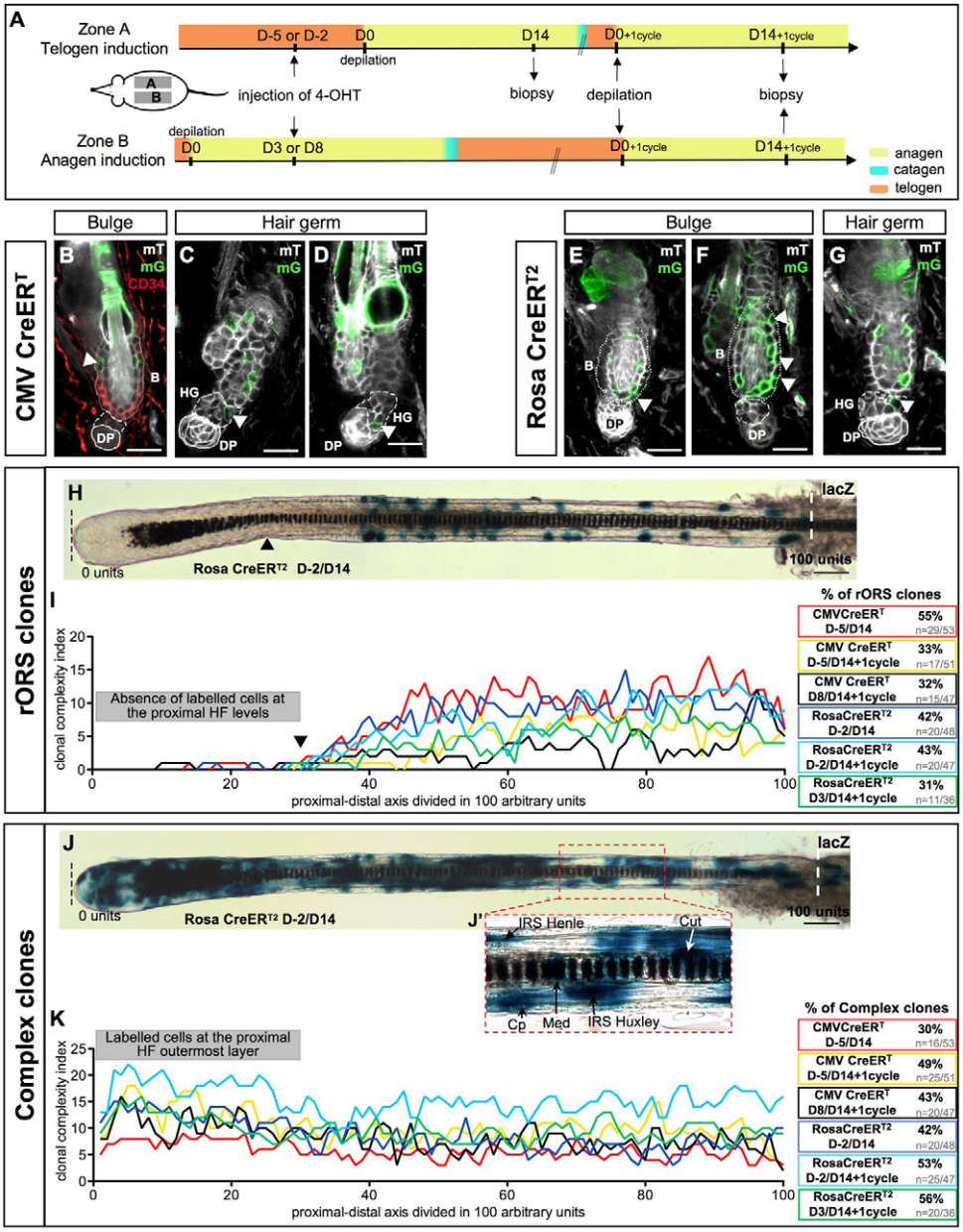

Fig. 1. rORS precursors preferentially contribute to the distal part of the ORS. (**A**) Experimental design for inductions in telogen (D-5 or D-2) or in anagen (D3 or D8), that were observed in the following anagens (Experiments CMV#1-2 and Rosa#1-3; supplementary material Table S1). D0 and D0+1 cycle represent the days of depilation. The times of 4-OHT injection (induction of clonal labelling) are indicated as days before or after depilation. D14 or D14+1 cycle indicate skin biopsies taken in the same or one complete cycle after depilation. To obtain from the same animal skin samples induced at two different stages of the HF cycle, zone A and B were delineated on the back of the mouse and treated separately. (**B-G**) Tests for CreER^T and CreER^{T2} activity in telogen cells. Inductions were with 4-OHT injections (40 μg/g) during telogen (P55) in CMVCreER^T and RosaCreER^{T2} lines and the patterns of labelling were observed 5 days following (which corresponds to the time of anagen activation, D0). CMVCreER^T (B-D) and RosaCreER^{T2} (E-G) expressed Cre (arrowheads) in all CD34$^+$ bulge (B,E,F) and hair germ (C,D,G) cells. The CD34$^-$ K6$^+$ innermost layer of the bulge [which is not involved in HF renewal (Hsu et al., 2011)] is also labelled. B, bulge; HG, hair germ; DP, dermal papilla. (**H**,**J**,**J'**) Whole-mount views of rORS (H) and complex (J) clones induced at D-2 and observed at D14 visualised by X-gal staining. (J') Higher magnification of complex clone in J. (**I**,**K**) The clonal complexity (*y*-axis) in the outer layer [the number of times a given proximal-distal (PD) level (the PD axis was divided into 100 arbitrary units, *x*-axis) is labelled by a clone]. Each line corresponds to a separate experiment using different inducer lines or times of induction. Boxes on the right refer to each experiment; '% of rORS clones' and '% of complex clones' correspond to the number of rORS or Complex clones relative to the total labelled HFs; *n*, total number of clones of the clonal category/total number of labelled HFs (supplementary material Table S1). IRS, inner root sheath; Cp, companion layer; Med, medulla; Cut, cuticle. Scale bars: 20 μm in B-G; 100 μm in H,J.

microscopy sections of the mid HF (Fig. 2E) indicate that the flattened, single layered, more electron dense cells exhibit the morphology seen for Cp cells (Ito, 1986; Ito, 1988; Ito et al., 1986; Orwin, 1971; Pinkus et al., 1981), whereas the cuboid cells exhibit that seen for the basal ORS. The contribution of all clones to the different radial positions (external cuboid, intermediary, internal flattened; Fig. 2E) and to each unit on the HF PD axis was examined (Fig. 2F). This analysis indicated that the flattened

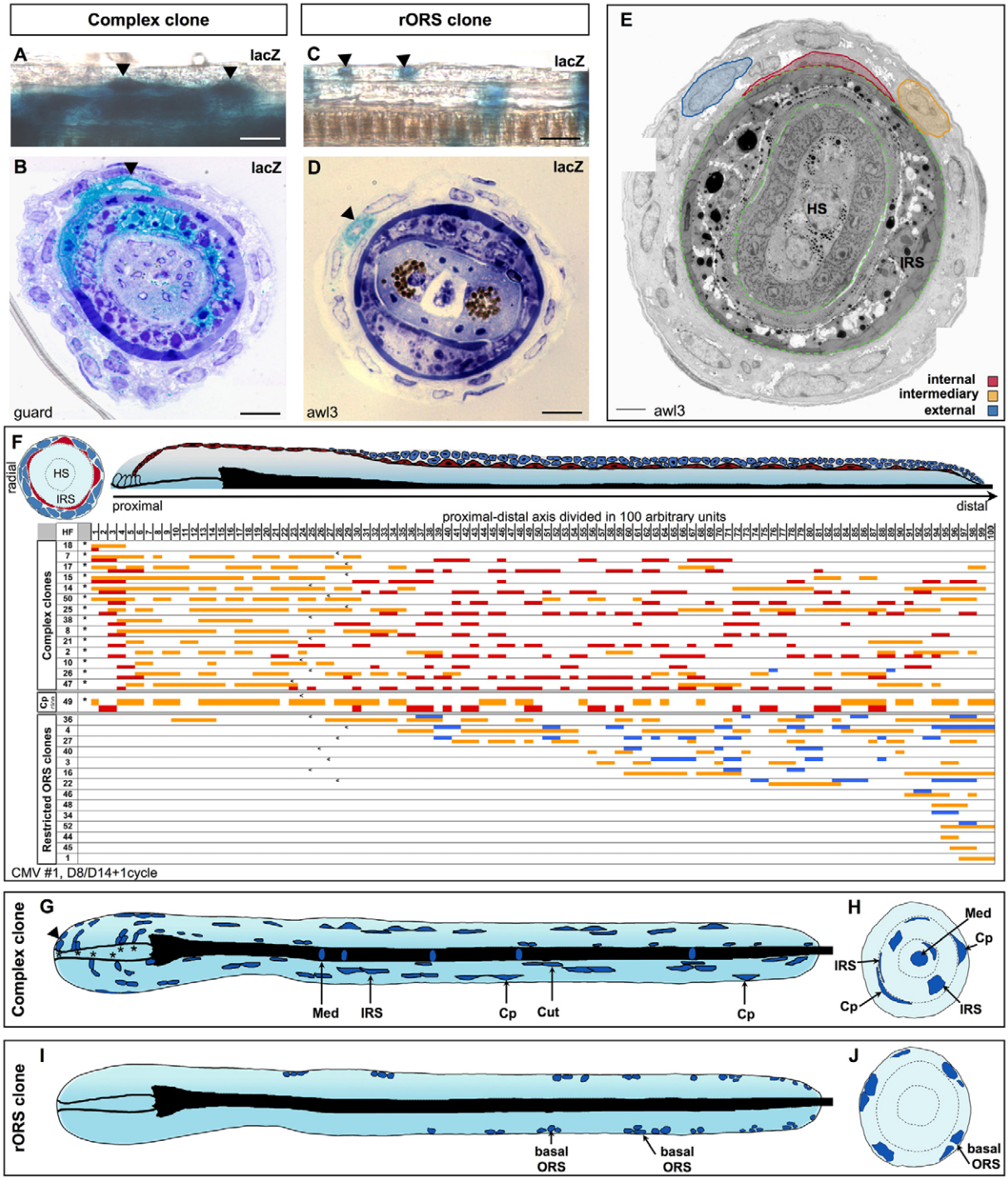

Fig. 2. Radial and proximal-distal contribution of rORS and complex clones to the HF outer layer. (**A-D**) X-Gal-stained whole-mounts (A,C) and Toluidine Blue-stained sections (B,D) of complex (A,B) or rORS (C,D) clones showing that they contribute to internal or external cells, respectively (arrowheads). (**E**) Electron micrograph of a transverse section through the HF showing internal (red), intermediary (orange) and external (blue) cells of the outer layer. (**F**) Radial-PD pattern of all clones that contribute to the outer layer (induced during anagen and analysed one complete cycle after at D14+1 cycle), arranged according to their most proximal contribution. The clones are classified into three categories: complex clones that contribute to the outer layer and to internal layers; rORS clones with restricted contribution to the ORS; and Cp clones that are restricted to the Cp. Each horizontal line corresponds to a clone. The coloured rectangles represent the radial position of labelled cells: red refers to the most internal, orange to the intermediary and blue to the most external cells. The numbers on the top row indicate the PD position of labelled cells according to the subdivision of the axis into 100 arbitrary units. Asterisk indicates that the clone contributes to a cell juxtaposing the dermal papilla, and < indicates the beginning of the basal ORS layer. (**G-J**) Schematic view of the two main clonal categories in longitudinal (G,I) and transverse (H,J) sections. (G,H) A complex clone (see supplementary material Movie 1 for *xz* virtual transverse views of confocal *z*-stack). Arrowhead (G) indicates the cells of the lower proximal cup. (I,J) An rORS clone. Dotted lines (E,F,H,J) delimitate hairshaft (HS) and IRS. IRS, inner root sheath; ORS, outer root sheath; HS, hair shaft; Cp, companion layer; Med, medulla; Cut, cuticle. Scale bars: 20 μm in A-D; 5 μm in E.

internal cells were only present in complex clones (Fig. 2F-H), whereas cuboid cells were present only in rORS clones (Fig. 2F,I,J). Therefore, complex clones and rORS clones correspond to two distinct radial-PD patterns.

The two clonal categories contribute separately to the basal ORS and the Cp

The distinct patterns of the two clonal categories suggest that they might contribute separately to the basal ORS and to the Cp cells. To test this hypothesis, we performed antibody staining against markers of the Cp (K75) (Wang et al., 2003; Winter et al., 1998) and of basal ORS (K14 and K5) (Coulombe et al., 1989; Langbein and Schweizer, 2005).

The keratin profile of the outer layer was first characterised (Fig. 3). Both K14 and K5 occupy the HF PD axis from unit 30 to the distal end (Fig. 3C-F, arrows). Their restriction to the area between the suprabulbar region and the distal HF was observed in all HF types of the mouse fur (supplementary material Fig. S1). This corresponds precisely to the limits of the rORS clone contribution (Fig. 1H,I, arrowhead). K75 occupies the HF PD axis from unit 16 to the distal end (Fig. 3A,B, white arrowheads; Fig. 4I, white arrowhead).

We then analysed the keratin profile at 3D cellular resolution in representative clones obtained with the mT/mG reporter line (Fig. 4). The rORS clone-derived cells were all K14$^+$ (Fig. 4A-C″; n=11/11 rORS clones; supplementary material Table S1, Rosa#2) and displayed an elongated shape that was oriented perpendicularly to the HF PD axis (Fig. 4J). The complex clone-derived cells occupying an internal position in the distal outer layer of the HF (Fig. 4D-E′; Fig. 2G,H; supplementary material Movie 1; n=10/10 complex clones; supplementary material Table S1, Rosa#2) were of a flattened hexagonal shape (Fig. 4K) and were all in the K75$^+$ domain (Fig. 4H). However, cells from these same clones were K75$^-$ when in the lower HF (Fig. 4I). Indeed, they lay beyond the proximal limit of the K75$^+$ layer (Fig. 3A,B, arrowheads; Fig. 4I, unit 16 on the PD axis). A few of these K75$^-$ cells abutted the dermal papilla in the proximal bulb (Fig. 4F,G, asterisks). This point is further analysed below with clones induced during anagen.

In summary, only the rORS clones participated in the K14$^+$ basal ORS layer, whereas the complex clones were the sole contributor to the K75$^+$ Cp layer. The K75$^+$ Cp clones also include K75$^-$ cells in the bulb. The basal ORS and the K75$^+$ Cp are thus two non-overlapping, distinct HF lineages.

The Cp originates in the germinative layer and is an integral part of the internal HF structure lineage

To clarify the problem raised by the K75$^-$ cells in the clones that also include cells abutting the dermal papilla, we analysed inductions during anagen (D8/D14, Fig. 5A), as this reveals individual sublineages (Legué and Nicolas, 2005). When we generated radial-PD graphs, two main families of clones emerged: those that contribute to the Cp (family A) and those that do not (family B) (Fig. 5B). Seventy-four percent of family A clones (n=17/23 Cp clones) correspond to long clones that supplied one cell juxtaposed to the dermal papilla lying immediately below the IRS progenitors in the germinative layer (Fig. 5B-E, asterisks). Analysis of clones obtained with RosaCreER^{T2}xmT/mG mice allowed us to show that this cell occupies positions 4-5 relative to the bulb proximal end (Fig. 5D-E; supplementary material Movie 2) and was never seen in a more proximal position. Distally from unit 30 of the PD axis, all cells of the family A clones are covered by K14$^+$ cells (Fig. 3B, white arrow; Fig. 3D, red arrowhead; Fig. 5D′,E, Cp cells in internal position; Fig. 4E, complex clone labelling Cp).

Thus, family A defines a concentric and flattened HF layer that originates in the germinative layer, then further upstream forms the K75$^+$ Cp (Fig. 5E; see Fig. 7). Three-dimensional reconstructions of the Cp clones and of the K75 immunostaining demonstrate that the Cp is a contiguous layer (Fig. 3B; Fig. 4K), despite the spacing of the nuclei. Remarkably, the clones induced during telogen that contribute to the Cp layer also contribute to other internal structures (Fig. 1J′; Fig. 2G,H; Fig. 4D-F). Therefore, the K75$^+$ Cp layer is an integral part of the lineage of internal HF structures.

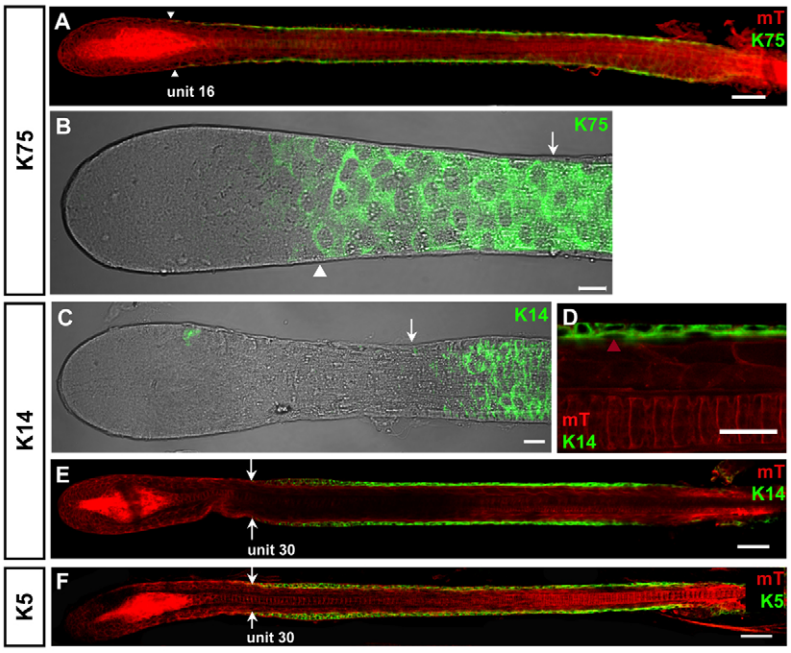

Fig. 3. HF outer layer characterisation by keratin immunostaining. In toto views of HFs from an mT/mG mouse (non-induced). (**A**,**B**) The proximal limit of K75 expression in Cp is the mid bulb (unit 16, white arrowheads). (**C-E**) The proximal limit of K14-expressing cells is above the bulb (unit 30, arrows). Distal to the arrows (B,C), the cells that express K75 are covered by K14-expressing cells (D, red arrowhead). (**F**) The proximal limit of K5-expressing cells is above the bulb (unit 30, arrows). (B,C) z-stack maximal projections. Scale bars: 50 μm in A-C,E,F; 20 μm in D.

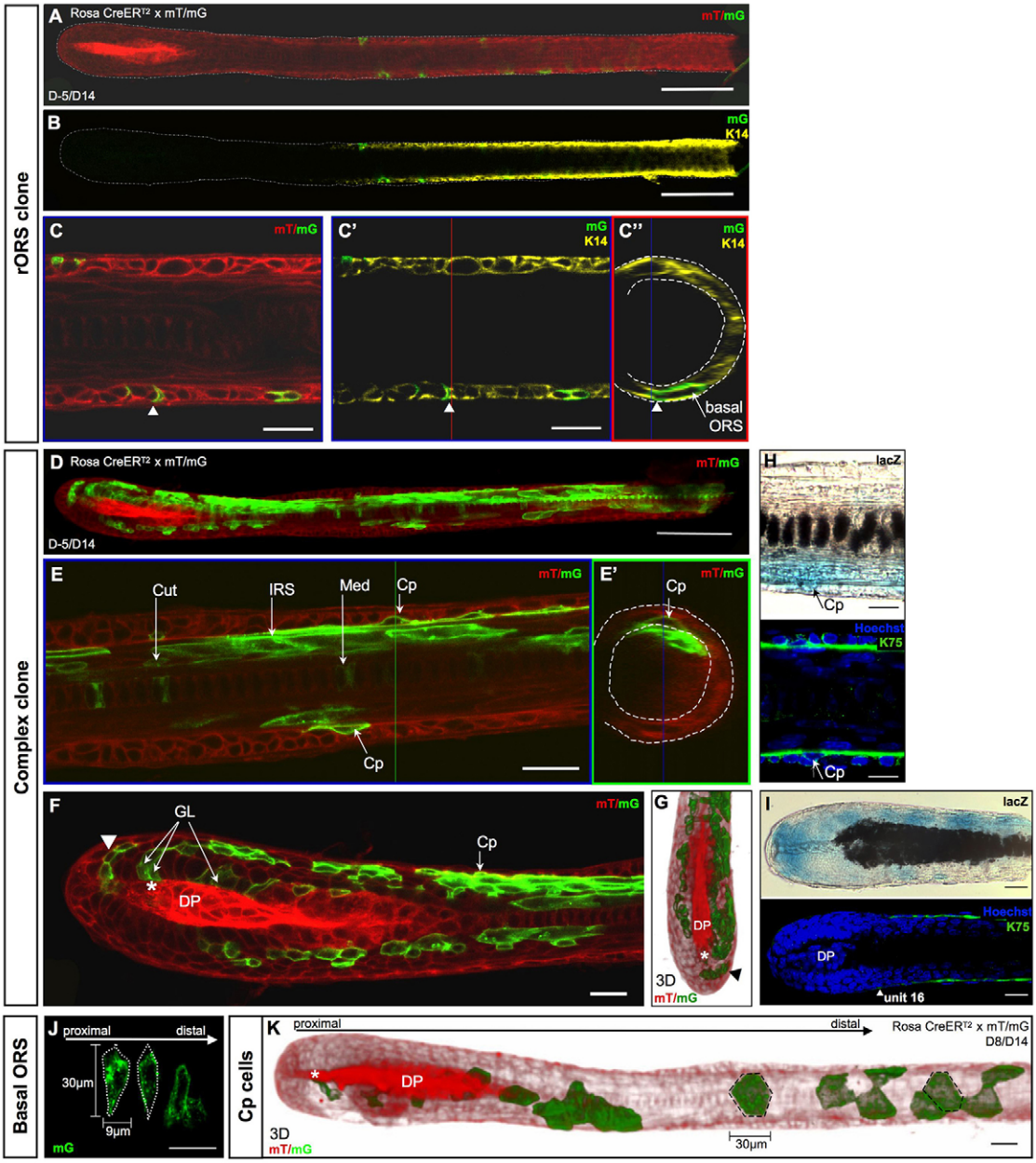

Fig. 4. Keratin profile characterisation of mT/mG rORS and complex clones. (**A-C″**) rORS clone immunostained for K14. mT/mG view (A; higher magnification in C) and mG/K14 view (B; higher magnification in C′; virtual transverse section in C″). Arrowhead (C-C″) indicates labelled cell of rORS clone in the K14^{+} domain. (**D-G**) Complex clone. mT/mG view (D; higher magnification in E; virtual transverse section in E′). (F) Enlargement of the bulb of the complex clone. LPC cells (arrowhead) and germinative layer cells (asterisk) both abut the dermal papilla. (G) Three-dimensional view of the bulb. (**H**,**I**) Complex clone stained for *lacZ* (top) and immunostained for K75 (bottom). K75 is expressed by the distal cells (H) but not by the proximal cells below unit 16 (I). (**J**,**K**) The shape of basal ORS (J) and Cp cells (K). IRS, inner root sheath; DP, dermal papilla; GL, germinative layer; Cp, companion layer; Med, medulla; Cut, cuticle. Scale bars: 100 μm in A,B,D; 20 μm in C,E,F,H-K.

The Cp shares the growth strategy used by other internal structures

Internal HF structures exhibit a stem growth mode from the germinative layer cells. Does this also apply to the Cp? Discriminative features of this mode of growth are as follows (Legué and Nicolas, 2005). First, the existence of anagen clones that do not include a cell in the germinative layer. Twenty-six percent of anagen family A clones (n=6/23 clones) did not include a cell that juxtaposes the dermal papilla, and 22% have only two cells (Fig. 5B). These are likely to correspond to the progeny of a transient-amplifying cell produced by asymmetric division of position 4-5 stem cells (Fig. 5C-E). Second, the systematic inclusion of a germinative layer cell in labellings examined after a complete HF cycle. We examined such labelling (D8/D14+1 cycle) and found that all clones contributing to the Cp supplied a cell juxtaposed to the dermal papilla at position 4-5 (Fig. 2F,G; Fig. 4F,G, asterisks). Third, telogen clones must contribute to the full length of the structure and anagen clones to only the newly formed proximal part of the structure. A comparison of the radial-PD patterns of anagen- and telogen-induced clones indicated that most telogen clones

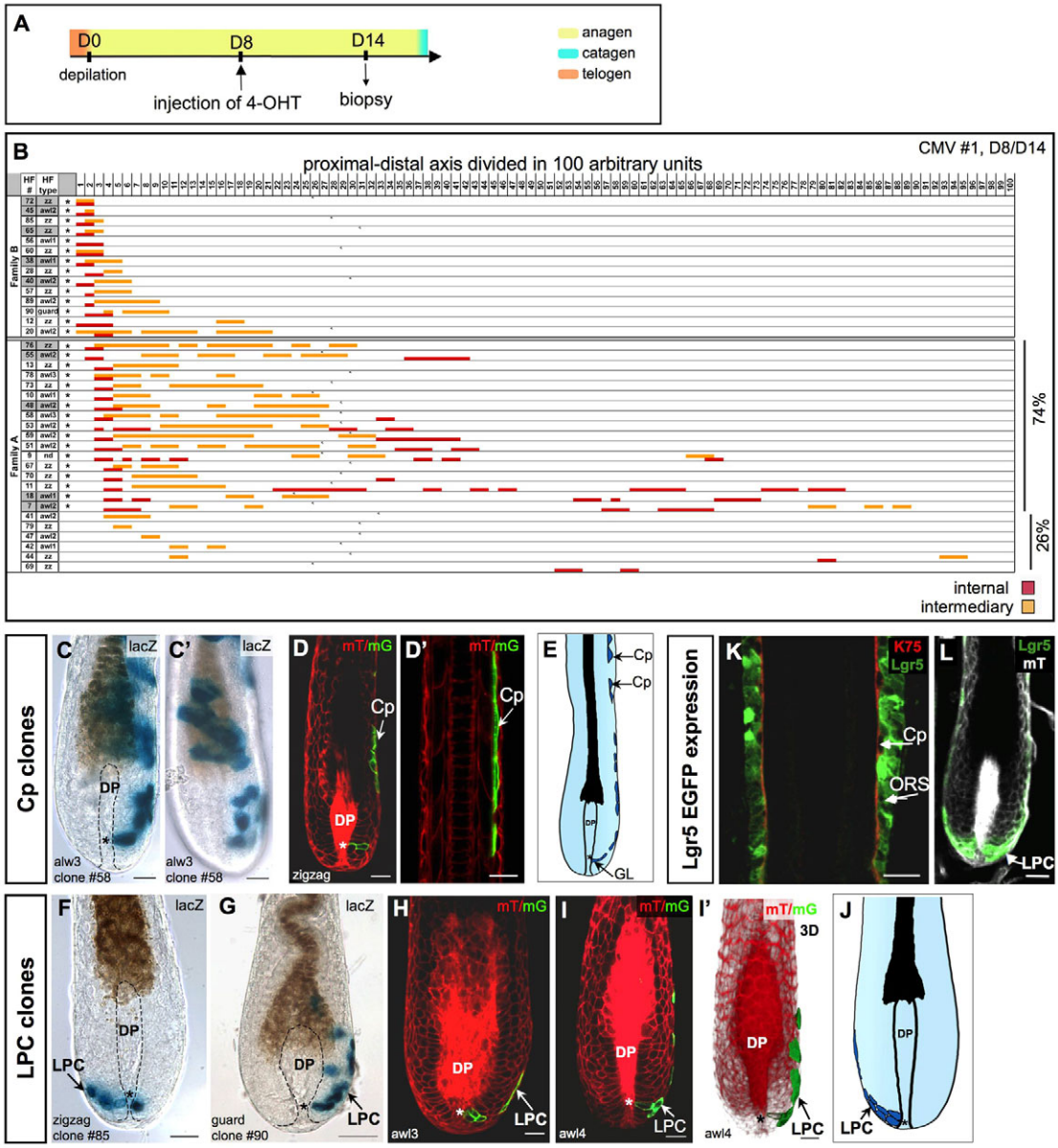

Fig. 5. Clonal organisation of the outermost layer of the HF bulb. (**A**) Experimental design for induction of clones during anagen 8 days (D8) after depilation (D0) that were observed in the same anagen (D14) (CMV#1, Rosa#3, D8/D14, supplementary material Table S1). (**B**) Radial-PD pattern of all D8/D14 clones (anagen) that contribute to the outermost layer of the HF proximal end, classified according to their most proximal contribution. Family A: long clones that contribute to both the proximal and distal parts of the outer layer. Family B: short clones that only contribute to the proximal end of the outer layer. Boxes on the left indicate the clone number and HF type. When the boxes are in grey, the labelling also concerned internal structures (combined labellings, supplementary material Table S1). The percentage values to the right indicate the frequency of clones including a cell that abuts the dermal papilla (74%) or not (26%). (**C-D'**) Contribution of the *lacZ* (C,C') and mT/mG (D,D') family A clones (Cp clones) in different HF types from mouse fur. (**E**) Schematic representation of a Cp clone. (**F-I'**) Contribution of the *lacZ* (F,G) and mT/mG (H-I') family B clones (LPC clones) in different HF types from mouse fur (zigzag, awl3, awl4 and guard hairs). (**J**) Schematic representation of an LPC clone. The asterisk indicates that the clone contributes a cell juxtaposing the dermal papilla. See also supplementary material Movie 2 for full confocal *z*-stack of a Cp clone and supplementary material Movie 3 for 3D reconstructions of an LPC clone. (**K**,**L**) Lgr5 expression pattern in an HF of the Lgr5EGFPIresCreER^{T2} mouse. (K) Upper HF immunostained for K75 (a Cp marker). (L) Bulb. ORS, outer root sheath; DP, dermal papilla; GL, germinative layer; Cp, companion layer; LPC, lower proximal cup. Scale bars: 20 μm.

contribute to the distal part of the HF and that the mean longitudinal clone extension increases 2-fold upon passage from anagen to telogen (Fig. 5B and Fig. 2F, respectively). Therefore, the Cp is formed using a stem growth mode, i.e. an asymmetric (stem) division followed by one or two symmetric divisions, similar to the other internal layers (Legué and Nicolas, 2005).

A novel HF lineage: the lower proximal cup

The unexpected family B anagen clones are very short clones that populate the most proximal part of the HF and do not contribute to the K75 layer (Fig. 5B). Clones belonging to this family frequently include cells that abut the dermal papilla at positions 1-3, but never more distally. Other cells from these clones contribute to the

outermost layer of the bulb (Fig. 5F-J; supplementary material Movie 3). The cells at positions 1-3 divide during anagen, but it is not clear whether they have a stem mode of division. The family B clones never contribute cells above unit 30 of the PD axis, in contrast to the family A clones, which all provide extremely distal cells (Fig. 5B). The family B clones show properties that differentiate them from the seven internal layers (including the Cp) and the basal ORS of the HF. The structure that they form constitutes a novel HF lineage that we have named the lower proximal cup (LPC), in reference to its shape and position. The LPC is found in all mouse fur HF types (Fig. 5B,F-I). As for labelling during telogen, all the clones that contributed to the LPC also contributed to the Cp (n=7/7 clones) and to internal structures (n=6/7 clones) (Fig. 2F-H; Fig. 4F,G, arrowheads), demonstrating that the LPC shares a common precursor with these structures. Therefore, the LPC belongs to the HF internal structure lineage.

The basal ORS and the LPC cells express Lgr5, unlike the Cp layer and its precursors

During anagen Lgr5 is expressed in the outer layer of the HF, including the bulb outer layer (Jaks et al., 2008). To clarify which cells in the outer layer express Lgr5, we performed antibody staining against K75 in anagen HF of Lgr5EGFPIresCreER^{T2} mice. Lgr5 expression includes the basal ORS but excludes the Cp K75^{+} cells (Fig. 5K). In the bulb we found that, as previously reported, the outer single-cell layer is Lgr5^{+}. The Lgr5 expression pattern also includes proximal cells juxtaposed to the dermal papilla with an upper limit at position 3 (Fig. 5L), the precursors of the Cp layer that occupy positions 4-5 being excluded (supplementary material Movie 2). This limit at position 3 coincides with the limit of the LPC (Fig. 5F-J).

In summary, the outermost layer of the HF bulb is formed by the Lgr5^{+} LPC cells (Fig. 7), which are integral components of the internal HF lineage. The distal HF outer layer is formed by a separate and complementary lineage comprising the Lgr5^{+} K14^{+} basal ORS cells that cover the differentiated Lgr5^{-} K75^{+} Cp cells (Fig. 7). The cells alongside the dermal papilla at positions 1-3 are Lgr5^{+} and belong to the LPC, whereas those at positions 4-5 are Lgr5^{-} and belong to the Cp layer.

Basal ORS growth occurs from its proximal end

The rORS clones obtained after induction during telogen had a longitudinal extension that varied from 75 units to 5 units (Fig. 6A, radial-PD pattern). Most of the clones (n=54/57 rORS clones) contributed extremely distally to the ORS. Strikingly, instead of being distributed regularly along the length of the HF, all the short clones were positioned in its distal portion. This specific clonal distribution provided clues concerning the growth mode of the basal ORS K14^{+} layer. Both strict regional and stem modes could clearly be excluded, and instead a mode of growth from a privileged proliferation zone (PPZ) (Fig. 6A, grey boxes) is proposed. In this scenario, the proliferation zone would progress in a distal (the site of contribution of most clones, including the shortest) to proximal direction, the fraction of cells left behind ceasing to divide (Fig. 6B, 1-3). According to this model, clones contributing to the PPZ should have more cells than clones with progenitors that have left the PPZ and stopped dividing. This prediction was fulfilled (Fig. 6C, compare the number of cells in ‘PPZ not labelled’ with ‘PPZ labelled’).

Another strong prediction that arises from this growth mode is that mitotic activity in the basal ORS layer must progress proximally from the distal end during growth, the distal regions becoming progressively less mitotically active over time. Ki67 labelling experiments during growth confirmed this prediction. During early HF growth, a zone of Ki67^{+} basal ORS cells progressed proximally from the distal cells (Fig. 6Da-c). The Ki67^{+} dividing cells corresponded only to the basal ORS (Fig. 6Dd, arrows), confirming that the Cp does not originate from the basal ORS layer, as they do not proliferate (these results) (Gu and Coulombe, 2007). Clearly, the basal ORS progenitors are located in the PPZ (Fig. 7).

DISCUSSION

Ultrastructural, biochemical and immunohistochemical studies have established that HFs are formed from eight distinct and well-characterised cell types (Hardy, 1992; Langbein et al., 2006) that are organised into three groups involved in different HF functions: hairshaft, IRS and ORS. These studies have failed to clarify the organisation of the bulb, which is the region of HF cell production and maturation. Furthermore, it is still ambiguous whether the clonal association of Cp cells is to the IRS or to the ORS. Our studies elucidate these points and lead to a novel model of HF organisation and growth (Fig. 7). This model applies at least to mouse fur HFs. We demonstrate that the Cp is an individual layer (Winter et al., 1998) that shares lineage and growth mode properties with the other HF internal layers (Fig. 5). We show that the bulb is structured by two elements: the germinative layer and the LPC. The germinative layer that produces the IRS and the hairshaft also produces the Cp. This germinative layer abuts the dermal papilla, but the three most proximal cells of the bulb juxtaposed to the dermal papilla belong to the second element of the bulb, the LPC. This novel HF entity includes the outermost bulb cells. These cells are distinct from both the ORS and the Cp and have their own clonal origin. They cover the proliferative cells of the bulb and are distally contiguous with the basal ORS. Both the basal ORS and the LPC are covered by the dermal sheath. Finally, we show that ORS formation employs a special growth mode that involves a privileged proliferation zone (PPZ). The PPZ is localised distal to the LPC, at the proximal end of the basal ORS domain. The implications of these discoveries for the functioning and dynamics of the HF are discussed below.

The companion layer

Analyses of the clones that contribute to the HF outer layer clearly show that it contains cells from two different clonal origins corresponding to the Cp cells or the basal ORS cells. The Cp cells have been considered by several researchers as belonging to the ORS (Ito, 1986; Ito, 1988; Pinkus et al., 1981) and by others as belonging to the IRS (Gu and Coulombe, 2007; Zhang et al., 2009). Our results demonstrate that they are not derived from the ORS or IRS but from unipotent progenitors in the bulb, more specifically from the cells proximal to the germinative layer stem cells (Legué and Nicolas, 2005) in positions 4-5 (Fig. 7), the function of which was until now undefined. This confirms that the PD organisation of the germinative layer prefigures the radial organisation of the HF (Legué and Nicolas, 2005), as the most proximal cells in the germinative layer produce the most radial cells in the mature part of the HF, the Cp cells. The stem growth mode of the Cp progenitors provides further evidence that this layer belongs to the same cell category as the other HF internal layers. Furthermore, all these internal layers share common progenitors during the HF renewal process.

The mode of production of the Cp and IRS explains three features of HF morphogenesis: the coordination of the number of cells of these layers as a consequence of the similar characteristics of their progenitors; the coupling of their proximal to distal movement (Poblet et al., 2005; Rogers, 1964; Rothnagel and Roop, 1995); and their progressive maturation after leaving the bulb (Langbein et al., 2006). These features certainly also optimise the

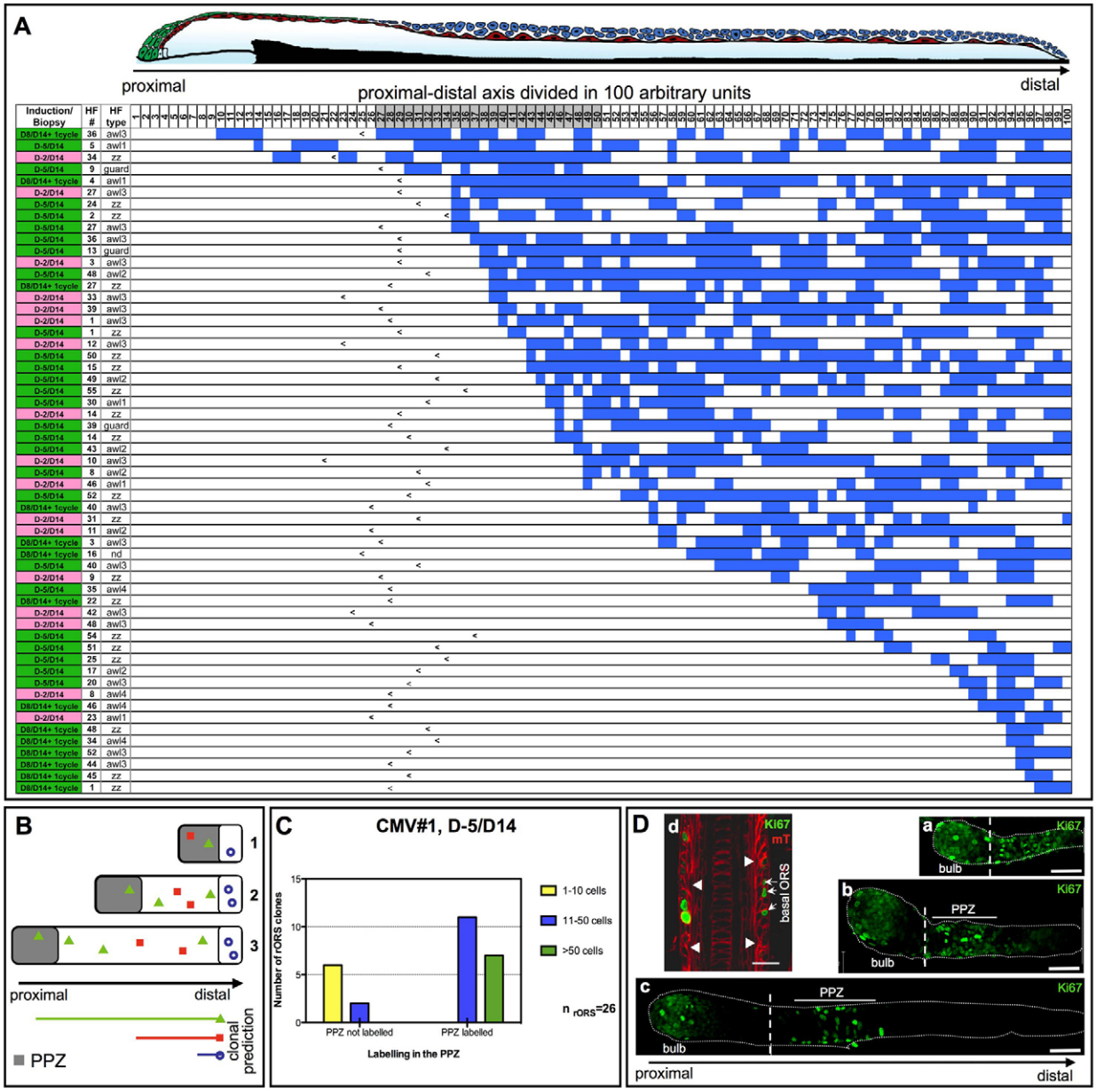

Fig. 6. The basal ORS lineage. (**A**) Radial-PD pattern of rORS clones induced before or during anagen and observed at D14 or D14+1 cycle. rORS clones are classified according to their most proximal contribution, as in Fig. 2F. Boxes on the left refer to the experiment (green, CMV#1; pink, Rosa#1). Grey positions indicate the privileged proliferation zone (PPZ). (**B**) Growth from a PPZ and clonal expectations. The structure grows from right to left (1-3). Only cells in the PPZ proliferate, producing the expanding white area where cells divide only rarely. Circle, a cell that will not be included in the PPZ and that produces a short distal clone; square, a cell producing progeny that will remain in the PPZ until the structure reaches 2, to give a medium sized clone; triangle, a cell for which at least one progeny remains in the PPZ, producing a long clone. The PPZ growth mode places cells from the structure that divides infrequently on the right. (**C**) Number of labelled cells in rORS clones that are labelled or otherwise in the PPZ. (**D**) Labelling for the Ki67 proliferation marker during HF growth. (a) At growth initiation, Ki67$^+$ nuclei appear uniformly among the ORS cells. (b,c) During anagen, the Ki67$^+$ cell population is located in the proximal portion of the basal ORS (as well as in the bulb). This cell population produces the distal postmitotic basal ORS cells and, in consequence, the PPZ is displaced proximally as the HF grows. At the same time, the proliferative cells in the bulb (on the left) produce internal structures and the bulb outer layer. The white dashed line corresponds to the proximal limit of the K14$^+$ basal ORS layer showing a constant localisation of the PPZ in the suprabulbar region. The two proliferation zones (bulb and PPZ) produce tissues in a proximal to distal direction. (d) The Ki67 proliferation marker is absent from the Cp cells (arrowheads); arrows indicate Ki67$^+$ cells in the basal ORS. Scale bars: 50 μm in Da-c; 20 μm in Dd.

establishment and maintenance of desmosomal junctions between the Cp and Henle cells (Gu and Coulombe, 2007; Ito, 1986; Orwin, 1971; Rogers, 1964).

The Cp layer belongs to the HF internal structures that share the same mode of production and development and have the same clonal origin. Together they form a distinct entity. The grouping of the seven internal layers that share the same clonal origin and mode of growth based on stem divisions of the germinative layer cells raises the possibility that these layers constitute a series of cell types that are phylogenetically related. It is tantalising to propose

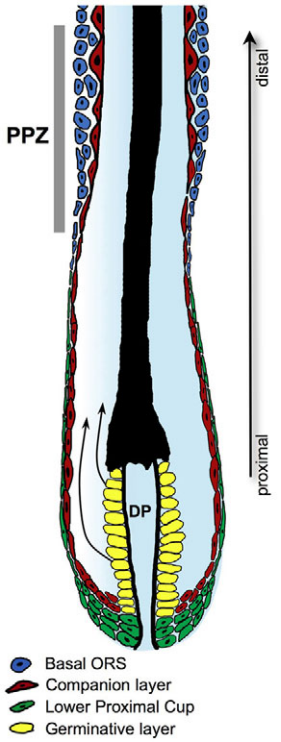

Fig. 7. A model of the outer layer of the HF. The outer layer originates from three clonal compartments: the K14+ basal outer root sheath (ORS) layer (blue); the companion layer (red) produced by proximal cells 4 and 5 in the germinative layer (yellow); and the lower proximal cup (green) that comprises bulb external cells and proximal cells 1 to 3 contiguous with the germinative layer. DP, dermal papilla; PPZ, privileged proliferation zone.

that they evolved by the duplication/diversification of cellular territories of the germinative layer.

The basal ORS and its growth

The second category of clones that contribute to the HF outer layer correspond to basal ORS cells. Therefore, the ORS has its own clonal origin. Other features of the ORS include the biochemical and immunological properties that it shares with the interfollicular epidermis with which it is contiguous (Hardy, 1992). Furthermore, during HF renewal the basal ORS layer has a specific mode of growth. This growth mode is neither stem nor strictly proliferative, as its functioning is anisotropic (it deposits behind it basal ORS cells that exhibit little mitotic activity) and involves only a subregion of the structure. This growth zone is located in the suprabulbar region. This result is in line with the high concentration of colony-forming cells present in this HF mid-region (Rochat et al., 1994). We propose to name it the privileged proliferation zone (PPZ) of the ORS (Fig. 7). Clearly, the ORS is excluded from the entity formed by the seven internal layers of the HF.

Two characteristics of the PPZ are remarkable. First, it moves passively downward due to mitotic pressure and the anisotropic production of cells, therefore accompanying the growth of the HF internal structures. The distal-to-proximal extension of the basal ORS might be the basis of the downward migration of cells described by several reports (Hsu et al., 2011; Jaks et al., 2008; Oshima et al., 2001; Panteleyev et al., 2001; Taylor et al., 2000). Second, the PPZ remains at a constant distance from the proximal end of the bulb independently of HF size. As a consequence, the newly produced ORS cells are adjacent to the newly produced cells of the internal layers. This may facilitate the establishment of junctions between the Cp and the basal ORS (Hanakawa et al., 2004). We propose that the maintenance and the strength of these junctions are increased because of the low mitotic activity of the ORS cells outside the PPZ, increasing the anchorage of the HF to the skin. These features support the idea of a crucial function of the ORS organisation in HF anchorage because when it is disturbed clefts develop between the IRS and the ORS and the anagen HF falls (Hanakawa et al., 2004).

The existence of a PPZ raises the question of its control. It is possible that early in anagen all ORS cells acquire the same division potential and that the PPZ is delimited by distal signals that confer quiescence to ORS distal cells. Another possibility is that the junctions between the Cp cells and the PPZ would sustain cell association and prevent their downward movement and division. The third possibility is that the mitotic activity of the PPZ is controlled by proximal elements of the HF. The exposure to dermal papilla activation signalling (Greco et al., 2009; Panteleyev et al., 2001) during anagen might be instrumental. Such a mechanism could also control (e.g. by modulating the concentration of a diffusible mitogenic factor) the size of the PPZ and its constant distance from the bulb, therefore coordinating the growth rate of the basal ORS and of the HF internal structures.

The bulb and the lower proximal cup

ORS anisotropic growth and the suprabulbar position of the PPZ both prevent this layer from covering the bulb, at least for mouse fur HFs. This is definitively demonstrated by the limit of the ORS clones and is consistent with *K14* mRNA (Coulombe et al., 1989) and Sox9 (Nowak et al., 2008; Vidal et al., 2005) expression. We show here that the outer layer of the bulb is formed of cells that have their own lineage. This structure includes not only the outermost single-cell layer of the bulb extending up to the basal ORS, but also the most proximal cells abutting the dermal papilla, at positions 1-3 in continuity with the germinative layer (Fig. 7). They cover the internal layers, in particular the Cp layer, and their progenitors inside the bulb. We propose to name this structure the lower proximal cup (LPC). The germinative layer cells formed by the progenitors of the seven internal layers (Legué and Nicolas, 2005; Legué et al., 2010) (these results) are contiguous with the most proximal cells of the LPC, which might or might not be their progenitor. Nevertheless, the LPC is clonally related to the internal layers of the HF and not to the ORS.

What is the function of the LPC? As the bulb cells are undifferentiated and poorly interconnected, it is likely that the shape of the bulb is determined at least in part by the shape of the dermal papilla and of the germinative layer. Because of its position, the LPC might also play a role in shaping the bulb by continuing the sheath that the ORS forms with dermal sheath cells. Notably, the dermal sheath covers both the LPC and the basal ORS. The fact that the LPC shares with the ORS characteristics such as Lgr5 expression (Jaks et al., 2008) (Fig. 5K,L) and therefore probably certain properties is very intriguing. These common properties might be involved in the mechanism that underlies their interaction with dermal cells to form a rigid sheath that extends to the most proximal part of the HF. Whether Lgr5 is a player in this mechanism remains to be established.

Finally, it has been shown that what we call the LPC is a source of FGF5 that controls features of the anagen including its duration (Hebert et al., 1994; Ota et al., 2002). More generally, it is possible that the LPC plays a wider role as a signalling centre, potentially interacting with the dermal papilla and the PPZ.

Acknowledgements
We thank Jacqueline Deschamps, Emilie Legué and Benjamin Steventon for comments on the manuscript; Christine Chevalier for contribution to the inductive system; Suzanne Capgras for excellent animal care; Lars Grotewold, Austin Smith, Daniel Metzger and Philippe Soriano for the gift of mouse lines; and we acknowledge IMAGOPOLE for imaging services and Ricardo Henriques for advice on image analysis.

Funding
This research was supported by Institut Pasteur [PTR grant no. 335]; Agence National de la Recherche [ANR-10-BLAN-121801 to J.-F.N] and Centre National Recherche Scientifique; I.S. was funded by Fundação para Ciência e Tecnologia and Association pour la Recherche contre Cancer; and J.-F.N. is from the Institut National de la Recherche Médicale.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.081091/-/DC1

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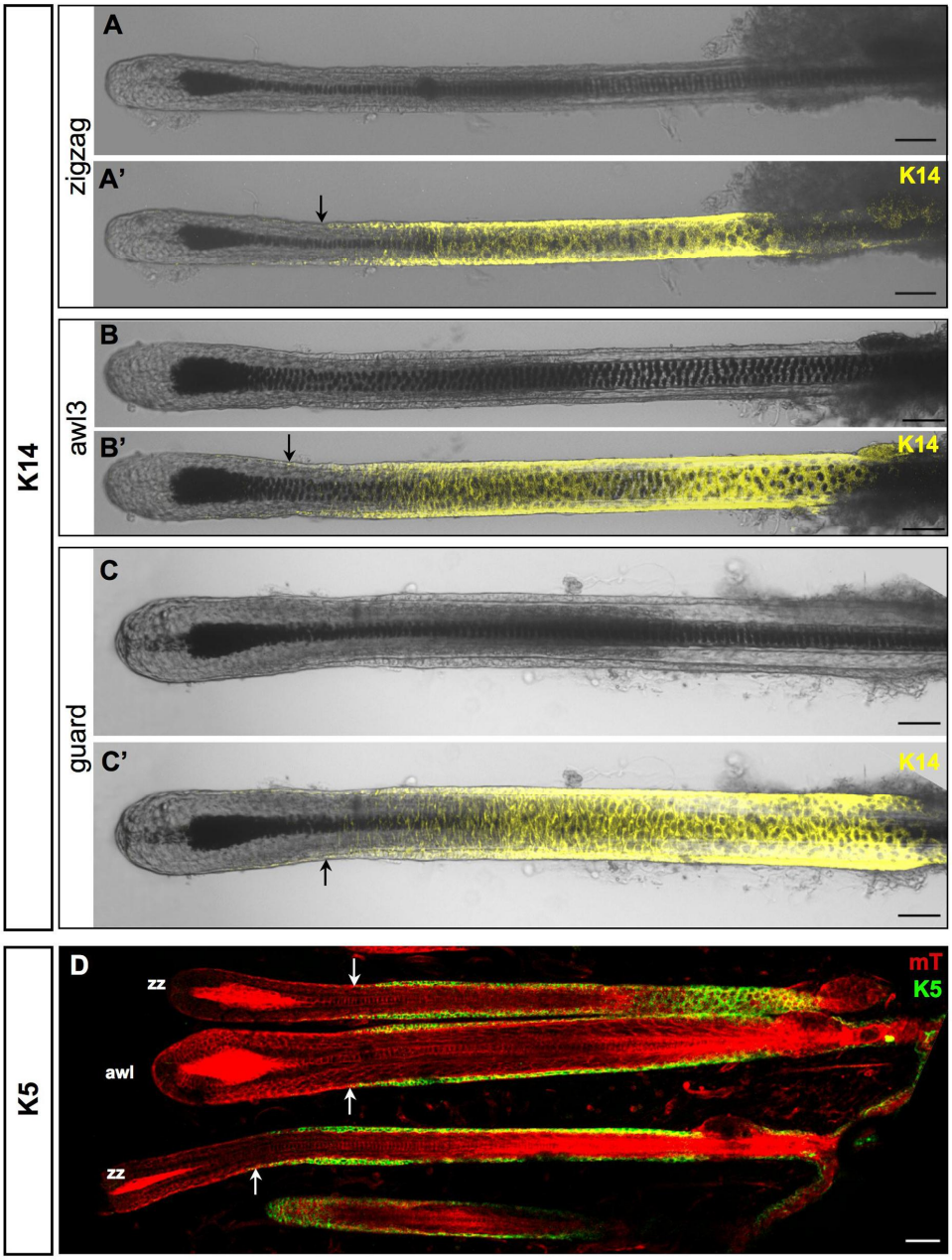

Figure S1 - Sequeira and Nicolas

Fig. S1. K14 and K5 expression in the outer layer of different HF types. In toto views of mouse back skin characterised by keratin immunostaining. (**A-C9**) K14 expression in zigzag (A,A9), awl3 (B,B9) and guard hairs (C,C9). (A,B,C) Bright-field view of each HF type. (A9,B9,C9) Bright-field view of each HF type and maximal projections of the K14 staining (yellow). (**D**) K5 expression (green) in HF sections from a mT/mG mouse with labelled membranes (red). All back skin HF types present a proximal limit of K14- and K5-expressing cells above the bulb (white arrows). Scale bars: 50 μm.

Table S1. Frequencies of labelling and categories of labelled HFs obtained after induction with different Cre and reporter lines

		CMV#1				CMV#2	Rosa#1			Rosa#2	Rosa#3	CMV#1	CMV#2	Rosa#1	Rosa#2
Animal	Inducer line	CMV CreER^{T}				CMV CreER^{T}	Rosa CreER^{T2}			Rosa CreER^{T2}	Rosa CreER^{T2}	CMV CreER^{T}	CMV CreER^{T}	Rosa CreER^{T2}	Rosa CreER^{T2}
	Reporter line	Rosa26R				mT/mG	Rosa26R			mT/mG	mT/mG	Rosa26R	mT/mG	Rosa26R	mT/mG
OHT dose		66.7 µg/g				3.33 µg/g	16.7 µg/g			1.67 µg/g	1.11 µg/g	0 µg/g			
Date of induction		**D8** (zone B)		**D-5** (zone A)		**D-5**	**D-2** (zone B)		**D3** (zone A)	**D-5**	**D8**	**Control** (non-induced)			
Date of biopsy		**D14**	**D14+1 cycle**	**D14**	**D14+1 cycle**	**D14**	**D14**	**D14+1 cycle**	**D14+1 cycle**	**D14**	**D14**	**P35**	**P35**	**P35**	**P35**
Frequency of labelling		**16.6%**	**8.10%**	**10.8%**	**9.37%**	**5.85%**	**3.20%**	**2.62%**	**2.87%**	**6.96%**	**4.57%**	**0.00%**	**0.11%**	**0.08%**	**0.00%**
Total number of HFs		686	864	1007	651	547	1875	2251	1430	359	1094	914	926	1230	977
Total number of labelled HFs		114	70	109	61	32	60	59	41	25	50	0	1	1	0
Number of analysed HFs		92	47	53	51	17	48	47	36	24	39	0	1	1	0
Labelling categories	rORS	12	15	29	17	9	20	20	11	11	4	0	0	0	0
	Complex	0	20	16	25	7	20	25	20	10	4	1	0	0	0
	Restricted Cp	18	2	1	1	0	1	1	0	0	6	0	0	0	0
	Oligopotent internal	8	6	6	5	0	6	1	5	2	12	0	0	0	0
	Restricted internal	28	4	1	3	0	1	0	0	1	10	0	1	0	0
	Restricted LPC	10	0	0	0	1	0	0	0	0	3	0	0	0	0
	Combined labellings	16	0	0	0	0	0	0	0	0	0	0	0	0	0

First line designates the experiment. The day of induction and the Cre inducer and reporter lines used are specified at the top of each column. The same experiment may have two different days of induction according to the area of the mouse skin considered (see Fig. 1A). All experiments were analyzed 14 days after depilation, in the same cycle (D14) or one cycle after induction (D14+1 cycle). 'Frequency of labelling' is the number of labelled HF/total number of

HF×100. 'Total number of HFs' refers to the number of HFs observed (labelled plus unlabelled). 'Number of analyzed HFs' refers to the number of labelled HFs that could be analyzed after dissection. For each cross, before the beginning of the experiment, control samples were analyzed at postnatal day 35 (P35), when dorsal pelage is in anagen (Control, non-induced). The labelled HFs were classified into seven categories: the rORS clonal patterns have labelling restricted to the basal ORS; the complex clonal patterns exhibit labelling in the Cp and internal structures of the HF; the oligopotent internal clonal patterns have labelled cells in several internal structures and the restricted internal clonal patterns with labelled cells in only one internal structure (restricted to IRS or restricted to HS). We checked whether the labellings were indeed generated by a single recombination event as opposed to a double or triple event. Combined labellings correspond to labelling in several structures (ORS, Cp and/or internal) with a frequency did not differ from the expected frequency of a double or triple recombination event. We therefore did not consider the HFs belonging to these categories as clones and represented them in the figures (Fig. 5B) with grey boxes.