A two-step mechanism generates the spacing pattern of the ciliated cells in the skin of *Xenopus* embryos

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SUMMARY

The skin of *Xenopus* embryos contains a population of specialized ciliated cells that are distributed in an evenly spaced pattern. Here we describe two successive steps that govern the differentiation and the generation of the spacing pattern of these ciliated cells. The first step occurs in the inner or sensorial layer of the non-neural ectoderm where a subset of cells are chosen to differentiate into ciliated-cell precursors. This choice is under the control of lateral inhibition mediated by a Suppressor of Hairless-dependent Notch signaling pathway, in which X-Delta-1 is the putative ligand driving the selection process, and a new Enhancer-of-Split-related gene is an epidermal target of Notch signaling. Because nascent ciliated-cell precursors prevent neighboring cells from taking on the same fate, a scattered pattern of these precursors is generated within the deep layer of the non-neural ectoderm. Ciliated-cell precursors then intercalate into the outer layer of cells in the epidermis. We show that the intercalation event acts as a second step to regulate the spacing of the mature ciliated cells. We propose that the differentiation of the ciliated cells is not only regulated by Notch-mediated lateral inhibition, but is also an example where differentiation is coupled to the movement of cells from one cell layer to another.

Key words: *Xenopus*, Ciliated cell, Spacing pattern, Lateral inhibition, Notch

INTRODUCTION

A common feature of epithelia in both plants and animals is that they contain specialized cells types, which are organized in ordered spatial patterns. Examples of such patterns include the trichomes (single-celled hairs) in the epidermis of *Arabidopsis Thaliana*, sensory organs with their associated bristles in insects, feather buds in the chicken skin and hair follicles in mouse skin. The common element in these examples is that the distance between the specialized cells tends to be maximized, giving rise to a so-called spacing pattern. Such spacing patterns are thought to arise developmentally by mechanisms involving lateral inhibition, a process in which one developing element produces an inhibitory signal that blocks the development of similar elements nearby (Doe et al., 1985; Wigglesworth, 1940).

One form of lateral inhibition studied extensively involves the receptors of the Notch family and their ligands, which mediate a common mechanism for restricting the number of cells that will take on a particular fate from a group of competent cells (Artavanis-Tsakonas et al., 1995). In this mechanism, differentiating cells express a transmembrane ligand of Notch, for example Delta, which binds the Notch receptor on neighboring cells. Activation of Notch signaling leads, via the conserved transcription factor, Suppressor of Hairless [Su(H), also known as CBF-1 or lag-1], to the transcriptional activation of Notch target genes whose products block the progression of cells into the differentiated state (Wettstein et al., 1997 and references therein). The role of Notch-mediated lateral inhibition in determining the spacing of differentiated cells has been studied extensively in *Drosophila* where sensory organs form with a characteristic spacing pattern in the epidermis (Hartenstein and Posakony, 1989; Simpson, 1990). The position of sensory organs is determined by the bHLH transcription factors encoded by the Achaete-Scute complex (AS-C), whose expression endows dispersed clusters of cells in the ectoderm with the competence to form sensory organs precursors (SOP). Within the cluster, a nascent SOP expresses Delta, which activates Notch in adjacent cells. Activation of the Notch pathway ultimately inhibits the activity of the AS-C genes in the other cells of the cluster and prevents these cells from also differentiating into SOPs (Ghysen et al., 1993; Heitzler et al., 1996). Blocking Notch-mediated lateral inhibition results in an overproduction of SOPs within each cluster, but the spacing of the clusters within the epidermis is unaffected (Heitzler and Simpson, 1991). Thus, in this example, Notch signaling is used to control spacing at a local level, by preventing SOPs from forming adjacent to each other, but does not appear to act to generate spacing in a more global context.

In vertebrates, the role of the Notch pathway in the patterning of non-neural epithelia has been examined during
the formation of feather buds (Crowe and Niswander, 1998; Viallet et al., 1998) Feather buds form with a regular spacing pattern, and activation of the Notch pathway at a critical stage, abolishes bud formation. However, the spacing pattern of buds appears to be preset through a reciprocal interaction between the ectoderm and underlying mesoderm, involving growth factors (Jung et al., 1998; Noramly and Morgan, 1998, reviewed in Oro and Scott, 1998).

In particular, BMPs are produced by the forming feather bud and acts as a long-range signal to restrict bud formation. In addition, recent evidence suggests that spacing of hair follicles in mammal skin may involve the Wnt signaling pathway (Gat et al., 1998). Thus, if Notch has a role in hair/feather follicle spacing, this role is more likely to be in controlling the size, polarity or the generation of cell types within the follicles, rather than their spacing within the epithelia.

Elucidation of the mechanisms generating hair and feather spacing patterns is complicated by the fact that they are complex multicellular structures. By contrast, the ciliated cells in the X. laevis embryonic skin constitute a simple example of spacing pattern involving single-celled epidermal appendages. Ciliated cells are arranged in the surface layer of the non-neural ectoderm in a strikingly ordered distribution (Fig. 1). Their function is enigmatic but, by beating together in the same direction, they could produce currents in the intravitelline medium, allowing the embryo to exchange oxygen or to distribute secreted products such as mucous or hatching enzymes. Mature ciliated cells are detected by stage 22, but by electromicroscopy, the precursors to the ciliated cells can be identified through numerous centrioles and precentriolar material accumulation as early as stage 13, when they appear to lie beneath the outer layer of cells (Billett and Gould, 1971; Steinman, 1968). The embryonic skin of X. laevis embryo is effectively composed of two layers of cells; the outer layer is made up of large columnar cells tightly adherent to each other while the inner, or sensorial layer is made up of smaller and loosely adherent cells. Tracing experiments have shown that ciliated cells originate from the inner layer: labeled grafts of inner cells produced labeled ciliated cells whereas labeled outer grafts were interspersed by non-labeled ciliated cells (Drysdale

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**Fig. 1.** Ciliated cells are arranged in a regularly spaced pattern and are marked by the expression of an \( \alpha \)-tubulin gene. (A,B) Scanning electron micrographs of the skin of a X. laevis embryo (stage 25) taken at a magnification of 200\( \times \) (A) and 750\( \times \) (B). Note the spacing pattern of cells with tufts of cilia in the outer layer of X. laevis skin (B, Arrow). (C) X. laevis embryo (stage 22) stained in whole mount using HRP immunohistochemistry with a mouse monoclonal antibody directed against acetylated \( \alpha \)-tubulin (6-11B1). (D) X. laevis embryo (stage 25) double-labeled by in situ hybridization with the \( \alpha \)-tubulin probe (light blue) followed by 6-11B1 antibody staining (brown). (E) Transverse section of the embryo shown in D, confirming that the same cells stain with both the \( \alpha \)-tubulin probe and the 6-11B1 antibody (arrows).

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**Fig. 2.** Expression of \( \alpha \)-tubulin in the skin of X. laevis embryos at different developmental stages. Expression of \( \alpha \)-tubulin detected by whole-mount in situ hybridization on X. laevis embryos at stage 13 (A-C), stage 16 (D-F) and stage 20 (G-I). Dorsal views (A,D,G) and lateral views (B,E,H) show that \( \alpha \)-tubulin expression occurs in the non-neural ectoderm (nne) and not in the neural plate (np). Expression is also absent from the cement gland (cg). (C,F,I) Sections of whole-mount-stained embryos show that cells expressing \( \alpha \)-tubulin are located in the inner layer (parenthesis or arrow labeled “i”) at stage 13, and in the outer layer (brackets labeled “o”) at stage 16 and older, indicating that the ciliated cells first arise in the inner layer before stage 13 and then subsequently move into the outer layer.
4717 Formation of the ciliated cells spacing pattern (and Elinson, 1992). Thus, ciliated cells appear to form in the inner layer and to migrate into the outer layer at neurula stages. How these cells are generated and achieve their final spacing pattern is not known.

Here, we examine the mechanisms that control the formation and spacing of the ciliated cells. We provide evidence that the mechanism responsible for their even distribution throughout the non-neural ectoderm of the embryo can be subdivided in two steps. The first step involves lateral inhibition via the Notch pathway, which restricts the number of cells in the sensorial layer that adopt the ciliated-cell fate. As this mechanism mostly precludes two precursors to be in direct contact, it presets the initial distribution pattern. The second step involves the migration of the ciliated-cell precursors from the inner to the outer layer of the epidermis. In itself, the intercalation into the tightly adherent layer of outer cells could prevent the migration of more than one inner cell per epithelial junction. Thus the ciliated cells distribution provides not only a new model system to study the lateral inhibition mechanism mediated by Notch and Delta in Xenopus embryos but also a simple example for studying the morphogenesis of an organized epithelial pattern.

MATERIALS AND METHODS

Embryos and injections

Embryos were obtained from Xenopus laevis adult frogs by hormone-induced egg laying and in vitro fertilization using standard methods. Xenopus embryos were staged according to Nieuwkoop and Faber (1967).

Constructs cloned in pCS2+ vectors (Turner and Weintraub, 1994) were transcribed into capped RNA using SP6 RNA polymerase. Templates for generating RNA encoding Notch-ICD, X-Su(H)-DBM and X-Delta1Stu were obtained as described, respectively, in Chitnis et al. (1995), Coffman et al. (1993) and Wettstein et al. (1997). Templates for generating RNA encoding ESR-6e, ESR-6eΔb, ESR-7 and ESR-7Δb were obtained by linearization of their respective CS2 constructs by NotI. For in situ hybridization analysis, synthetic n-lacZ RNA (20 pg), encoding a nuclear-localized form of β-galactosidase, along with 1-2 ng test RNAs (stated if otherwise) were injected into single blastomeres of albino embryos at the 2-cell stage as described previously (Coffman et al., 1993). For animal caps, 2-cell-stage Xenopus embryos were injected in the animal region of each blastomere with 1-2 ng of the indicated capped synthetic RNAs.

Fig. 3. X-Delta-1 is expressed in the ventral ectoderm in a punctate pattern that anticipates α-tubulin expression. (A-C) Xenopus embryo (stage 11) stained in whole mount for the expression of X-Delta-1 by in situ hybridization. View of the ventral side is shown in A, a lateral view in shown in B and a transverse section is shown in C. Note that X-Delta-1 is expressed by scattered cells within the inner layer of the ventral ectoderm (A,C), in addition to cells (B, arrowheads) that are localized to stripes within the neural plate where primary neurons will form (np). (D,E) α-tubulin expression is first detected by in situ hybridization at stage 12 in the ventral ectoderm (D). Lateral view shows that α-tubulin is expressed by scattered cells in the non-neural ectoderm (nne) but not in the neural plate (np). bl, blastopore.

Fig. 4. Notch signaling restricts the number of α-tubulin-expressing cells. Albino embryos were injected at 2-cell stage in one blastomere with (A-C) nLacZ RNA alone, (D-F) Notch-ICD and nLacZ RNAs, (G-I) X-Su(H)-DBM and nLacZ RNAs, or (G-L) X-Delta-1Stu and nLacZ RNAs. At stage 17-18 (neurulae), the embryos were fixed, stained in whole mount with X-gal (light-blue reaction product) and then labeled by in situ hybridization for the expression of α-tubulin (dark purple). Left panels show a lateral view of the uninjected side, the middle panels a lateral view of the injected side, and the right panels show a dorsal view. Note that Notch-ICD suppresses α-tubulin expression in 93% of injected embryos (57 embryos in four representative experiments) while the density of α-tubulin-expressing cells increases in embryos injected with X-Su(H)-DBM (55/64 injected embryos) or with X-Delta-1Stu (64/80 injected embryos) in three independent experiments.
Whole-mount in situ hybridization, immunostainings and histology

Whole-mount in situ hybridization of *Xenopus* embryos was performed according to Harland (1991) with modifications described by Knecht et al. (1995) using digoxigenin-labeled antisense RNA probes for *X-Delta-1* (Chitnis et al., 1995), *Xenopus α-tubulin* and *ESR-6e* (full-length sequences were subcloned in pBS-SK, linearized, respectively, by *Not* or *BamH*I and transcribed by T7 RNA polymerase). For detection of acetylated α-tubulin, embryos were stained with the monoclonal antibody (Ab) 6-11B1 (Sigma) as described (Bradley et al., 1998). Prior to in situ hybridization or immunostaining, injected embryos were stained for β-galactosidase activity with X-gal or Magenta-gal ( Biosynth) to localize the tracer. Embryos to be sectioned were embedded in Paraplast, serially sectioned into 10 μm thick sections, dewaxed, stained with eosin and finally photographed under a phase-contrast microscope.

Animal caps experiments and RNase protection assays (RPA)

Animal caps were dissected at stage 9 and cultured on agarose-coated Petri dishes in 0.5x MMR containing gentamycin until sibling controls reached the stage noted. In experiments where the two ectodermal layers were separated, outer ectodermal layers of whole stage 9 embryos animal caps were removed manually and set aside on agarose coated culture dishes in 0.5x MMR. The inner layer of the animal cap was then cut off from the rest of the embryo and cultured likewise in another dish. The inner layer preparations were visually checked for absence of contamination by pigmented outer layer cells. RNA was isolated and analyzed by RNase protection assay using 32P-labeled antisense RNA probes as previously described (Kintner and Dodd, 1991; Kintner and Melton, 1987). The template used for the α-tubulin probe corresponds to the EcoRV-HindIII fragment contained in the second half of the open reading frame, while that for X-Delta-1 has been described previously (Chitnis and Kintner, 1996). 6-11E6 probe corresponds to the fragment of the bHLH domain initially isolated by RT-PCR, which was subcloned between the *Xhol* and *BamH*I sites in pBS-SK(Stratagene). RNA samples isolated from 10 animal caps, or from 2 animal caps for α-tubulin assays, were analyzed simultaneously along with the *EF1*-α probe to control for sample recovery during the assay. Quantification was carried out on a PhosphorImager (Molecular Dynamics) and, for each lane, specific band intensities were normalized to the amount of *EF1*-α RNA.

Isolation of a new marker of the ciliated cells and their precursors

As observed by scanning electromicroscopy performed at any stage after stage 22, the epidermis of *Xenopus* embryos contains specialized cells with tufts of cilia that are arranged in a regular spacing pattern (Fig. 1A,B). From stage 20 onwards, the ciliated cells can be detected using the antibody 6-11B1, which reacts with acetylated α-tubulin (Fig. 1C), a stable form of α-tubulin found in microtubules (Chu and Klymkowsky, 1989). Staining with 6-11B1 occurs only when the ciliated cells begin assembling considerable amounts of microtubules required for cilia growth, thus making it a relatively late marker for ciliated cells. However, while screening a cDNA library for genes expressed during neurogenesis, we fortuitously isolated an α-tubulin cDNA, which is expressed by evenly spaced cells in the non-neural ectoderm at earlier stages (Fig. 2). The cells that stain with the α-tubulin probe also stain with the 6-11B1 antibody in double-labeled embryos, indicating that the ciliated cells express α-tubulin (Fig. 1D,E).

In order to establish a time course for α-tubulin expression, embryos at different developmental stages were stained with α-tubulin probe using whole-mount in situ hybridization. α-tubulin expression is first detected in late gastrula-stage embryos (stage 12) as a diffuse but scattered pattern of staining in the animal pole ectoderm (Fig. 3D,E). By early neurula stages (stage 13), expression of α-tubulin is clearly restricted to the non-neural ectoderm (Fig. 2A,C). Between stages 13 and 16, the α-tubulin mRNA accumulates dramatically in a subset of cells within the non-neural ectoderm and, from stage 16 onwards, the α-tubulin-expressing cells are clearly organized in a spacing pattern (Fig. 2D-I). The neural plate and cement gland are devoid of α-tubulin-positive cells at all stages. α-tubulin expression stays on at tadpole stages (stage 30) and remains restricted to cells within the epidermis with the characteristic spacing pattern of ciliated cells (data not shown).

The ciliated cells originate from the inner layer of the ectoderm and intercalate to the surface

Since the α-tubulin marker allowed us to detect the cells that eventually become ciliated much earlier than the 6-11B1 staining, we were able to follow the differentiation of these cells, from gastrula stages when they are first generated, to...
early tadpole stages when they are completely differentiated as ciliated cells. In tissue sections of stained embryos, the α-tubulin-expressing cells are initially located only in the sensory layer of the ectoderm (Fig. 2C). From stage 16 onwards, the α-tubulin-expressing cells are located exclusively in the superficial layer (Fig. 2F,I), suggesting that they have intercalated from the lower layer outwards. By stage 20, the α-tubulin-expressing cells have completed their migration (Fig. 2I) and, by stage 23, they are capped by the characteristic tuft of cilia (Fig. 1E). These observations indicate that the ciliated cells first arise in the deep layer at gastrula stages, intercalate into the superficial layer around stage 15, and then fully differentiate by stage 23.

A lateral inhibition process restricts the number of ciliated-cell precursors

The results obtained with the α-tubulin marker indicate that only a subset of the inner layer cells appear to be committed to the ciliated-cell fate. Furthermore, staining for α-tubulin expression performed around stage 12-13 (Fig. 2A-C) indicated that, even at these early stages, the ciliated-cell precursors are not adjacent to each other, suggesting that their formation is regulated by cell-cell interactions that restrict the number of these precursors. Given the ubiquitous role of the Notch pathway in selecting out specialized cell types from a field of equipotential cells, we asked whether the specification of the ciliated-cell fate is restricted by lateral inhibition as mediated by X-Notch-1 and its ligand X-Delta-1.

To examine the role of the Notch pathway in the specification of ciliated cells, we first examined the expression of X-Delta-1 using whole-mount in situ hybridization at stages when the ciliated-cell precursors are likely to form (stages 11-12). This analysis showed that a population of cells located in the non-neural ectoderm, opposite to the blastopore, expresses X-Delta-1 in a scattered pattern that prefigures the cells that will express α-tubulin (Fig. 3A,B). In addition, tissue sections demonstrated that these X-Delta-1-positive cells are located in the inner layer of the ectoderm where the α-tubulin-expressing cells are apparently generated (Fig. 3C). At stages 12-13, when α-tubulin expression first occurs (Fig. 3D,E), X-Delta-1 expression is downregulated in the non-neural ectoderm. By stage 13, X-Delta-1 is no longer detectable outside of the neural plate (data not shown). Thus, X-Delta-1 expression occurs transiently within the sensory layer of the non-neural ectoderm and corresponds to the time in development when the ciliated-cell precursors are likely to form.

To assess whether the formation of the ciliated-cell precursors is regulated by the Notch pathway, we used several reagents that have proven effective at activating or inhibiting Notch signaling in primary neurogenesis (Chitnis et al., 1995; Wettstein et al., 1997). Activation of the Notch pathway can be achieved by overexpressing the intracellular domain of Notch (Notch-ICD), which constitutively translocates to the nuclei where, in association with Suppressor of Hairless [X-Su(H)], it transactivates Notch target genes. Expressing Notch-ICD in embryos blocks the formation of primary neurons. Alternatively, Notch signaling can be inhibited by expressing a DNA binding mutant of Su(H), X-Su(H)-DBM, or by expressing a form of the X-Delta-1 ligand that lacks an intracellular domain, called X-Delta-1Stu. Expressing X-Su(H)-DBM or X-Delta-1Stu in embryos by RNA injection causes more primary neurons to form than normal. Thus, we injected RNA transcripts encoding Notch-ICD, X-Su(H)-DBM and X-Delta-1Stu into one blastomere of 2-cell-stage embryos, along with a tracer RNA, nLacZ (see Materials and Methods) and tested for α-tubulin expression.

Injecting embryos with just nLacZ RNA, (Fig. 4A-C) did not affect the density or the distribution of the α-tubulin-expressing cells. By contrast, injecting embryos with RNA encoding the constitutively active form of X-Notch1, Notch-ICD, resulted in a complete loss of the α-tubulin-expressing cells, as predicted if Notch signaling negatively regulates the formation of ciliated-cell precursors (Fig. 4D-F). The RNA encoding Notch-ICD inhibited the formation of α-tubulin-expressing cells over a range of 2 ng to 125 pg of injected RNA per embryo. Injection of RNAs encoding the molecules that block Notch signaling, X-Su(H)-DBM or X-Delta-1Stu, led to a dramatic increase in the density of the ciliated cells precursors (Fig. 4G-L) so that the α-tubulin-expressing cells filled the whole injected region of the inner ectodermal layer and were no longer separated by non-expressing cells. In some embryos, more severe phenotypes were observed where the ectodermal cell layer was thickened and filled by several layers of α-tubulin-expressing cells (Fig. 8C). Thus, these results indicate that the number of ciliated-cell precursors generated during gastrula stages is regulated by the Su(H)-dependent Notch pathway.

Previous studies have shown that overexpressing the wild-type form of X-Delta-1 in the neural plate suppresses the formation of primary neurons, as expected for a ligand that activates Notch signaling. However, when X-Delta-1 was overexpressed in the non-neural ectoderm, it did not inhibit the ciliated-cell fate but, on the contrary, caused an overproduction of α-tubulin cells with phenotypes resembling those caused by X-Delta-1Stu RNA injection (not shown). The penetrance of the X-Delta-1 phenotype was weaker: only 45% of injected embryos on a total of 94 (in 4 independent experiments) displayed the phenotype compared to 80% for X-Delta-1Stu. While this result is at odds with the idea that X-Delta-1 acts as an inhibitory ligand during the formation of the α-tubulin-expressing cells, it is reminiscent of similar results in Drosophila, where overexpressing Delta usually leads to a reduction of Notch signaling, suggesting a dominant negative effect (see Discussion).

Lateral inhibition regulates X-Delta-1 expression within the non-neural ectoderm

In Drosophila, expression of Delta is positively regulated by the proneural genes, whose activity, in turn, is negatively regulated by the Notch pathway. In principle, therefore, the levels of Delta expression between cells undergoing lateral inhibition is modulated so that Delta expression reaches high levels in differentiating cells and low levels in cells whose differentiation is blocked by the Notch pathway. To examine whether X-Delta-1 expression is similarly modulated during the generation of ciliated-cell precursors, we examined the expression of X-Delta-1 in embryos where the Notch pathway had been activated or inhibited. As shown in Fig. 5, when embryos were injected with Notch-ICD RNA, the expression of X-Delta-1 was suppressed, whereas injecting RNA encoding X-Su(H)-DBM produced the opposite effect: a large increase in the number of X-Delta-1-expressing cells. To confirm these
**Fig. 6.** ESR-6e encodes a WRPW-bHLH protein expressed epidermally and activated by the Notch pathway. (A) ESR-6e encodes a WRPW-bHLH protein related to other Xenopus ESR genes that are activated by the Notch pathway. ESR-1 and ESR-7 are expressed in the developing nervous system, while ESR-5 is expressed in the paraxial mesoderm during segmentation. Percentages of similarity between the bHLH domains of ESR-6e and other ESR factors identified in Xenopus are given as well as the overall similarity for the full-length proteins.

(B) Expression of ESR-6e RNA in stage 14 (neural plate) Xenopus embryos as detected by whole-mount in situ hybridization. Note that ESR-6e expression is mostly localized to the non-neural ectoderm as shown by the white dashed line marking the border of the neural plate. A high level of expression is also observed in the cement gland (white arrowhead) and a low level in the central neural plate (black arrowhead). (C) Total RNA extracted from embryos at the indicated stages was assayed by RPA for the levels of ESR-6e RNA and EF1-α RNA as a loading control. The levels of ESR-6e at different stages are plotted in a bar graph after normalizing to the levels of EF1-α RNA. EF1-α RNA is first transcribed at the mid-blastula transition, reaching steady levels at stage 12. Thus, the levels of ESR-6e RNA are likely to be overestimated after normalizing to the levels of EF1-α RNA at stage 8 and 10. (D) Notch-ICD RNA was coinjected with nLacZ RNA in one blastomere of 2-cell-stage embryos. At stage 14, the embryos were stained with X-Gal (light blue) and for ESR-6e expression using in situ hybridization (dark blue). Note that Notch-ICD RNA induces high levels of ESR-6e expression but only in the non-neural ectoderm in 48 injected embryos on a total of 54. In this example, the Notch-ICD-injected area (circled by the black dashed line) extends into the neural plate but ESR-6e overexpression stays confined to the prospective epidermis (white dashed line) as in B. Note also that the embryo shown in D has been stained for less time than the one in B, as indicated by the reduced signal from the basal expression of ESR-6e. (E,F) Transverse sections of a Notch-ICD-injected embryo as in D. (G) RNase protection assay to measure the levels of ESR-6e RNA in ectoderm isolated from embryos injected with RNA encoding Notch-ICD or X-Su(H)-DBM as described in the legend to Figure 5. RNA was extracted at stage 11 from intact ectoderm (w. caps) or from the inner layer (inner) or outer layer (outer) after manual separation. Note that Notch-ICD induces higher levels of ESR-6e expression in both layers. X-Su(H)-DBM also increases the levels of ESR-6e in the inner layer but not in the outer layer.
results, we also measured the levels of X-Delta-1 mRNA in an animal cap assay. Ectodermal explants dissected out before mid-gastrula stages form atypical epidermis only and no neural ectoderm. Although this isolated tissue is not properly organized into two layers, it nevertheless develops ciliated cells and express epidermal markers such as epi and XK81 (Akers et al., 1986; Miyatani et al., 1986; Wilson and Hemmati-Brivanlou, 1995; Xu et al., 1997). Embryos were injected at 2-cell stage with 2 ng of Notch-ICD or X-Su(H)-DBM RNA in the animal pole of each blastomere. Animal caps were dissected at stage 8 and left to develop until sibling embryos reached stage 11, when total RNA was extracted. RNase protection assays (RPA) with X-Delta-1 probe confirmed that the expression of Notch-ICD blocks X-Delta-1 expression while expression of X-Su(H)-DBM increases it (Fig. 5E). We also verified that the variations of X-Delta-1 mRNA levels correlated with those of a-tubulin mRNA as measured by RPA (Fig. 5D). These complementary results are a strong indication that Notch signaling effectively regulates X-Delta-1 expression during the process of selecting out the ciliated-cell precursors. Furthermore, these results indicate that Notch signaling regulates the activity of an unknown upstream factor that not only promotes the differentiation of ciliated-cell precursors, but also the expression of X-Delta-1.

A novel bHLH-WRPW transcription factor activated by the Notch pathway

In many cases where Notch acts during Drosophila development, its downstream signaling activates genes in the E(spl)-C. Many of them encode bHLH proteins that share several characteristic structural features, namely a conserved proline in the basic region and a C-terminal WRPW tetrapeptide necessary for interaction with the co-repressor Groucho (Bailey and Posakony, 1995). To further examine how the Notch pathway might regulate the formation of a-tubulin-expressing cells, we used RT-PCR to amplify sequences that encode WRPW-bHLH proteins from RNA isolated from Notch-ICD-expressing animal caps. This approach led to the identification of a cDNA that encodes a novel WRPW-bHLH protein with significant homology to other Enhancer of Split-related factors (ESR) known to act downstream of Notch signaling in Xenopus. Based on this homology, and its regulation by the Notch pathway (see below), the new Xenopus protein was termed ESR-6e (Xenopus Enhancer of Split-Related-6 epidermal). Compared to the other ESR proteins identified so far, ESR-6e shares the highest level of similarity with ESR-1 (D. L. Turner, personal communication), a WRPW-bHLH protein expressed in the neural plate, with 76% homology between their bHLH domains (Fig. 6A).

The expression of ESR-6e was localized in early Xenopus embryos by whole-mount in situ hybridization at neurula stages, when ESR-6e is strongly expressed in the non-neural ectoderm, including the cement gland. By contrast, ESR-6e expression was undetectable in the neural plate except for extremely weak expression in the medial neural plate (Fig. 6B). In addition, ESR-6e staining in the non-neural ectoderm is mostly localized to the outer layer of cells (Fig. 6F) where it is uniformly expressed (Fig. 6B). Even though ESR-6e expression could be detected weakly in the inner layer of the non-neural ectoderm (Fig. 6F), whole-mount in situ hybridization proved not to be sensitive enough to detect ESR-6e expression before neurula stages or to allow the visualization of a pattern of expression that could be related to X-Delta-1 or early a-tubulin expression patterns. With the aim to establish if ESR-6e could play a role during the formation of a-tubulin-expressing cells, we used a more sensitive method, RPA, to detect ESR-6e RNA expression in...
embryos and found that expression could be detected soon after the mid-blastula transition, with levels peaking at stage 10 to 12 and then decreasing through tadpole stages (Fig. 6C). Moreover, using RPA, we could detect ESR-6e RNA at stage 11-12 in both the inner and outer ectodermal layers when assayed separately after manual separation (Fig. 6G). Thus, RPA results indicate that ESR-6e RNA is also expressed at earlier stages, within the inner layer, at the time and place where the ciliated-cell precursors form.

**ESR-6e is regulated by the Notch pathway**

We next examined whether ESR-6e expression in the ectoderm is regulated by the Notch pathway. In embryos injected with RNA encoding an activated form of the receptor, Notch-ICD, ESR-6e expression was dramatically increased, as expected for a Notch target gene (Fig. 6D-G). Strikingly, ESR-6e expression increased markedly in the non-neural ectoderm but not detectably in the neural ectoderm, even when Notch signaling was activated in a large region of the embryo extending into the neural plate (Fig. 6D). By comparison, other Notch target genes, such as ESR-1 or ESR-7, respond to activated Notch by upregulating in both the non-neural and neural ectoderm (data not shown). Transverse sections demonstrated that ESR-6e responded to Notch-ICD RNA injection by increasing in both the outer layer, as well as in the inner layer, where expression is normally more difficult to detect (Fig. 6E,F). RPA confirmed that the expression of ESR-6e was induced in both the inner and outer cell layer of ectoderm isolated from embryos injected with Notch-ICD RNA (Fig. 6G). Thus, these results indicate that the expression of ESR-6e can be promoted by the Notch signaling pathway.

To determine if Notch signaling via Su(H) is necessary for ESR-6e expression, we also assayed, at stage 11-12, the levels of ESR-6e in the inner and outer layer of isolated ectoderm from embryos injected with X-Su(H)-DBM RNA. The results show that expression of X-Su(H)-DBM did not affect the levels of ESR-6e RNA in intact ectoderm (Fig. 6G), which reflects the results obtained with the outer layer as a significant portion of ESR-6e expression is accounted by expression in the outer layer (Fig. 6E). However, in the deep cell layer, the expression of X-Su(H)-DBM appeared to increase the levels of ESR-6e RNA. Similar results have been obtained with the E(spl)-C genes that are regulated by the Notch pathway in Drosophila (see Discussion), suggesting that the expression of ESR-6e is not only regulated by Notch signaling but by other factors as well.

**ESR-6e functions to restrict the number of ciliated precursors**

If ESR-6e acts downstream of Notch to restrict the number of ciliated cells, then altering the activity of ESR-6e should change the number of α-tubulin-expressing cells that form. To address this point, we increased ESR-6e activity by injecting ESR-6e RNA into embryos. Conversely in order to decrease ESR-6e activity, we designed a potential dominant negative mutant (ESR-6eΔb) in which key residues of the basic domain were modified (8EKMRRDRIN165→REMEEIDDID), producing a form that should not bind DNA. The design of the mutant was based on the premise that these proteins act primarily as dimers, and that a DNA-binding mutant subunit would inactivate endogenous wild-type molecules. Injection of RNA encoding ESR-6e or ESR-6eΔb into embryos produced a marked decrease and increase, respectively, in the number of α-tubulin-expressing cells as predicted if ESR-6e acts downstream of Notch to regulate the formation of ciliated-cell precursors (Fig. 7A-D).

As a specificity control for these experiments, we also injected RNA coding for another ESR family member, ESR-7, which is activated by the Notch pathway but is expressed in the neural plate in the three domains where the primary neurons form, and not in the non-neural ectoderm (data not shown). The bHLH domains of ESR-7 and ESR-6e share 70% identity (Fig. 6A). We therefore exploited the fact that these two very related WRPW-bHLH proteins, which are both activated by the Notch pathway, have different expression domains to test the specificity of the ESR-6eΔb mutant. In these experiments, RNAs encoding wild-type and basic domain mutants of ESR-6e and ESR-7 were injected into embryos. Ectodermal caps were removed at stage 9 and assayed for expression of X-Delta-1 at stage 11 and α-tubulin at stage 19 by RPA. Injection of either ESR-6e or ESR-7 RNA led to a decrease in the levels of X-Delta-1 and α-tubulin RNA (Fig. 7E,F) in animal caps, mirroring the effects seen when the Notch pathway is activated by ICD. Significantly, injection of ESR-6eΔb RNA caused an increase in the expression levels of X-Delta-1 and α-tubulin RNA (Fig. 7E,F) while injection of ESR-7Δb RNA did not (Fig. 7F). Moreover, co-injecting ESR-6eΔb RNA partially rescued the loss of α-tubulin expression caused by ESR-6e but not by ESR-7 (Fig. 7F). These results establish ESR-6eΔb as a specific dominant negative form of ESR-6e and argue strongly in favor of ESR-6e acting downstream of X-Notch-1 in the lateral inhibition process that restricts the number of ciliated cells precursors.

The intercalation of ciliated cells precursors is the second step responsible for establishment of the final spacing pattern

The results described above indicate that the α-tubulin-expressing cells are initially generated in a fine-grained pattern as a result of lateral inhibition. As development proceeds and ciliated-cell precursors intercalate into the outer layer and differentiate, the pattern becomes increasingly organized into a spacing pattern. For example, in a majority of embryos, the α-tubulin-expressing cells are produced in a higher number anteriorly: they are denser and smaller at the limit of the open head folds and around the cement gland (Fig. 2E). However, by stage 20, the distribution of the ciliated-cell precursors appears more homogeneous throughout the whole embryo even if their density is often higher on the forming head (Fig. 2G,H). This observation suggests that even if lateral inhibition presets a salt and pepper distribution pattern of ciliated-cell precursors, other mechanisms contribute to the final organization of the ciliated-cell pattern in later embryos. If this is indeed the case, when lateral inhibition fails, a certain degree of organization in the pattern of the ciliated cells should remain.

To test this idea, we blocked lateral inhibition in the non-neural ectoderm by injecting embryos with RNA encoding X-Su(H)-DBM. When an aliquot of these embryos were processed at stage 16, the density of α-tubulin-expressing cells on the injected side was increased to a varying degree as shown above (Fig. 8A-C). In embryos with a mild phenotype, α-tubulin-expressing cells were located adjacent to each other in
the sensorial layer while, in the more severe cases, the \( \alpha \)-tubulin-expressing cells were not only adjacent, but formed several layers in thickness (Fig. 8C). We then examined how the mild and severe increases in \( \alpha \)-tubulin-expressing cells affected the final spacing pattern of the ciliated cells later in development, by processing the remaining embryos around stage 25. These embryos were fixed, stained for the tracer distribution with Magenta-gal (Fig. 8, magenta staining) and double-labeled with the \( \alpha \)-tubulin probe by in situ hybridization (Fig. 8, light-blue staining) and with the 6-11B1 antibody to detect the differentiated ciliated cells (Fig. 8, brown staining). In embryos with the mild phenotype (Fig. 8D-H), all the \( \alpha \)-tubulin-expressing cells appeared to have differentiated in mature ciliated cells by stage 25, as marked by 6-11B1 staining (Fig. 8F,G). These differentiated ciliated cells were still organized into a spacing pattern in the outer layer, but their density was approximately doubled, compared to the uninjected side (Fig. 8F-H). This result indicates that increasing the number of ciliated-cell precursors increases the density of differentiated ciliated cells. In embryos with a severe phenotype, the number of \( \alpha \)-tubulin-expressing cells increased to the point where the \( \alpha \)-tubulin staining in whole mount appeared relatively uniform in the injected area compared to uninjected area or to embryos with milder phenotypes (compare Fig. 8I and D). The spacing of these cells in the outer layer was difficult to assess in whole mount because the brown color corresponding to 6-11B1 Ab staining was obscured due to the high overexpression of \( \alpha \)-tubulin (Fig. 8I). Nonetheless, some dark-blue cells (blue and brown stainings combined) emerged from the homogeneous light-blue staining in a regular pattern (Fig. 8I arrow and Fig. 8L), suggesting that these cells were still in a spacing pattern, despite the overproduction of ciliated-cells precursors. This interpretation was confirmed when these regions were examined in tissue section. In these sections, the inner layer of the ectoderm, which is normally devoid of \( \alpha \)-tubulin-expressing cells at this stage, was filled with cells that not only stained with \( \alpha \)-tubulin, but also mostly stained for the 6-11B1 antibody (Fig. 8K,N arrowheads). This overabundance of \( \alpha \)-tubulin-expressing cells accounted for the relatively uniform \( \alpha \)-tubulin staining observed when these severely affected embryos were examined in whole mount (compare Fig. 8D,I and F,L). These sections also confirmed that, in the surface layer of the skin, differentiated ciliated cells were surrounded by non-ciliated cells (Fig. 8N asterisks) and organized in a spacing pattern. Thus, this result indicates that when lateral inhibition is blocked and ciliated-cell precursors are produced in excess, they still form a normal spacing pattern in the outer layer, while excess precursors are left in the inner layer where they inappropriately differentiate. This inappropriate differentiation apparently explains the defects in the morphology of the epidermis of severely affected embryos (Fig. 8I,K). Finally, we note that, in these embryos, some of the excess “inner ciliated cells” appeared to be undergoing intercalation at stage 25 (Fig. 8N arrows), suggesting that intercalation can occur over a protracted period of time, as the embryo grows and the epidermis expands.

**DISCUSSION**

Here we describe two successive steps that govern the spacing pattern of ciliated cells in the skin of *Xenopus* embryos (Fig. 9). The first step occurs in the inner layer of the non-neural ectoderm where a subset of cells are chosen to differentiate into ciliated-cell precursors. This choice is under control of lateral inhibition mediated by Notch signaling, in which X-Delta-1 is the putative ligand driving the selection process and a new ESR gene is an epidermal target of Notch signaling. The second step involves intercalation of the ciliated precursors into the outer ectodermal layer when they move outwards to reach their definitive position at the surface of the embryonic skin. The intercalation imposes a restriction on every differentiated ciliated cell to be surrounded by non-ciliated cells, thus generating the final spacing pattern.

**A mechanism of lateral inhibition in the non-neural ectoderm of *Xenopus* embryos**

\( \alpha \)-tubulin is an early marker of the ciliated cells that has allowed us to follow these cells along their differentiation path. We have shown unambiguously that ciliated-cell precursors arise from a subset of cells in the inner layer of the non-neural ectoderm as suggested earlier from transplantation studies (Drysdale and Elinson, 1992). Several lines of evidence presented here indicate that the ciliated-cell precursors are selected by a lateral inhibition mechanism mediated by Notch. First, expression of X-Delta-1 is transiently detected in a scattered pattern in the sensorial layer of the non-neural ectoderm with a peak of expression occurring shortly before the onset of \( \alpha \)-tubulin expression (stage 11). This relationship reflects that seen between Delta and neuronal markers in developing neurons in the spinal cord or in the otic epithelium (Adam et al., 1998; Chitnis et al., 1995; Ericson et al., 1992; Haddon et al., 1998; Henrique et al., 1995; Membreg and Hall, 1995; Tsuchida et al., 1994). Thus it is likely that the cells expressing the highest level of X-Delta-1 at the time of the specification are inner layer cells that will adopt the ciliated-cell fate while repressing their neighbours’ differentiation. This interpretation is supported by the results of functional experiments in which an activated form of X-Notch-1 (Notch-ICD) suppresses the formation of ciliated cells while blocking Notch signaling using the dominant-negative form of X-Su(H), X-Su(H)-DBM, increases dramatically the number of \( \alpha \)-tubulin-expressing cells.

In addition, the activity of the Notch pathway during this process appears to be under control of a negative feedback loop. Expressing the activated form of X-Notch-1 or overexpressing a downstream target like ESR-6e led to X-Delta-1 downregulation. Conversely, blocking Notch signaling or its downstream target with the dominant negative forms of X-Su(H) or ESR-6e, respectively, caused X-Delta-1 upregulation. Thus, these observations fit the proposed model of lateral inhibition in which differentiating cells not only inhibit other cells from differentiating, but the selection of the cells that will differentiate depends on a negative feedback loop that generates winners and losers during lateral inhibition (Ghysen et al., 1993; Heitzler et al., 1996).

Unexpectedly, X-Delta-1 overexpression led to an increase in \( \alpha \)-tubulin-expressing cells in nearly half of the injected embryos, which reproduces, with a lower penetrance, the dominant negative phenotype as obtained by injection of X-Delta-1\(^{sta} \) RNA. Overexpressing X-Delta-1 in the non-neural
ectoderm does not activate Notch signaling as it does in the neural plate where it suppresses neuronal differentiation (Chitnis et al., 1995) but rather prevents it. Consistently, in Drosophila, much evidence has accumulated recently to support the idea that high levels of Notch ligands within a cell may make that cell unresponsive to Notch signaling (Jacobsen et al., 1998, and references therein). Another possibility would be that overactivating Notch through its ligand before the time of the actual cell fate decision would produce a high level of signaling, eventually leading to a desensitization in the responding cells at a time when the Notch pathway is normally required to control differentiation.

Outer ectodermal cells appear not to give rise to α-tubulin-expressing cells even when Notch signaling is blocked (data not shown) and the reason why this layer does not produce ciliated-cell precursors will be of interest in the future. In contrast, all cells in the inner layer of the non-neural ectoderm appear to be capable of differentiating as α-tubulin-expressing cells (Fig. 8C), suggesting that they all express a factor that drives ciliated-cell differentiation. The distribution of this unknown factor would be initially relatively uniform within the inner cell layer of the ectoderm and the outcome of Notch signaling would be to silence this instructive factor in the subset of cells that are not allowed to differentiate. In an example that is comparable to the ciliated cells selection, the different cell types of the Drosophila endoderm segregate from

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**Fig. 8.** Spacing of ciliated cells is imposed by intercalation. Notch signaling was blocked in the ectoderm of Xenopus embryos by injecting 2 ng of X-Su(H)-DBM RNA into two blastomeres of 4-cell-stage embryos, along with nLacZ RNA as a tracer. (A-C) At stage 17, a sample of embryos were fixed and the distribution and density of the α-tubulin-expressing cells was assessed by in situ hybridization with α-tubulin probe (dark blue) after detection of the tracer by X-gal staining (light blue). (C) A section through the embryo shown in A,B illustrates that blocking Notch signaling can result in several layers of α-tubulin-expressing cells. (B) The white dashed line indicates an area of the uninjected side of the embryo that has been populated by cells descending from the injected blastomeres as a result of cell mixing. (D-N) The remaining embryos were fixed at stage 25. In these embryos, the distribution of injected RNA was measured by staining with Magenta-gal (magenta), the density of α-tubulin-expressing cells was measured by in situ hybridization using just BCIP for detection (light blue), and the status of differentiated ciliated cells was measured by staining with the 6-11B1 antibody using HRP immunohistochemistry (brown). Note that BCIP detection gives rise to a higher level of background (diffuse sky blue staining) compared to NBT-BCIP detection. (D-H) Example of an embryo with a relatively mild increase in the number of α-tubulin-expressing cells. Lateral views are shown with anterior to the left. (F,G) Magnified views of the area framed in D and E, respectively, showing that all the α-tubulin-expressing cells have differentiated since they are also stained with 6-11B1. The density of the differentiated ciliated cells approximately doubled on the injected side without affecting the morphology of the skin. (H) Transverse section through the embryo shown in D and E showing that the extra α-tubulin-expressing cells have reached the surface, resulting in a spacing pattern twice as dense on the injected side: seventeen ciliated cells can be counted on the injected side, versus nine on the uninjected side. nc, notochord; sm, somitic mesoderm; lm, lateral mesoderm; epi, epiderm. (I-N) An embryo with a severe overproduction of α-tubulin-expressing cells was assessed by in situ hybridization with α-tubulin probe (dark blue) after detection of the tracer by X-gal staining (light blue). (C) A section through the embryo shown in A,B illustrates that blocking Notch signaling can result in several layers of α-tubulin-expressing cells. (B) The white dashed line indicates an area of the uninjected side of the embryo that has been populated by cells descending from the injected blastomeres as a result of cell mixing. (L,M) Magnified views of the area framed in I and J. Note the continuous pattern of α-tubulin staining on the injected side (compare I to J and L to M). The arrow points to a region where 6-11B1 Ab staining can be distinguished despite the high level of blue staining due to α-tubulin overexpression. (K,N) Sections through the embryo shown in I and J allow the detection of the 6-11B1 staining much better than the whole-mount view. (K) The injected area, marked by purple-stained nuclei, lies between the two arrowheads. Note that overproduction of α-tubulin-expressing cells in this area leads to a thickening and altered morphology of the epidermis. (N) A high-power view of an adjacent section shows that most of the α-tubulin-expressing cells have differentiated since they are also stained by 6-11B1 antibody whichever position they have in the ectoderm. The differentiated ciliated cells (blue and brown) that have reached the surface of the skin are surrounded by non-ciliated cells (asterisks). A fraction of the double-labeled cells were retained in the inner layer of the epidermis (arrowheads). Note that some of them are likely to be migrating to the surface (arrows) indicating that intercalation is an ongoing process.
a equipotential field which is the whole endodermal germ layer (Tepass and Hartenstein, 1995). The bHLH proneural genes were shown to be expressed homogeneously throughout the endoderm, to be required for determination of cell fates and to interact with the neurogenic genes in the same way as they do in the neural ectoderm. Thus, it seems likely that cells forming the ciliated-cell precursors are selected by a feedback loop that would operate through an upstream factor, a proneural-like factor expressed by the whole inner layer, driving X-Delta-1 expression and instructing the ciliated-cell fate. Further understanding of how ciliated-cell precursors are selected depends largely on identifying this upstream factor.

**ESR-6e is a Notch target gene in the non-neural ectoderm**

We have identified a novel *Xenopus* WPRW-bHLH gene, called *ESR-6e*, which encodes a protein with all the conserved features shared by the Hairy-E(spl)-related proteins including a basic Helix-Loop-Helix region that has 70% homology with ESR-1 bHLH region, a conserved proline residue in the basic domain and the C-terminal WRPW tetrapeptide, which binds the transcriptional repressor Groucho (Fisher and Caudy, 1998; Fisher et al., 1996). *ESR-6e* is primarily expressed within the non-neural ectoderm where it appears to have two aspects to its expression pattern. *ESR-6e* is detected at relatively high and uniform levels in the outer layer of the non-neural ectoderm after gastrulation is complete. In addition, *ESR-6e* is also detected at low levels in the inner layer of ectoderm during gastrulation when ciliated-cells precursors form and when X-Delta-1 is expressed. This latter aspect of *ESR-6e* expression is consistent with a role as a Notch target gene during the selection of ciliated-cell precursors. Indeed expression of *ESR-6e* in both layers can be upregulated by an activated form of Notch, indicating that Notch signaling is one input promoting the expression of *ESR-6e*. Notably, *ESR-6e* expression cannot be induced in the neural plate by Notch-ICD, suggesting that Notch signaling is not sufficient for *ESR-6e* expression in all regions of the ectoderm. These results parallel those obtained for other WRPW-bHLH genes that are targets of Notch signaling in *Xenopus* in that, in most cases, these genes are only expressed in a particular tissue when Notch signaling is active. For example, *ESR-1* and *ESR-7* are activated by Notch signaling in the neural plate during primary neurogenesis, while *ESR-4* and *ESR-5* are activated by Notch signaling in the paraxial mesoderm during segmentation (Jen et al., 1999).

Because these different Notch target genes have tissue specificity, other factors likely modulate their ability to respond to Notch signaling. In the case of *ESR-6e*, it will be interesting to know why Notch-ICD preferentially induces its expression in the non-neural ectoderm and how this preference relates to the role of the BMP pathway in directing ectoderm into a non-neural fate.

The expression of *ESR-6e* in the inner cell layer occurs at a time when X-Delta-1 is expressed, prefiguring the appearance of ciliated-cell precursors. In addition, expression of *ESR-6e* can be induced in the inner cell layer by Notch-ICD, suggesting that it is a Notch target gene. However, X-Su(H)-DBM, an inhibitor of Notch signaling, activates the expression of *ESR-6e* in the inner layer. On one hand, this result was unanticipated for a target of a Su(H)-mediated Notch signaling, since X-Su(H)-DBM blocks the expression of other *Xenopus* ESR genes such as *ESR-1*, *ESR-4*, *ESR-5* and *ESR-7* (Jen et al., 1999; Wettstein et al., 1997) and not shown). On the other hand, this result is fully consistent with observations made on the regulation of the E(spl)-C genes in *Drosophila*, which were shown to be direct targets of both the Notch-activated Su(H) and the proneural bHLH proteins Achaete and Scute (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Singston et al., 1994). Like *ESR-6e*, the expression of these genes increases when Notch signaling is blocked, presumably because loss of Notch function leads to higher levels of ac-sc expression, which in turn drive higher expression levels of the E(spl) genes via E-boxes present in their promoters. From these observations and the extensive analysis of the E(spl)-C gene promoters, Posakony and colleagues have proposed that dual transcriptional activation by Su(H) and bHLH proteins is the rule for eight out of nine genes in the E(spl) complex (Nellesen et al., 1999) and that this dual regulation is conserved amongst divergent *Drosophila* species. By analogy, we propose that the

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**Fig. 9.** Schematic representation of the two-step mechanism spacing the ciliated cells. (1) Lateral inhibition takes place in the inner ectodermal layer at late gastrula stages and drives a subset of cells to express higher levels of X-Delta-1 (green cells). These cells inhibit the differentiation of their neighbours (orange cells) and take on the ciliated-cell fate as marked by expression of α-tubulin (blue cells). (2) At neurula stages, the inner layer cells organize into a monolayer and the α-tubulin-expressing cells intercalate into epithelial junctions of the outer layer (columnar red cells). Differentiation of the ciliated cells occurs at early tadpole stages while they reach their definitive position in the epithelium. At the same time, division of the surface layer cells produces new interstitial locations available for intercalation of additional ciliated cells.
expression of ESR-6e in the inner cell layer is also under dual control by Su(H) and another upstream factor driving the ciliated-cell differentiation, and that this explains why its expression increases in response to X-Su(H)-DBM. We note that, in addition to this hypothetical dual control in the inner layer, there is likely to be a third level of control that accounts for the high and relatively uniform expression of ESR-6e in the surface layer. The regulation and role of ESR-6e expression in the outer ectodermal layer present an interesting aspect of the development of the non-neural ectoderm that we did not address here. It would be of interest to determine if the high level of ESR-6e expressed in the outer layer plays a role in the establishment of any of its specific characteristics relative to the inner layer. In first analysis, ESR-6e does not seem to be required to keep the outer layer refractory to α-tubulin-positive cells differentiation since impairing ESR-6e function by injection of ESR-6eΔb RNA did not allow outer ectodermal cells to give rise to ciliated-cell precursors (not shown).

ESR-6e acts downstream of Notch to regulate ciliated-cell differentiation

To analyze the role of ESR-6e in the formation of ciliated-cell precursors, we first overexpressed ESR-6e and found that it suppressed the formation of α-tubulin-expressing cells, indicating that ESR-6e negatively regulates ciliated-cell differentiation. As a control for the specificity of this effect, we used ESR-7, which is related to ESR-6e but normally expressed in the neural plate. Overexpression of ESR-7 in the non-neural ectoderm suppressed the formation of α-tubulin-expressing cells as efficiently as ESR-6e. It is perhaps not surprising that ESR-7 can substitute for ESR-6e when misexpressed in the non-neural ectoderm if all the Hairy-E(spl)-related factors bind to similar N-boxes (Ohsako et al., 1994; Van Doren et al., 1994), and the ESR-7 basic domain differs from ESR-6e basic domain only by the substitution of Ile7 by Val. Based on this gain-of-function phenotype alone, it is difficult to know whether ESR-6e normally acts to regulate ciliated-cell differentiation.

To test more directly the role of ESR-6e, we generated a basic domain mutant, ESR-6eΔb, by changing basic residues that had been shown in MyoD to be crucial for DNA binding (Davis et al., 1990). Since bHLH proteins bind DNA as dimers only, DNA-binding mutants act as dominant negative mutants by forming nonfunctional dimers with wild-type proteins (Shirakata et al., 1993). The specificity of these mutants hinges on whether or not they preferentially form homodimers and whether they heterodimerize with other members of the family or with a common heterodimerization partner. Because the WRPW-bHLH proteins are not known for certainty to have a preference for homodimers versus heterodimers, we generated a similar DNA-binding mutant for ESR-7 as a specificity control. Expression of RNA encoding ESR-7Δb in animal caps did not alter the formation of ciliated-cell precursors as marked by α-tubulin expression. By contrast, RNA encoding ESR-6eΔb was very potent at promoting α-tubulin expression. These results indicate that the WRPW-bHLH proteins have a preference for homodimerization over heterodimerization.

Since the “lack-of-function” phenotypes that we consider are based on overexpression of dominant negative mutants, we cannot rule out that ESR-6e would act in parallel and not downstream of X-Notch-1. However, based on the premises that ESR-6e and ESR-6eΔb overexpressions result in phenotypes associated respectively with activation or inhibition of Notch signaling, that Notch-ICD activates ESR-6e expression and that altering ESR-6e activity feeds back on X-Delta-1 expression, we propose that ESR-6e is a regulator of ciliated-cell differentiation acting downstream of Notch.

Spatial rearrangement is required to set up the final spacing pattern of ciliated cells

When Notch signaling is blocked in the ectoderm, α-tubulin-expressing cells form in greater numbers and at higher density. The fate of these excess ciliated-cell precursors is informative in terms of how the spacing pattern of differentiated ciliated cells arises in the outer layer. Indeed, producing an excess of ciliated precursors (as marked by α-tubulin staining) in the inner cell layer results in an increase in the number of differentiated ciliated cells (as marked by 6-11B1 Ab staining) in the outer layer. However, even though supernumerary precursors were adjacent to each other, clusters of differentiated ciliated cells were not observed, arguing that distribution and intercalation must occur simultaneously and that the mature ciliated cells are not distributed based on the scattered pattern of the precursors generated via lateral inhibition in the inner layer. In addition, precursors of the ciliated cells appear to be normally limiting, since the number of ciliated cells in the outer layer can increase by increasing the number of precursors. However, the upper limit in the density of ciliated cells in the outer layer suggests that the spacing is due to a mechanism that restricts the ability of precursors to laminate into the outer layer. In addition, the fact that excess precursors still undergo intercalation at stage 25 (Fig. 8N) indicates that intercalation would be an ongoing process taking place as long as precursors are present in the inner layer and as long as interstitial positions in the epithelium exist that are not already occupied by a ciliated cell. This latter restriction imposes the preservation of a spacing pattern of mature ciliated cells in the epithelium even in cases of severe overproduction of precursors. As the embryo grows and outer epithelial cells divide, more epithelial junctions are generated and made available for intercalation of additional ciliated cells (Fig. 9). Thus, the preservation of a spacing pattern is a consequence of the restriction imposed on every mature ciliated cell to be surrounded by non-ciliated cells.

Several mechanisms could explain how the intercalation of ciliated precursors is regulated to produce a spacing pattern. Ciliated cells in the outer layer could secrete a repulsive signal that prevent intercalation of equivalent cells within a certain perimeter. Alternatively, Drysdale and Elinson (1992) proposed a model based on differential cell adhesion where intercalating cells have to increase their adhesion to outer layer cells and release their adhesion to inner cells. One can extend this idea by proposing that inner layer cells can only thread their way into the outer layer at points where no ciliated cells are present, the limitation due to adhesion differences. Imposing a restriction on where cells can intercalate would effectively direct precursors, which are relatively mobile laterally in the inner layer, into position where no other ciliated cells exists, thus spacing these cells out in the appropriate pattern. This model suggests that the analysis and identification
of molecules, adhesion or repulsive factors, involved in the intercalation/distribution mechanism will be important in determining how a spacing pattern arises in the outer layer.

Our results indicate that the number of ciliated-cell precursors that form in the inner cell layer is determined by lateral inhibition. Moreover, among the first properties acquired by a ciliated-cell precursor is the ability to leave one cell layer where cells are loosely attached to intercalate into an occluding epithelium. Lateral inhibition via Notch signaling has also been shown to regulate the differentiation of cells in a neurogenic epithelium into neuroblasts or neurons that differentiate by delaminating from this epithelium. Thus, in this case, Notch signaling keeps cells in an epithelium while in the case of the ciliated-cell precursor, Notch signaling keeps cells from moving into an epithelium. In both cases, Notch controls differentiation but the link between cell differentiation and cell rearrangement remains poorly understood. The results presented here show that the spacing pattern of the ciliated cells in *Xenopus* embryonic skin provides a new and accessible model system for studying Notch-mediated lateral inhibition as well as how differentiation is linked to cell rearrangements during tissue formation.

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