

Delta-like 1 and lateral inhibition during hair cell formation in the chicken inner ear: evidence against cis-inhibition

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SUMMARY

The formation of the salt-and-pepper mosaic of hair cells and supporting cells in the sensory epithelia of the inner ear is regulated by Notch signalling and lateral inhibition, but the dynamics of this process and precise mode of action of delta-like 1 (Dll1) in this context are unclear. Here, we transfected the chicken inner ear with a fluorescent reporter that includes elements of the mammalian *Hes5* promoter to monitor Notch activity in the developing sensory patches. The *Hes5* reporter was active in proliferating cells and supporting cells, and Dll1 expression was highest in prospective hair cells with low levels of Notch activity, which occasionally contacted more differentiated hair cells. To investigate Dll1 functions we used constructs in which Dll1 expression was either constitutive, regulated by the *Hes5* promoter, or induced by doxycycline. In support of the standard lateral inhibition model, both continuous and *Hes5*-regulated expression of Dll1 promoted hair cell differentiation cell-autonomously (in cis) and inhibited hair cell formation in trans. However, some hair cells formed despite contacting Dll1-overexpressing cells, suggesting that some progenitor cells are insensitive to lateral inhibition. This is not due to the cis-inhibition of Notch activity by Dll1 itself, as induction of Dll1 did not cell-autonomously reduce the activity of the *Hes5* reporter in progenitor and supporting cells. Altogether, our results show that Dll1 functions primarily in trans to regulate hair cell production but also that additional mechanisms operate downstream of lateral inhibition to eliminate patterning errors in the sensory epithelia of the inner ear.

KEY WORDS: Lateral inhibition, Inner ear, Delta-like 1, Notch, Pattern formation, Cis-inhibition

INTRODUCTION

Lateral inhibition is a key mechanism for cell diversification and patterning in a wide variety of embryonic and adult tissues. In this process, cells adopting a given developmental fate inhibit their neighbours from doing likewise through direct cell-to-cell communication mediated by the Notch signalling pathway (reviewed by Chitnis, 1995; Lewis, 1998; Artavanis-Tsakonas et al., 1999). The signal-sending cells that adopt the primary fate express ligands of the Delta/Serrate (also named Jagged in mammals)/Lag2 (DSL) family that bind to and activate Notch receptors in the neighbouring signal-receiving cells. This triggers a series of γ -secretase-dependent cleavages that release the intracellular domain of Notch (NICD). The NICD then translocates to the nucleus and induces expression of transcriptional repressors of the Hairy and Enhancer of Split (HES) family, which inhibit adoption of the primary fate (reviewed by Bray, 2006). Hence the signal-sending and signal-receiving cells end up with low and high levels of Notch activity, respectively, and adopt opposite fates.

In the standard model of lateral inhibition with feedback, the expression of DSL ligands is repressed by Notch activity (Heitzler and Simpson, 1991; Heitzler et al., 1996); consequently, a signal-receiving cell will lose over time its ability to act as a signal-sending cell. By amplifying small and random variations in the expression of DSL ligands, this intercellular negative-feedback loop is thought to be sufficient to drive initially equivalent cells into either high or low Notch states (Collier et al., 1996). However, in many cases lateral inhibition is biased by intrinsic factors that

impact on the ability of cells to send or receive Notch signals (reviewed by Schweisguth, 2004). There is also growing evidence that, in addition to their effects in trans, DSL ligands can inhibit Notch activity cell-autonomously (in cis) (de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997; Jacobsen et al., 1998) (reviewed by del Álamo et al., 2011). The great variety of cellular contexts in which lateral inhibition operates could be matched by equal diversity in its molecular mechanisms.

One striking outcome of lateral inhibition is found in epithelial sheets, in which interacting cells differentiate into a salt-and-pepper mosaic of alternate cell types. Examples of such epithelia are found in the lung endoderm (Morimoto et al., 2010), the *Xenopus* epidermis (Deblandre et al., 1999), the zebrafish pronephros (Liu et al., 2007) and in the inner ear. The inner ear comprises several pseudostratified sensory epithelia that contain thousands of mechanosensory hair cells that are activated by the displacement of their apical bundle of stereocilia. Hair cells are located in the apical layer and are interspersed by the apical processes of several non-sensory supporting cells (Fig. 1A,B) with cell bodies that rest on the basal lamina. The formation of this cellular mosaic from bipotent progenitor cells is regulated by lateral inhibition. Newly formed hair cells express delta1-like 1 (Dll1) and jagged 2 (Jag2) ligands that activate Notch1 receptors in neighbouring progenitor cells; in the latter, high levels of Notch activity induce *Hes1/5* expression, which in turn suppress expression of the pro-hair cell transcription factor *Atoh1* (Fig. 1C) (Bermingham et al., 1999). When Notch signalling is disrupted at the time of hair cell formation, overproduction of hair cells occurs (Haddon et al., 1998; Lanford et al., 1999; Zine et al., 2000; Zine et al., 2001; Kiernan et al., 2005; Brooker et al., 2006). Modelling studies suggest that the emergent properties of lateral inhibition with feedback are sufficient to pattern inner ear epithelia (Fig. 1D) (Collier et al., 1996; Webb and Owen, 2004). However, nothing is known about the actual dynamics of this process in vivo, and the way that Dll1

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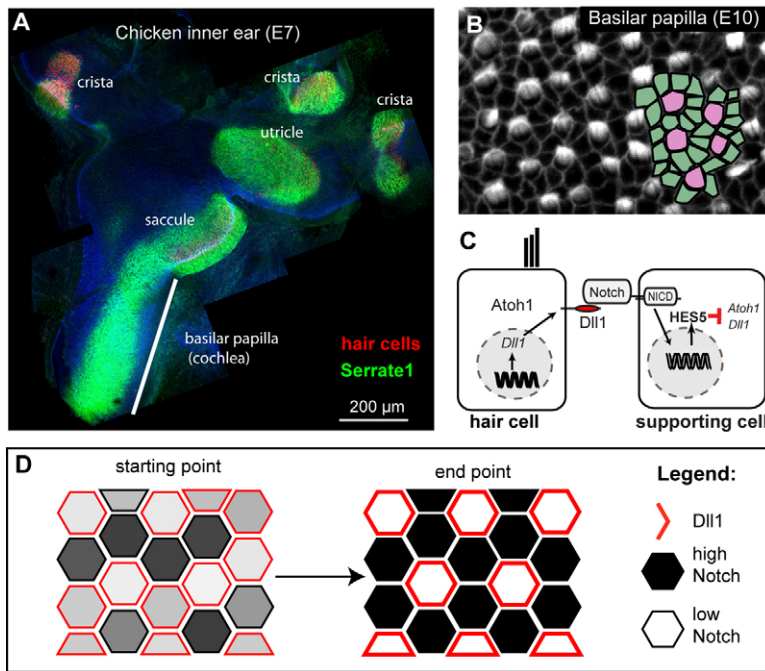


Fig. 1. Lateral inhibition in the chicken inner ear.

(A) Whole-mount preparation of an E7 inner ear immunostained for Serrate1 (green) and the hair cell markers HCA and HCS1 (red). The saccule, the utricle and three cristae form the vestibular system; the sensory epithelium of the cochlea is the basilar papilla. (B) Surface view of an E10 basilar papilla stained with fluorescent phalloidin. Hair cells (magenta overlay) have an actin-rich apical bundle of stereocilia and each hair cell is surrounded by the apical surfaces of supporting cells (green overlay). (C) A simplified model of lateral inhibition in the inner ear. The signal-sending cell expresses Atoh1 and the Notch ligand Dll1 and differentiates into hair cell. In the signal-receiving cell, Notch activity results in NICD translocation to the nucleus, which induces the expression of Hes family genes. This antagonises Atoh1 and Dll1 in the signal-receiving cell and promotes supporting cell differentiation. (D) Patterning of epithelial sheets by lateral inhibition with feedback. In starting conditions, equivalent cells exhibit random differences in their endogenous levels of Notch activity and Delta expression (red outline). The intercellular negative-feedback loop regulating Delta expression generates over time a salt-and-pepper mosaic of cells with either high or low levels of Notch activity and Delta.

functions to regulate hair cell fate decisions remains unclear. In fact, it has been reported that artificial overexpression of Dll1 does not affect hair cell formation (Eddison et al., 2000) and that transient contacts between immature hair cells can occur in the embryonic chick inner ear (Goodyear and Richardson, 1997) (supplementary material Fig. S1). These observations suggest that patterning of inner ear epithelia by lateral inhibition is more complicated than the standard model proposes.

Here, we used a Hes5 reporter to monitor Notch activity in the developing chicken inner ear. We then used different gain-of-function strategies to investigate Dll1 function during hair cell formation and its impact on Notch activity. In agreement with the standard model, our results show that Dll1 promotes hair cell fate cell-autonomously and represses hair cell formation in trans. However, some progenitor cells are not prevented from becoming hair cells by neighbouring Dll1-expressing cells. This is unlikely to result from cis-inhibition of Notch activity by Dll1 itself, given that overexpression of Dll1 does not prevent activation of the Hes5 reporter cell-autonomously. Altogether, our data show that Dll1 is a key determinant of hair cell fate decisions and that it functions primarily in trans. Our findings also support the idea that additional mechanisms must operate downstream of lateral inhibition to eliminate patterning errors in the sensory epithelia of the inner ear.

MATERIALS AND METHODS

Animals

Fertilised White Leghorn chicken (*Gallus gallus*) eggs were obtained from Henry Stewart UK and incubated at 37.8°C for the designated times. Embryonic stages are either from Hamburger-Hamilton (HH) tables (Hamburger and Hamilton, 1992) or refer to embryonic days (E), with E1 corresponding to 24 hours of incubation. Embryos older than E5 were sacrificed by decapitation. All procedures were approved by University College London and by the UK Home Office.

Plasmids

The following plasmids were used: pHes5::d2EGFP (Takebayashi et al., 1995); RCAN(B) (Petropoulos and Hughes, 1991); RCAS-cDelta1 (Henrique et al., 1997); pCAS-Delta1-IRES-EGFP (Fior and Henrique,

2005); pDSRED2-C1 (thereafter named pDsRed; Clontech); TOPO-nTomato (Collins et al., 2010); pCAGGS-T2TP encoding the Tol2 transposase; pT2K-CAGGS; pT2K-CAGGS-rtTA²M2, a Tol2 construct encoding the tet-on activator; pT2K-TRE-B1-eGFP (herein named pTRE-EGFP), which consists of a cassette of bidirectional transcriptional units (one controlling transcription of *EGFP*, the other empty) under the control of a tetracycline-responsive element (TRE) between the left and right ends of Tol2 (Sato et al., 2007; Watanabe et al., 2007); pTurbo-FP635 (Evrogen).

The following plasmids were generated using standard cloning procedures: pHA-NICD-IRES-mRFP1, driving constitutive expression of an HA-tagged version of the intracellular domain of chicken Notch1 and mRFP1; pT2K, a promoterless version of pT2K-CAGGS; pT2K-Hes5::d2EGFP and RCAN(B)-Hes5::d2EGFP; pT2K-Hes5::nd2EGFP; pT2K-CAGGS-Delta1-IRES-EGFP (thereafter named pT2K-Delta1-GFP); pT2K-Hes5::Delta1-IRES-EGFP (thereafter named pT2K-Hes5::Delta1-GFP); pT2K-CAGGS-nTomato (pT2K-nTomato); pT2K-TRE-B1-FP635 (herein named pTRE-FP635), which is a modified version of pTRE-EGFP in which Turbo-FP635 has been cloned in place of EGFP; pT2K-TRE-B1-FP635-Delta1 (pTRE-FP635-Delta1), which contains the coding sequence of chicken Dll1 in the second transcriptional unit of pTRE-FP635.

In ovo electroporation

Microelectroporation of the otic cup of E2 chick embryos was performed using a BTX ECM 830 Electro Square Porator as previously described (Daudet and Lewis, 2005). Doxycycline (Dox) treatments were performed in ovo by injecting around the embryos ~500 µl 20 µg/ml Dox in PBS.

Organotypic cultures and time-lapse confocal microscopy

The sensory epithelium was dissected at room temperature in DMEM/F12 medium and immobilised at the bottom of Mattek dishes using either a platinum harp or 1% low-gelling-temperature agarose. Approximately 2–3 ml of DMEM/F12 medium without Phenol Red (Invitrogen) was added and the specimens were maintained for a minimum of 3 hours in a tissue culture incubator (5% CO₂, 37°C) before live imaging. The γ -secretase inhibitor DAPT {N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester; Calbiochem} was used at 20 µM. Time-lapse confocal microscopy was performed using a Nipkow spinning-disc confocal microscope and a Zeiss LSM510 inverted confocal microscope equipped with an environmental chamber. Frame intervals for time-lapse experiments ranged from 10 to 20 minutes. Confocal stacks were analysed using ImageJ or iQ (Andor). For quantification of fluorescence levels in the DAPT experiments, average

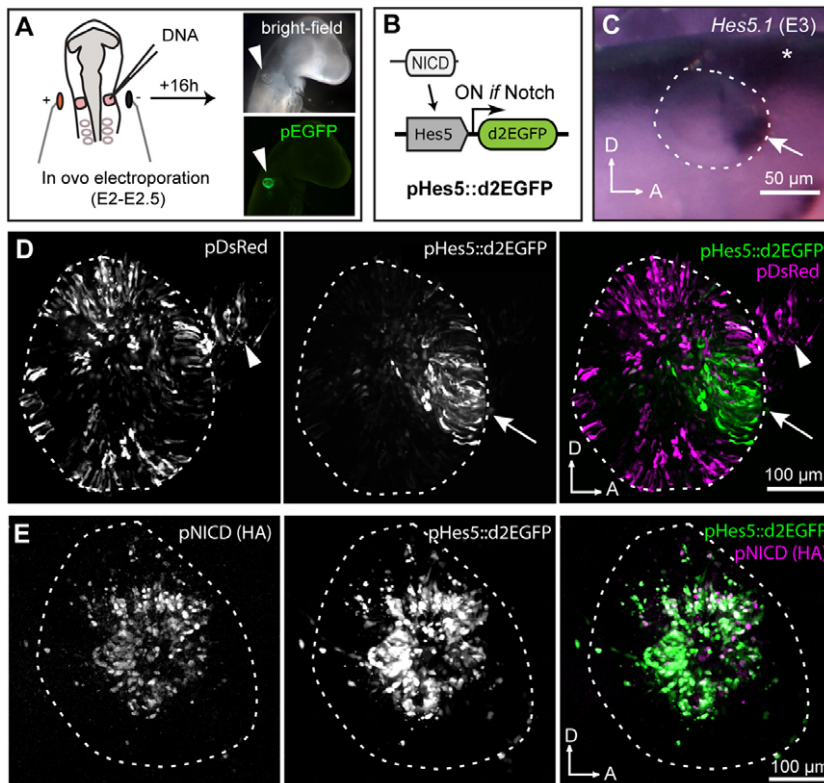


Fig. 2. Initial characterisation of the pHes5::d2EGFP reporter. (A) The experimental setup for in ovo electroporation of the chicken inner ear. Plasmid DNA is injected onto the surface of the otic placode/cup and electrical pulses are applied across the embryo. Strong fluorescence (arrowhead) is seen throughout the otocyst 16 hours after electroporation with pEGFP. (B) The Hes5 reporter drives d2EGFP expression in Notch-active cells. (C) Strong expression of *Hes5.1* is seen in the neural tube (asterisk) and within the anterior neurogenic patch of the otocyst (dashed outline). (D) E3 otocyst analysed 16 hours after co-electroporation with pDsRed and pHes5::d2EGFP plasmids. pDsRed fluorescence is mosaic but found throughout the otocyst, whereas high EGFP levels are restricted to the anterior neurogenic domain (arrows). Cells delaminating from the anterior region of the otocyst express DsRed but not d2EGFP (arrowheads). (E) Co-electroporation with the pHA-NICD-IRES-mRFP1 plasmid strongly elevates Hes5::d2EGFP levels throughout the otocyst. Transfected cells identified by positive HA immunostaining exhibit high levels of Hes5::d2EGFP fluorescence. A, anterior; D, dorsal.

projections of the z-stack (12-bit images) were made at each time point. At least two regions of interest (ROIs) were selected within each sample. Arbitrary fluorescence units were obtained by subtracting background levels of fluorescence from ROI values at each time point.

Immunocytochemistry and in situ hybridisation

Immunocytochemistry and in situ hybridisation were performed as described previously (Daudet and Lewis, 2005). The following antibodies were used: monoclonal mouse IgG1 anti-HCA (hair cell antigen; supernatant used at 1:1000-1:2000) (Bartolami et al., 1991); monoclonal mouse IgG2b anti-HCS1 (recognising otoferlin; 1:250) (Goodyear et al., 2010); rabbit anti-Serrate1 (Adam et al., 1998); and rabbit anti-Delta1 (Henrique et al., 1997). Goat anti-mouse IgG or anti-rabbit IgG secondary antibodies conjugated to Alexa 405, 488, 546, 633 or 647 (Invitrogen) were used at 1:1000. Specimens were examined on a Zeiss LSM510 inverted confocal microscope.

Quantification of Hes5::nd2EGFP fluorescence in Dll1-FP635 and FP635 induced cells

For comparison of Hes5::nd2EGFP levels in cells transfected with either pTRE-FP635 or pTRE-Delta1-FP635, the inner ear of E5.5 embryos treated with Dox for 21 hours was dissected and imaged on a spinning-disc Nipkow confocal microscope equipped with a 40 \times oil-immersion objective. Measurements of mean intensity values for the red (FP635) and green (EGFP) channels (12-bit confocal stacks of 40-50 optical slices) were made on randomly selected Hes5::nd2EGFP-positive nuclei using ImageJ. FP635-positive nuclei were defined by a mean value of FP635 fluorescence that exceeded 400, a threshold value corresponding approximately to the mean $FP635_{background} + (10 \times s.d. \text{ of } FP635_{background})$. The mean EGFP levels of each nucleus were standardised using Z-scoring [$z = (x - \text{mean})/s.d.$] then pooled across each experimental condition (FP635 or Delta1-FP635 transfected). Similar analysis of Hes5::nd2EGFP levels was performed on five samples electroporated with pTRE-Delta1-FP635 and treated at E6 with Dox for 14 hours, then immunostained for Delta1 expression to identify Dll1-induced and non-induced cells. Statistical analyses were computed using SPSS 19 for Mac. The data did not follow a normal distribution, so nonparametric tests were used; all *P*-values are two-tailed.

RESULTS

Initial characterisation of the Hes5::d2EGFP reporter in the embryonic chicken inner ear

To investigate the spatiotemporal pattern of Notch activity during chicken inner ear development, we used a reporter of Notch activity that contains a 0.8 kb fragment of the promoter of the mouse *Hes5* gene regulating the expression of a destabilised form of EGFP, pHes5::d2EGFP (Takebayashi et al., 1995). When analysed 16-20 hours after in ovo electroporation of the otic placode/cup at E2 (Fig. 2A,B), pHes5::d2EGFP elicited a pattern of EGFP fluorescence very similar to the endogenous pattern of Notch activity. At this developmental stage, otic neurogenesis occurs in a restricted, anterior region of the otic cup, in which high levels of Dll1 and Hes5 expression are present (Fig. 2B) (see Adam et al., 1998; Abelló et al., 2007; Daudet et al., 2007). In specimens ($n=20$) transfected with pHes5::d2EGFP, strong fluorescence was restricted to the neurogenic patch despite widespread transfection of the otic cup as shown by pDsRed expression (Fig. 2D). Furthermore, co-electroporation of pHes5::d2EGFP with a plasmid driving constitutive expression of either an active form of the chicken Notch1 receptor (HA-tagged NICD, which activates Notch signalling in cis and independently of ligand binding) or the Notch ligand chicken Dll1 (which activates Notch signalling in trans) induced strong and widespread EGFP fluorescence in the otocyst (Fig. 2E; $n=6$; data not shown). This indicated that the Hes5::d2EGFP reporter is sensitive to canonical Notch activity in the chicken inner ear.

RCAN retrovirus and Tol2 transposon enable stable integration of the Hes5::d2EGFP reporter throughout the developing chicken inner ear

Hair cell formation starts at around E4 in vestibular patches, peaks at E9-E12 in the utricle and carries on at a slower pace until post-hatch stages (Goodyear et al., 1999). In the basilar papilla, hair cell formation occurs between E5 and E10 only (Katayama and

Corwin, 1989; Goodyear and Richardson, 1997). In order to monitor Notch activity with the *Hes5* reporter at these late developmental stages, the *Hes5* reporter was cloned into the RCAN retroviral construct (RCAN-*Hes5*::d2EGFP) and into a

promoterless version of the Tol2 transposon (pT2K-*Hes5*::d2EGFP). Both types of vector enable stable integration of transgenes into the genome of chicken cells (Petropoulos and Hughes, 1991; Sato et al., 2007). Following in ovo electroporation

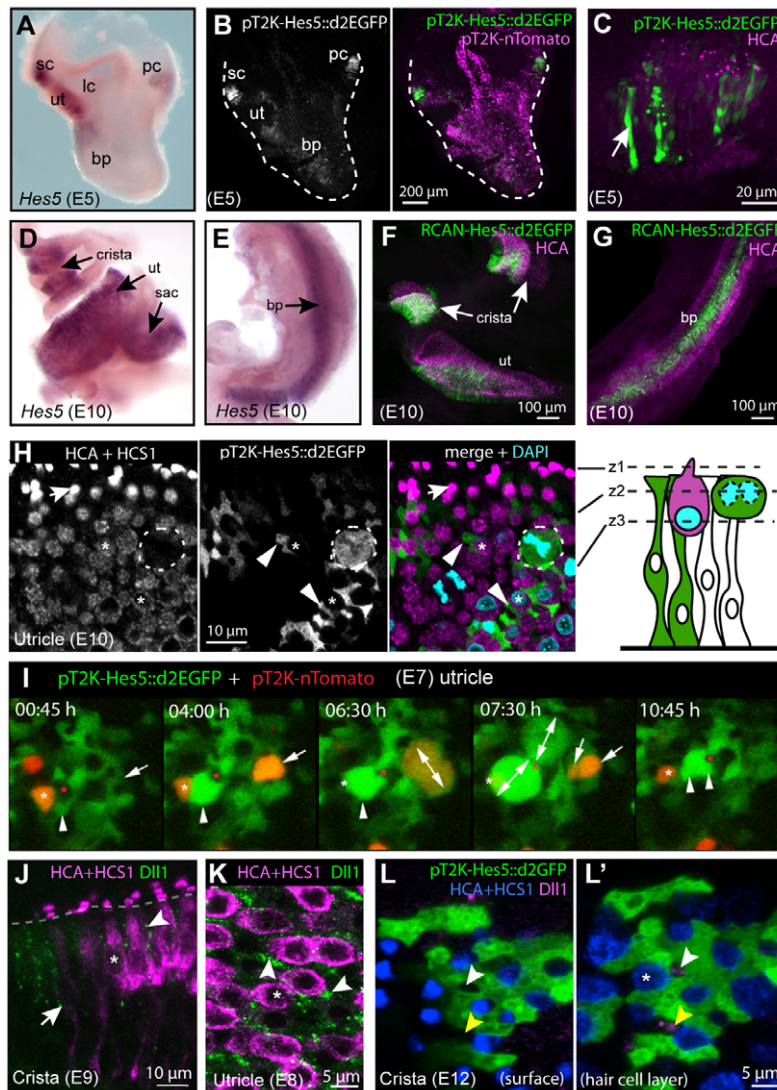


Fig. 3. Activity of the *Hes5*::d2EGFP reporter in the developing inner ear. (A) At E5, *Hes5* gene expression is high in the crista and utricle region and lower in the prospective basilar papilla. (B) E5 inner ear transfected with pT2K-*Hes5*::d2EGFP and pT2K-nTomato. The pattern of *Hes5*::d2EGFP activity resembles that of the *Hes5* gene; in this example, the utricle does not contain transfected cells, as seen by the absence of nTomato expression. (C) Lateral view of a pT2K-*Hes5*::d2EGFP-transfected crista. Very few hair cells have differentiated and express HCA at their apical surface. The d2EGFP-positive cells have elongated cell bodies that span the entire height of the epithelium (arrow). (D, E) *Hes5* gene expression is detected in all sensory epithelia of the E10 inner ear. (F, G) Whole-mount view of an E10 sample transfected with RCAN-*Hes5*::d2EGFP, demonstrating specific activation of the reporter in the sensory epithelia. (H) Single optical slice from a utricle transfected with pT2K-*Hes5*::d2EGFP and immunostained for HCA and HCS1. The plane of section is not exactly parallel to the luminal surface: the upper part of the panel shows the hair cell bundles with HCA staining (arrow) and the bottom part shows deeper planes of the epithelium in which hair cell soma (HCS1-positive) and nuclei (asterisks) are visible. *Hes5*::d2EGFP is found in cells with small polygonal surfaces (arrowheads) in between hair cells. Mitotic cells with rounded EGFP-positive cell bodies (dashed outline) are seen in the superficial plane of the epithelium. The drawing illustrates the approximate z-location of the cellular features visible on the single optical slice. (I) Live imaging (surface view) of an E7 utricle transfected with pT2K-*Hes5*::d2EGFP and pT2K-nTomato (still images from supplementary material Movie 1). Cells with high levels of d2EGFP fluorescence delineate presumptive hair cells (stars), which appear as darker cell bodies of circular form with an apically localised nucleus (with nTomato in cases in which the cell was co-transfected, e.g. white star). The nuclei of mitotic cells always migrate to the surface of the epithelium before cell division (white arrow). Mitosis (double-headed arrows) results in major reorganisations and intercalation of new cells within the epithelial mosaic. In this sequence, note the change in the relative position of the cells labelled with the white and red asterisks after the mitosis of the progenitor cell marker by the arrowhead. (J, K) E9 crista and E8 utricle immunostained for Dll1 expression and HCA/HCS1. Dll1 protein is detected in small intracellular vesicles within cells that appear to intercalate between differentiated hair cells (arrowheads). (L, L') Consecutive optical slices from a confocal z-stack of an E12 crista transfected with pT2K-*Hes5*::d2EGFP and immunostained for Dll1 and HCA/HCS1. Note that the cells that express Dll1 (arrowheads) have low levels of d2EGFP, do not express HCA and HCS1, and can be located next to differentiated hair cells (asterisk). sc, superior crista; lc, lateral crista; pc, posterior crista; ut, utricle; bp, basilar papilla.

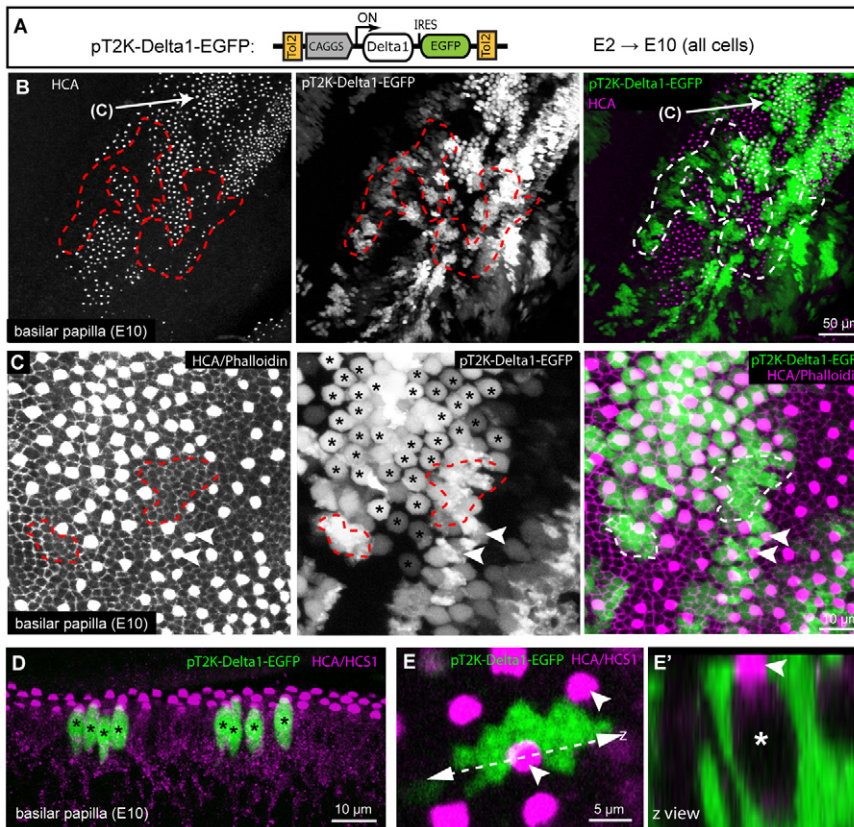


Fig. 4. Effects of constitutive Dll1 on hair cell formation. (A) The pT2K-Delta1-EGFP plasmid. (B) Low-magnification view of a transfected basilar papilla analysed at E10 and immunostained for HCA. Some regions have a reduced density of hair cells (red outlines), whereas hair cell formation appears to occur normally in other highly transfected regions (arrow). (C) High-magnification view of the region indicated by the arrow in B. No hair cells form within relatively large clusters of transfected cells (red outlines). By contrast, many of the EGFP-positive cells are hair cells (asterisks) in other highly transfected regions; their cell bodies are usually surrounded by a darker outline, suggesting that these are surrounded by untransfected supporting cells. Note also the presence of hair cells in direct contact with Dll1-overexpressing cells (arrowheads). (D) Lateral view of a transfected basilar papilla with few transfected cells, which have all differentiated into hair cells (asterisks). (E) Surface view of a group of transfected supporting cells in contact with untransfected hair cells (arrowheads). (E') Transverse z-projection of the region shown in E, demonstrating absence of EGFP in the cytoplasm of the hair cell (asterisk) with a well-differentiated stereociliary bundle (arrowhead).

of either construct at E2, strong EGFP fluorescence was detected in the developing sensory patches at E5 and E10 (Fig. 3). At E5, the levels of Hes5::d2EGFP fluorescence were more intense in the developing crista than in the other sensory patches (Fig. 3B,C); this fits with the endogenous pattern of Hes5 expression (Fig. 3A) and the temporal progression of hair cell production in the embryonic chick inner ear. At E10, many hair cells identifiable by HCA and otoferlin (HCS1) immunostaining had formed within sensory patches, where high levels of *Hes5* gene expression (Fig. 3D,E) and Hes5::d2EGFP fluorescence (Fig. 3F,G) were observed.

In whole-mount preparations examined at high magnification (Fig. 3H), high levels of EGFP were restricted to elongated cells with small polygonal apical surfaces intercalated in between hair cells, which are typical features of progenitor cells and supporting cells. A few EGFP-positive cells were undergoing mitotic division, as demonstrated by DAPI staining of their condensed nuclear DNA in apical planes of the epithelium. Preliminary time-lapse observations of transfected inner ear samples (Fig. 3I; supplementary material Movies 1, 2; data not shown) confirmed that a large number of Hes5::d2EGFP-positive cells were indeed mitotic progenitor cells. Their nuclei underwent a characteristic interkinetic migration, with mitosis occurring exclusively at the apical surface and generating transient movements of neighbouring cells during anaphase and telophase. By contrast, cells with very reduced Hes5::d2EGFP fluorescence, a rounded and constricted apical surface, and a more steady and apically located nucleus (visualised by co-transfecting with pT2K-nTomato in some samples) were not proliferating; these were likely to correspond to the hair cells previously identified in fixed specimens.

We next analysed Dll1 expression in relation to hair cell markers and Notch activity. Immunostaining for Dll1 was visible in small intracellular vesicles – a subcellular localisation that is due to the

internalisation of DSL ligands in signalling cells (Itoh et al., 2003; Matsuda and Chitnis, 2009). However, the cells with highest levels of Dll1 protein were not the HCA/HCS1-positive hair cells, but cells located in between these well-differentiated hair cells (Fig. 3J,K). In Hes5::d2EGFP-transfected samples, the Dll1-positive cells had low levels of EGFP fluorescence, a rounded and constricted apical surface and no HCA/HCS1 expression (Fig. 3L,L') or HCA expression only (supplementary material Fig. S2); these characteristics suggested that Dll1-expressing cells were prospective hair cells. Interestingly, at least some of these cells appeared to contact more mature-looking hair cells (yellow arrowhead in Fig. 3L,L' and supplementary material Fig. S2), an observation that is in line with that of transient contacts between mature and immature-looking hair cells reported by Goodyear and Richardson (Goodyear and Richardson, 1997).

Dll1 promotes hair cell fate cell-autonomously and inhibits hair cell fate in trans

To directly test Dll1 function during hair cell formation, we used two different Tol2 constructs. In the first, a constitutively active promoter drives chicken Dll1 in all transfected cells (pT2K-Delta1-EGFP; Fig. 4A). In the second construct, Dll1 expression is regulated by the mouse *Hes5* promoter (pT2K-Hes5::Delta1-EGFP; Fig. 5A) so as to investigate specifically the effects of Dll1 in 'Notch-active' progenitor cells, which are normally prevented from differentiating into hair cells. Both constructs included an IRES-EGFP sequence to identify transfected cells. Following electroporation at E2, the inner ear was analysed at stages when hair cells have differentiated, between E7 and E12. Highly transfected ears exhibited morphological defects, possibly owing to interference of the artificial Dll1 expression with the early function of Notch activity in prosensory specification (data not

shown). For clarity, we focus here on the effects of Dll1 on hair cell differentiation only. These were more clearly seen in the basilar papilla than in vestibular epithelia, as hair cell production is almost complete by E10 in the former tissue (Katayama and Corwin, 1989; Goodyear and Richardson, 1997).

In pT2K-Delta1-EGFP-transfected basilar papilla ($n=7$), levels of EGFP fluorescence were variable but transfected hair cells and supporting cells were easily recognisable by their shape as well as by immunostaining with HCA/HCS1. Within the same sample, two contrasting types of transfected region were found: those with a clear reduction in hair cell density (outlined in Fig. 4B), and those that appeared relatively unaffected (arrow in Fig. 4B). The inhibition of hair cell formation only occurred in regions in which relatively large clusters of cells (~10 or more) were transfected and contacted one another (outlined in Fig. 4C). In EGFP-positive regions without any reduction in hair cell density, the majority of hair cells were in fact transfected; although these appeared densely packed, the EGFP-positive hair cell bodies were in general surrounded by a darker outline, indicating the presence of non-transfected supporting cells separating them. This was confirmed by examination of the surface of the epithelium stained with fluorescent phalloidin. Hence, the majority of isolated transfected cells were hair cells, a trend that was more obvious in regions containing fewer transfected cells (Fig. 4D). Despite this, some hair cells, transfected (e.g. arrowheads in Fig. 4C) or otherwise (Fig. 4E,E'), were also found in direct contact with Dll1-overexpressing cells of supporting cell morphology. This suggests that some progenitor cells can differentiate into hair cells despite contacting neighbouring cells that express Dll1.

Similar observations were made in pT2K-Hes5::Delta1-EGFP-transfected samples, in which Dll1 expression was artificially induced in Notch-active cells only (Fig. 5). The reduction in hair cell density was only seen in regions containing large clusters of transfected cells (Fig. 5B,C), whereas a large proportion of isolated transfected cells differentiated into hair cells (Fig. 5D). Out of 171 isolated EGFP-positive cells scored in the basilar papilla ($n=6$; E11-E12), 114 were hair cells (66%). By comparison, only 30% of hair cells were scored among cells transfected with pT2K-nTomato in control experiments ($n=355$ cells; hair cell to supporting cell ratio of 1:2.45). This value fits within the 1:1.71 to 1:3.9 range of hair cell to supporting cell ratio determined in the basilar papilla of E12 chicken embryos (Goodyear and Richardson, 1997).

With the Hes5::Delta1-EGFP construct, the intensity of EGFP fluorescence was lower in hair cells than in supporting cells (Fig. 5D), suggesting that transfected hair cells were no longer expressing the Hes5::Delta1-EGFP transgene at the time of analysis. To verify this, we performed immunostaining for Dll1 in Hes5::Delta1-EGFP-transfected samples. None of the weakly EGFP-positive hair cells (arrowheads in Fig. 5E) expressed detectable levels of Dll1 in their cytoplasm. This confirmed that transfected progenitor cells that differentiate into hair cells stop expressing the Delta1-EGFP transgene once they no longer experience Notch activity. By contrast, isolated cells or clusters with high levels of EGFP fluorescence and a supporting cell-like morphology expressed high levels of Dll1 protein (arrow in Fig. 5E). In such clusters, high levels of Notch activity must be present in order to maintain expression of the Delta1-EGFP transgene. Finally, as previously reported with the constitutively expressed Delta1-EGFP transgene, we noted that some hair cells (transfected or otherwise) were contacting Dll1-expressing cells.

Altogether, these results showed that Dll1 represses hair cell differentiation in trans and promotes adoption of the hair cell fate

in cis, including for progenitor cells that experienced Notch activity. However, the trans-inhibition of hair cell formation by Dll1 was not systematic; the occurrence of contacts between hair cells and Dll1-overexpressing cells as well as the formation of hair cells within highly transfected regions suggested that some progenitor cells might be insensitive to Dll1-mediated lateral inhibition.

Dll1 does not inhibit Notch activity cell-autonomously

The occurrence of reciprocal contacts between hair cells and the differentiation of hair cells next to Dll1-expressing cells could in theory be explained by cis-inhibition, i.e. if Dll1 inhibited Notch activity cell-autonomously, in addition to its effects in trans. The persistence of high levels of expression of Delta1-EGFP when driven by the *Hes5* promoter in clusters of transfected cells suggests that if Dll1-mediated cis-inhibition occurs then it is not as efficient as the trans-activation of Notch receptors. However, Hes5-regulated gene expression is dynamically regulated by Notch activity; therefore, Hes5::Delta1-transfected cells might in fact be oscillating between ON and OFF phases of Dll1 expression and cis-inhibition.

In order to determine whether Dll1 functions by cis-inhibition in the inner ear, we investigated the consequences of a time-controlled induction of Dll1 expression for the activity of the Hes5 reporter. If Dll1 were able to inhibit Notch activity cell-autonomously, we expect that cells in which Dll1 is induced would have reduced levels of Hes5::nd2EGFP fluorescence ~8-12 hours after the onset of inhibition – a delay that is necessary for reduction of Hes5::d2EGFP fluorescence to basal levels after artificial blockade of Notch activity with DAPT in organotypic cultures (see supplementary material Fig. S3 and Movie 3). To test this, we co-transfected inner ear samples with the pT2K-Hes5::nd2EGFP reporter (which includes a nuclear-localised variant of d2EGFP to facilitate analysis of fluorescent signals) and two additional Tol2 constructs: pTRE-Delta1-FP635, in which the expression of Dll1 (and the red fluorescent protein FP635) is regulated by a tetracycline-responsive element (Fig. 6A), and pT2K-CAGGS-rTA²M2, which encodes the tet-on transactivator (Sato et al., 2007).

Electroporated embryos were incubated until E9, then treated with doxycycline (Dox) in ovo and sacrificed 6, 24 and 48 hours later ($n=3$ for each time point). The basilar papillae were dissected and immunostained for Dll1 expression and hair cell markers. Six hours after Dox treatment (Fig. 6B,C), Dll1-induced cells were recognisable after immunostaining by their elevated levels of intracellular and membrane-localised Dll1 protein. In contrast to previous experiments, the majority of Dll1-induced cells had supporting cell characteristics (no HCA staining and elongated cell bodies) and the pattern of hair cell differentiation was normal in transfected samples (Fig. 6C,C'), indicating that the Dll1 transgene was not expressed in the absence of Dox. The pattern of transfection was mosaic and the cells overexpressing Dll1 represented only a subset of Hes5::nd2EGFP-positive cells. This allowed a comparison of the levels of Hes5::nd2EGFP within cells that overexpressed Dll1 with those that did not.

The cells with the highest levels of nuclear-localised Hes5::nd2EGFP fluorescence were located in regions where the Dll1 protein was overexpressed, confirming the efficient induction of Notch activity by ectopic Dll1. The levels of Hes5::nd2EGFP fluorescence varied greatly from cell to cell; however, high levels of Notch activity were detected in both Dll1-induced and non-induced

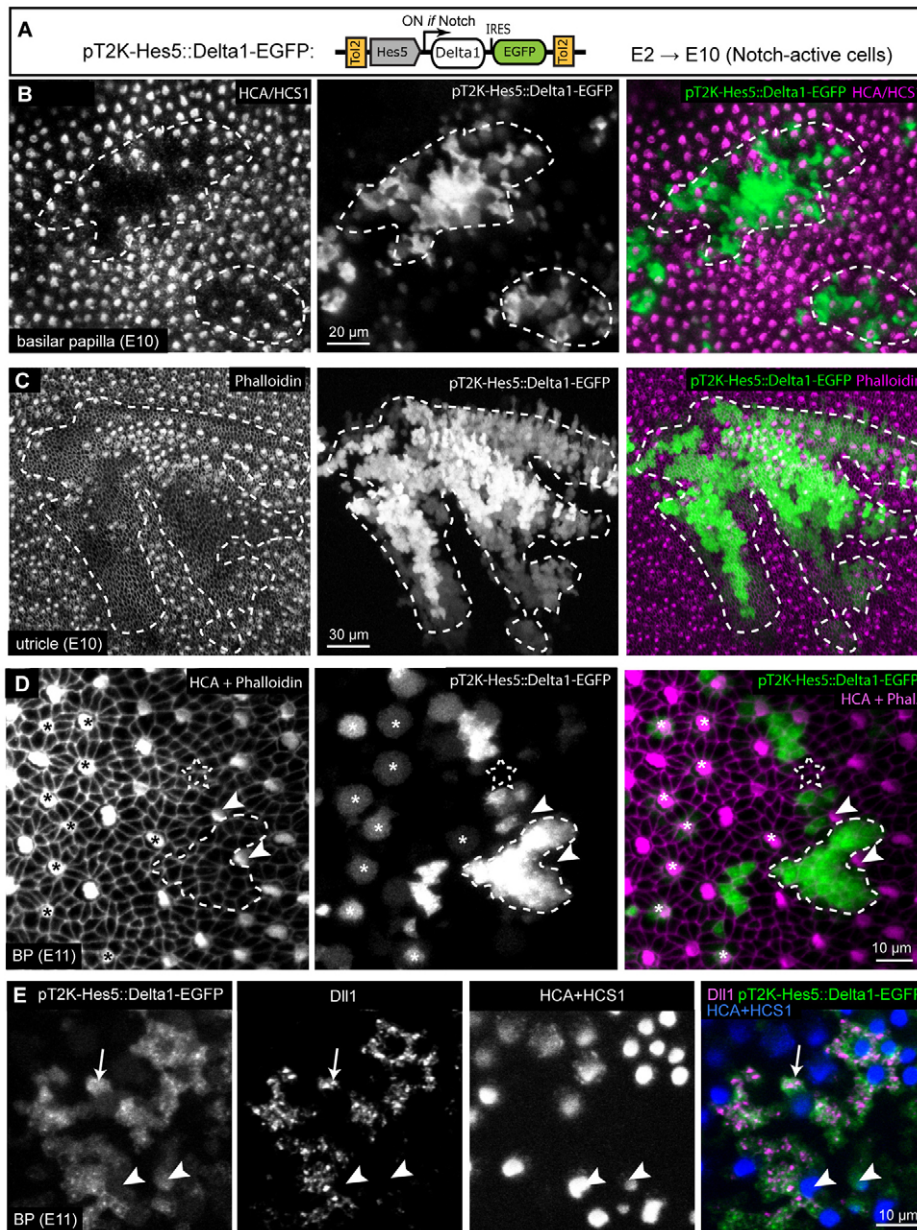


Fig. 5. Effects of Dll1 expression in Notch-active cells. (A) The pT2K-Hes5::Delta1-EGFP plasmid. (B,C) Samples transfected with pT2K-Hes5::Delta1-EGFP and examined at E10. Hair cell density is reduced in regions with large clusters of transfected cells (outlined). (D) High-magnification view of an E11 basilar papilla with a very mosaic pattern of transfection. Hair cells are missing within large clusters of EGFP-positive cells (outlined) and next to transfected regions (star with dashed outline). However, many isolated transfected cells have differentiated into hair cells (asterisks), which exhibit low levels of EGFP fluorescence. Note also that some hair cells can form in direct contact with EGFP-positive cells (arrowheads). (E) E11 basilar papilla transfected with pT2K-Hes5::Delta1-EGFP and immunostained for Dll1. High levels of Dll1 expression are present within cells with high EGFP fluorescence and supporting cell morphology (arrow). Note that transfected hair cells do not express Dll1 (arrowheads).

cells. At 24 (Fig. 6D) and 48 (not shown) hours after Dox treatment, there was still very strong expression of Dll1 within induced cells, the majority of which had supporting cell morphology. Once again, the fluorescence of the Hes5::nd2EGFP reporter varied from cell to cell, being stronger in Dll1-induced regions, and was particularly high in some of the Dll1-induced cells.

Similar results were obtained in experiments performed at an earlier developmental stage in vestibular sensory patches. The Dox treatment was started at E5.5 and the inner ear tissue analysed 21 hours later. As previously noted, there was strong induction of Hes5::nd2EGFP fluorescence around Delta1-FP635-induced cells, but some Delta1-FP635-expressing cells were also Hes5::nd2EGFP positive (Fig. 6E). Despite some caveats linked to the mosaicism of transfection, the effects of Dll1 in trans, and some uncertainties about the differentiation status of FP635-positive cells at these early developmental stages, we tried to quantify the effect of ectopic Dll1 on the activity of the Hes5 reporter. We measured and compared the mean intensity of Hes5::nd2EGFP fluorescence

within individual FP635-positive cells (see Materials and methods) that were either transfected with pTRE-Delta1-FP635 ($n=461$ out of a total of 1416 nuclei in three samples) or with pTRE-FP635 ($n=959$ out of a total of 1697 nuclei in five samples) as a control. Statistical analysis of the data showed that the distribution of the normalised values of mean fluorescence intensities (Fig. 6F) was not significantly different between the two categories of transfected cells (Mann-Whitney $U=212,380$; $P=0.231$), suggesting that the induction of Dll1 did not significantly alter the intrinsic levels of Notch activity. As a further test, we repeated this analysis on E6 samples treated in ovo with Dox for 14 hours ($n=5$), and immunostained for Dll1 to identify Dll1-overexpressing cells (Fig. 6G,H). The mean levels of Hes5::nd2EGFP activity varied greatly from cell to cell and were not reduced in Dll1-induced cells. On the contrary, statistical analysis of the normalised values of mean nd2EGFP fluorescence showed that Hes5::nd2EGFP activity was significantly elevated in Dll1-induced cells ($n=327$) compared with non-induced cells ($n=305$; Mann-Whitney $U=56,318$; $P=0.05$).

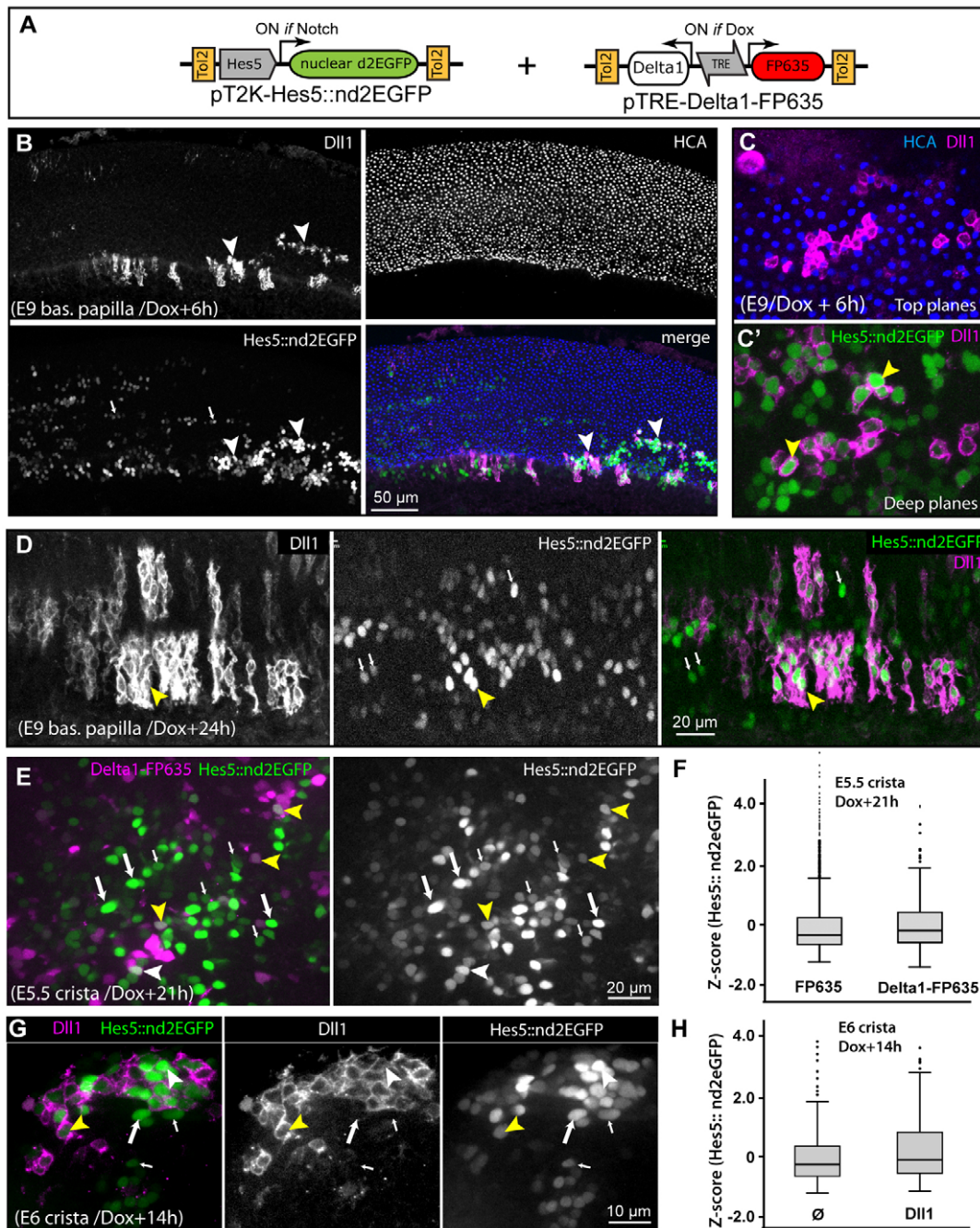


Fig. 6. Dll1 does not inhibit Notch activity cell-autonomously. (A) The pT2K-Hes5::nd2EGFP and pT2K-Delta1-FP635 tet-on plasmid for Dox-inducible expression of Dll1. (B) Basilar papilla examined 6 hours after Dox administration at E9 and immunostained for Dll2. Levels of nd2EGFP fluorescence are elevated in regions with high Dll1 expression (arrowhead) when compared with regions in which Dll1 is not induced (arrows). (C, C') Dll1-expressing cells have supporting cell morphology and intercalate between hair cells at the surface of the epithelium (C); their cell bodies extend to basal planes (C') and some cells exhibit high nd2EGFP fluorescence (arrowheads). (D) Lateral view of a basilar papilla 24 hours after Dox treatment at E9. The majority of Dll1-overexpressing cells have supporting cell morphology and some exhibit high levels of Hes5::nd2EGFP fluorescence (e.g. yellow arrowhead) when compared with cells that do not overexpress Dll1 (arrows). (E) Representative view of an E6 sensory crista analysed 21 hours after Dox treatment in ovo. In cells that are FP635 negative, levels of nd2EGFP fluorescence vary greatly, from very high (thicker arrows) to very low (thin arrows). Cells that are FP635 positive exhibit variable levels of Hes5::nd2EGFP fluorescence in their nuclei (yellow arrowheads). (F) Z-scores for mean values of Hes5::nd2eGFP fluorescence in FP635-positive cells transfected with either pTRE-FP635 (FP635, $n=959$) or pTRE-Delta1-FP635 (Delta1-FP635, $n=461$), analysed 21 hours after Dox treatment in ovo. (G) Example of an E6 sensory crista immunostained for Dll1 expression 14 hours after Dox treatment in ovo. Induced cells have elevated levels of Dll1 protein at their membrane (arrowheads) when compared with non-induced cells (arrows). In both categories of cells, the nuclear levels of nd2EGFP fluorescence are very variable. (H) Z-scores for the mean values of Hes5::nd2eGFP fluorescence in cells transfected with pTRE-Delta1-FP635 and analysed 14 hours after Dox treatment in ovo at E6. Cells were immunostained for Dll1 expression and categorised into either non-induced (\emptyset , $n=305$) or induced (Dll1, $n=327$) cells. (F, H) Outliers, minimum, first quartile, median, third quartile and maximum are displayed.

Altogether, these data indicate that Dll1 acts primarily in trans to activate Notch receptors, and strongly suggest that expression of Dll1 does not inhibit Notch activity cell-autonomously in the developing inner ear.

DISCUSSION

Much of our understanding of tissue patterning by lateral inhibition derives from mathematical modelling and in vitro studies, but comparatively little is known about the actual dynamics of this process in vivo, in particular in vertebrates. In this study, we tested some of the fundamental assumptions of the lateral inhibition model in the sensory epithelia of the chicken inner ear. In agreement with the standard model, we show that elevation of Dll1 expression in progenitor cells is crucial for adoption of the hair cell fate, and that Dll1 functions primarily in trans to activate Notch activity and to repress hair cell formation. However, we also found that some cells can differentiate into hair cells while contacting other Dll1-expressing cells. Although this indicates that some progenitor cells are not sensitive to lateral inhibition, we argue that this is unlikely to result from the cis-inhibition of Notch activity by Dll1 itself.

Dll1 is a key determinant of hair cell fate decisions

Although hair cells express several DSL ligands, Dll1 is thought to play the most significant role in the lateral inhibition of hair cell formation in the inner ear. In fact, the absence of Dll1 produces a much more severe overproduction of hair cells (Kiernan et al., 2005; Brooker et al., 2006) than that of Jag2 (Lanford et al., 1999), whereas *Dll3* mutant mice have no discernible ear phenotype (Hartman et al., 2007). However, some questions remain about the mode of action of Dll1 in hair cell fate decisions. Are hair cell fate decisions dictated by progenitor cell-to-cell competition for Dll1 expression? Alternatively, does Dll1 operate only after hair cells are committed in order to protract hair cell formation? Is Dll1 able to inhibit the reception of Notch activity in cis within progenitor cells of the inner ear?

Here, our gain-of-function studies showed that the formation of hair cells was reduced within clusters of Dll1-expressing cells, but that a large proportion of isolated Dll1-expressing cells differentiated into hair cells. This confirms that Dll1 represses hair cell fate in trans, and that cells that express Dll1 have a greater chance of adopting the primary fate than those that do not. In standard lateral inhibition, ‘signal-sending’ and ‘signal-receiving’ cells compete for Dll1 expression, which is repressed by Notch activity in signal-receiving cells. We found that reversing this negative-feedback loop into a positive one using a *Hes5* promoter-regulated Dll1 construct can convert the majority of signal-receiving into signal-sending cells. This shows that cells in which Notch is active can differentiate into hair cells if they succeed in elevating their endogenous levels of Dll1, and that the intercellular competition for Dll1 expression is one of the key determinants of hair cell fate decisions.

However, some data did not seem to fit with the standard model of lateral inhibition. First, not all isolated Dll1-expressing cells differentiated into hair cells. One possible explanation is that some progenitor cells are not competent to adopt the hair cell fate; ultimately, it is the expression of proneural genes, such as *Atoh1* in the case of hair cells (Bermingham et al., 1999), that determines the adoption of the primary fate during lateral inhibition. Second, some cells (transfected with Dll1 or not) could differentiate into hair cells despite contacting other Dll1-expressing cells. The

complete inhibition of hair cell formation was only observed within relatively large clusters of Dll1-expressing cells, which suggests that efficient repression of the hair cell fate is only achieved when several signal-sending cells cooperate to activate Notch in any given signal-receiving cell. It is also possible that some of the progenitor cells are refractory to Notch activity induced by Dll1. The underlying mechanisms could be extremely diverse, ranging, for example, from the reduction of cell surface levels of Notch receptors to interference with downstream components of the Notch signalling cascade. In addition, recent studies have highlighted the capacity of DSL ligands to cell-autonomously inhibit Notch activity; however, as we discuss below, several results suggest that Dll1 does not act in this way in the inner ear.

Dll1 is unlikely to function by cis-inhibition of Notch activity in the inner ear

Gain-of-function studies have revealed that, in some developmental contexts, DSL ligands can inhibit Notch activity cell-autonomously (reviewed by del Álamo et al., 2011). Recent in vitro experiments using cell lines transfected with a Notch reporter have also shown that varying the levels of Dll1 in cis can indeed affect their response to Dll1 in trans (Sprinzak et al., 2010). Although the underlying mechanisms remain unclear, the commonly accepted model is that, when high levels of DSL ligands are present at the surface of a cell, they can bind in cis to Notch receptors, thereby preventing their activation in trans by other cells – a titration effect. Modelling studies have suggested that cis-inhibition could improve the robustness of cell fate decisions and might be essential for efficient patterning of epithelial sheets by lateral inhibition (Barad et al., 2010; Sprinzak et al., 2010), but is this the case in the inner ear?

In the present experiments, formation of hair cells within highly Dll1-transfected regions could be interpreted as evidence for cis-inhibition; however, hair cells within such regions were, in general, surrounded by untransfected supporting cells. Hence, mosaicism of transfection as well as cell mixing could explain why hair cell formation was not reduced in such regions. The reduction of hair cell density within *Hes5::Delta1*-transfected clusters provides additional evidence that, if cis-inhibition occurs, it does not prevent the reception of trans-inhibition; in fact, if Notch activity were blocked by Dll1 expression cell-autonomously, *Hes5*-regulated Dll1 expression would not be occurring in such clusters. Finally, the use of a tet-on inducible system confirmed that the strong elevation of Dll1 expression does not significantly reduce the activity of the *Hes5* reporter in progenitor and supporting cells. Although this does not exclude the possibility that other intrinsic factors or DSL ligands expressed in progenitor cells inhibit Notch activity cell-autonomously, the present results provide strong evidence that Dll1 does not. Our data suggest instead that the ability of Dll1 to promote hair cell differentiation in cis results from its inhibitory impact on Dll1 expression in trans, according to the standard model of lateral inhibition with intercellular feedback.

Pattern formation by lateral inhibition: a two-step process?

Expression of Dll1 protein is absent or very low in Notch-active cells, peaks in cells with characteristics of very immature hair cells, but is downregulated in differentiated hair cells expressing HCA and otoferlin. This is in agreement with other studies showing temporally restricted waves of expression of Dll1, Jag2 and Dll3 in the auditory hair cells of mammals (e.g. Hartman et al., 2007) and suggests that Dll1/Notch-mediated lateral inhibition is short lived,

occurring primarily between progenitor cells and immature hair cells. As hair cells differentiate, they might not deliver sufficient inhibition to prevent neighbouring cells from becoming hair cells. This would explain the transient contacts between mature-looking and immature hair cells in the immature basilar papilla (Goodyear and Richardson, 1997). One advantage of short-lived lateral inhibition is that it could facilitate the addition of new hair cells to inner ear epithelia over an extended developmental period, or even throughout life, as is the case in the avian vestibular system (Jørgensen and Mathiesen, 1988). However, this implies that additional mechanisms must operate downstream of lateral inhibition to eliminate patterning errors such as reciprocal hair cell contacts.

One attractive hypothesis is that differential adhesion properties of hair cell and supporting cells could lead to a progressive refinement of their relative position through homo- and heterotypic interactions (Goodyear and Richardson, 1997; Podgorski et al., 2007). In support of this idea, a recent study has shown that the cell adhesion molecules of the nectin family regulate cell patterning in the organ of Corti (Togashi et al., 2011). Other cell adhesion molecules, including Eph/ephrins and cadherin family members (see Warchol, 2007), can mediate cell sorting in other tissues (Steinberg and Takeichi, 1994; Xu et al., 1999) and may also contribute to the fine-grained patterning of the inner ear epithelia. A two-step model such as this would resemble the mechanism described for patterning of the *Xenopus* epidermis: lateral inhibition within the inner layer of the non-neural ectoderm establishes a pre-pattern of differentiation, which is then refined as ciliated cells migrate and intercalate into the superior layers of the epidermis (Deblandre et al., 1999). The mechanistic separation of cell fate decisions and fine-grained patterning events could also be advantageous during epithelial regeneration, for example after hair cell loss in the avian inner ear. In the damaged adult basilar papilla, Dll1 expression is upregulated in the newly formed hair cells, but not in the surviving ones (Stone and Rubel, 1999; Daudet et al., 2009). In the case of limited hair cell loss and regeneration, fine-grained patterning via differential cell affinities could provide a solution for the accurate positioning of a limited number of new cells within a pre-established cellular mosaic independently of Notch signalling.

A simultaneous occurrence of inductive (such as lateral inhibition) and morphogenetic (such as cell proliferation or cell adhesion) mechanisms, or ‘morphodynamic’ patterning strategy (Salazar-Ciudad et al., 2003), operates in the inner ear sensory epithelia. This raises new questions regarding the influences of one set of mechanisms over the other(s), in the inner ear as well as in other tissues in which Notch signalling operates. To answer these questions, live-imaging approaches as well as computational models in which the interaction of inductive and morphogenetic processes can be investigated (Podgorski et al., 2007) will be instrumental.

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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.074476/-/DC1>

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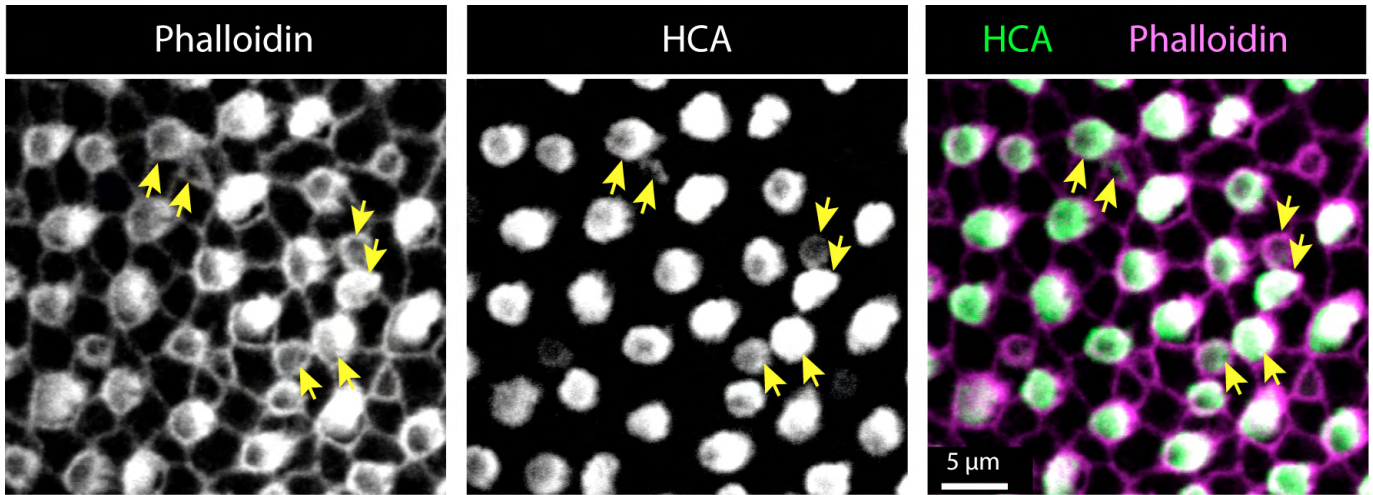


Fig. S1. Surface view of an E10 basilar papilla (distal region) immunostained for HCA and labelled with fluorescent phalloidin. Note the presence of contacts between hair cells with strong HCA staining and some hair cells with very faint or no expression of HCA (arrows), but a characteristic enrichment of actin at their periphery.

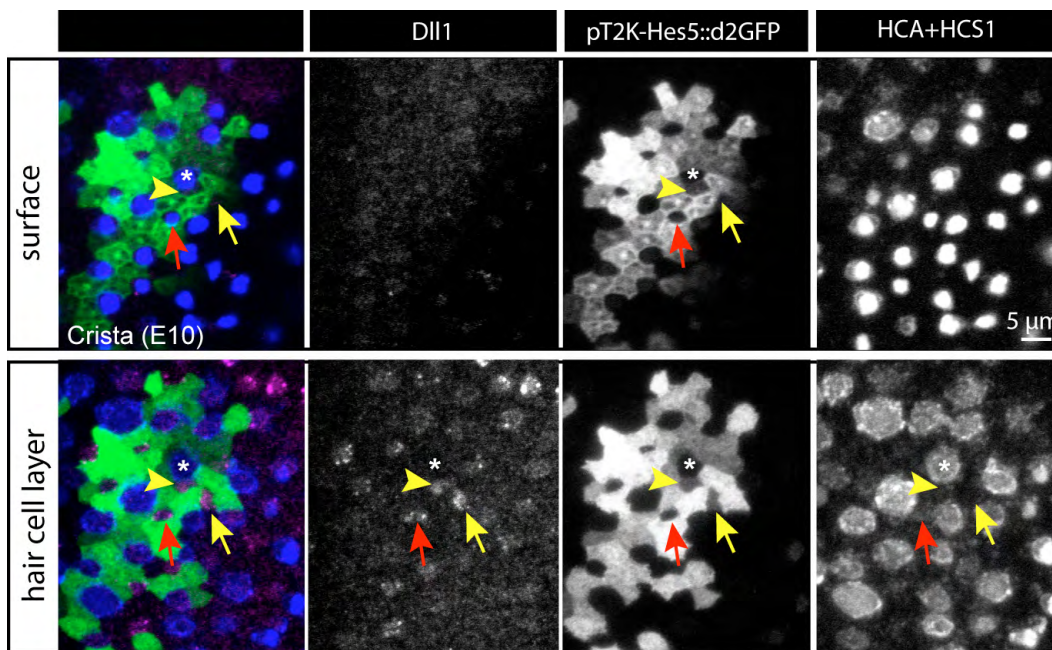


Fig. S2. Immunostaining for Dll1, HCA and otoferlin (HCS1) in relation to Hes5::d2EGFP activity in an E10 sensory crista. Two different optical planes from the same region, and parallel to the luminal surface, are shown. The expression of Dll1 is reduced in differentiated hair cells (asterisk) expressing HCA and otoferlin. The Dll1-positive cells (yellow arrow and arrowhead and white arrow) have low levels of Hes5::d2EGFP, can be HCA positive (red arrow), but do not express otoferlin. Note that one of these cells (yellow arrowhead) is in direct contact with a more differentiated hair cell (asterisk).

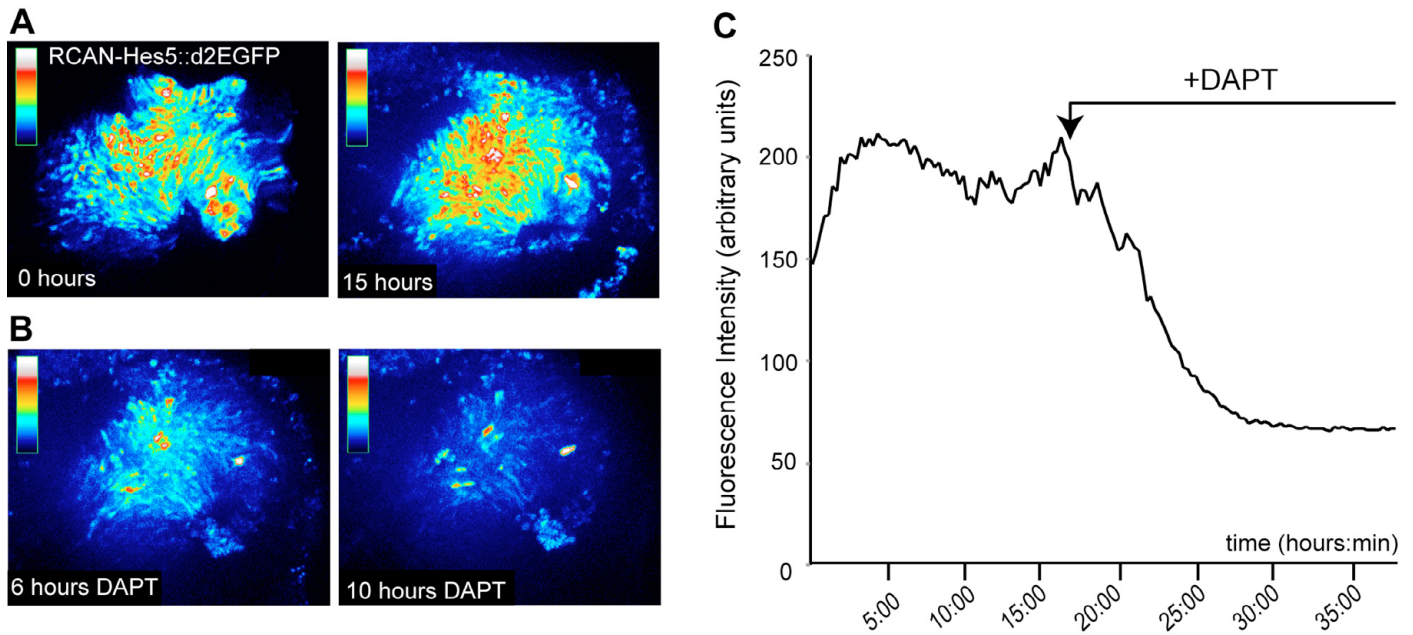


Fig. S3. Treatment with the γ -secretase inhibitor DAPT (20 μ M) reduces EGFP fluorescence levels in organotypic cultures of E10 crista stably transfected with Hes5::d2EGFP. (A) Low-magnification surface view of a crista transfected with RCAN-Hes5::d2EGFP and imaged at 15-minute intervals on a Nipkow spinning-disc confocal microscope (see Movie 1). In control medium, levels of fluorescence show slight fluctuations but remain relatively stable over 15 hours. (B) The same crista following DAPT addition to the medium. After a delay of \sim 2 hours, fluorescence levels start to decrease, reaching a minimum level in \sim 10 hours ($n=3$). Similar results and timecourse for reduction in fluorescence were obtained in samples transfected with pT2K-Hes5::d2EGFP and with pT2K-Hes5::nd2EGFP ($n=5$; not shown). (C) Quantification of mean fluorescence intensities (arbitrary units) before and after DAPT addition in the crista shown in A,B.