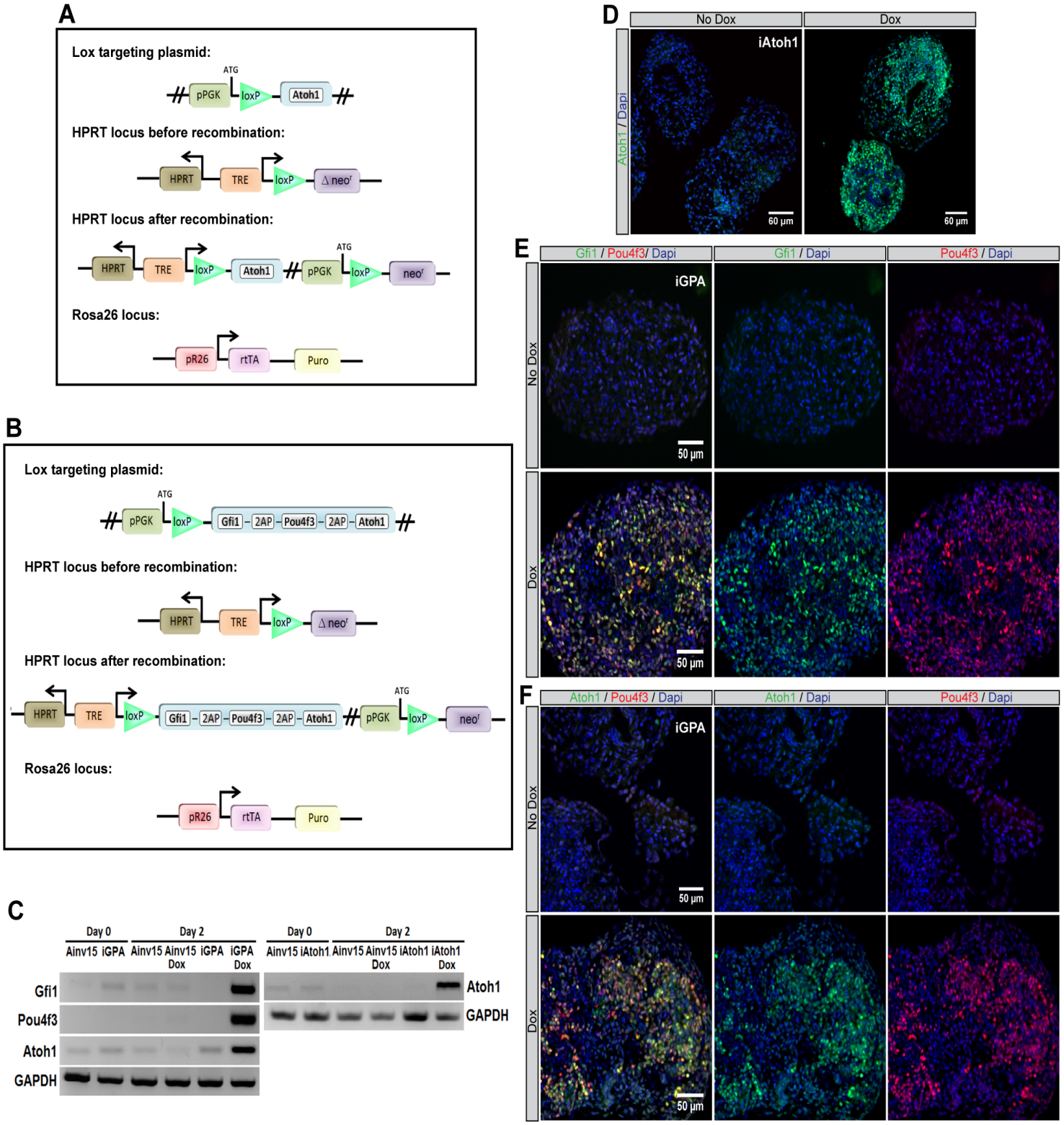


**Fig. S1. Generation and characterization of iAtoh1 and iGPA ES lines.**

(A and B) Schematic representation of experimental procedures used for the generation of mESC lines in which Doxycycline-induced expression of Atoh1 (A) and co-expression of Gfi1, Pou4f3 and Atoh1 (B) can be achieved.

(C) RT-PCR analysis reveals strong expression of *Gfi1*, *Pou4f3* and *Atoh1* mRNAs after 48h of 2µg/ml Dox treatment in the iGPA line, but not in the parental Ainv15 line. Similarly, the iAtoh1 line also shows increased *Atoh1* expression upon Dox treatment.
(D) Immunostaining for Atoh1 expression in day 6 EBs generated from iAtoh1 mESCs, cultured with or without Dox for 24h.
(E and F) Immunostaining analysis of Pou4f3/Gfi1 (E) and Pou4f3/Atoh1 (F) in EBs derived from iGPA mESCs in the presence or absence of Dox for 24h.
Abbrevaitions: pPGK, phospho-glycero-kinase promoter; HPRT, hypoxanthine phosphoribosyltransferase 1 gene; TRE, tetracycline responsive element; rtTA, reverse tetracycline transactivator; Puro, puromycin resistance gene; pR26, promoter of the ROSA26 locus; 2AP, 2A peptide sequence; ATG, start codon for the neo gene; loxP, Cre-recombinase recognition sequence; Δ $neo^r$, deletion mutant of the neomycin (G418) resistance gene; //, plasmid sequence.

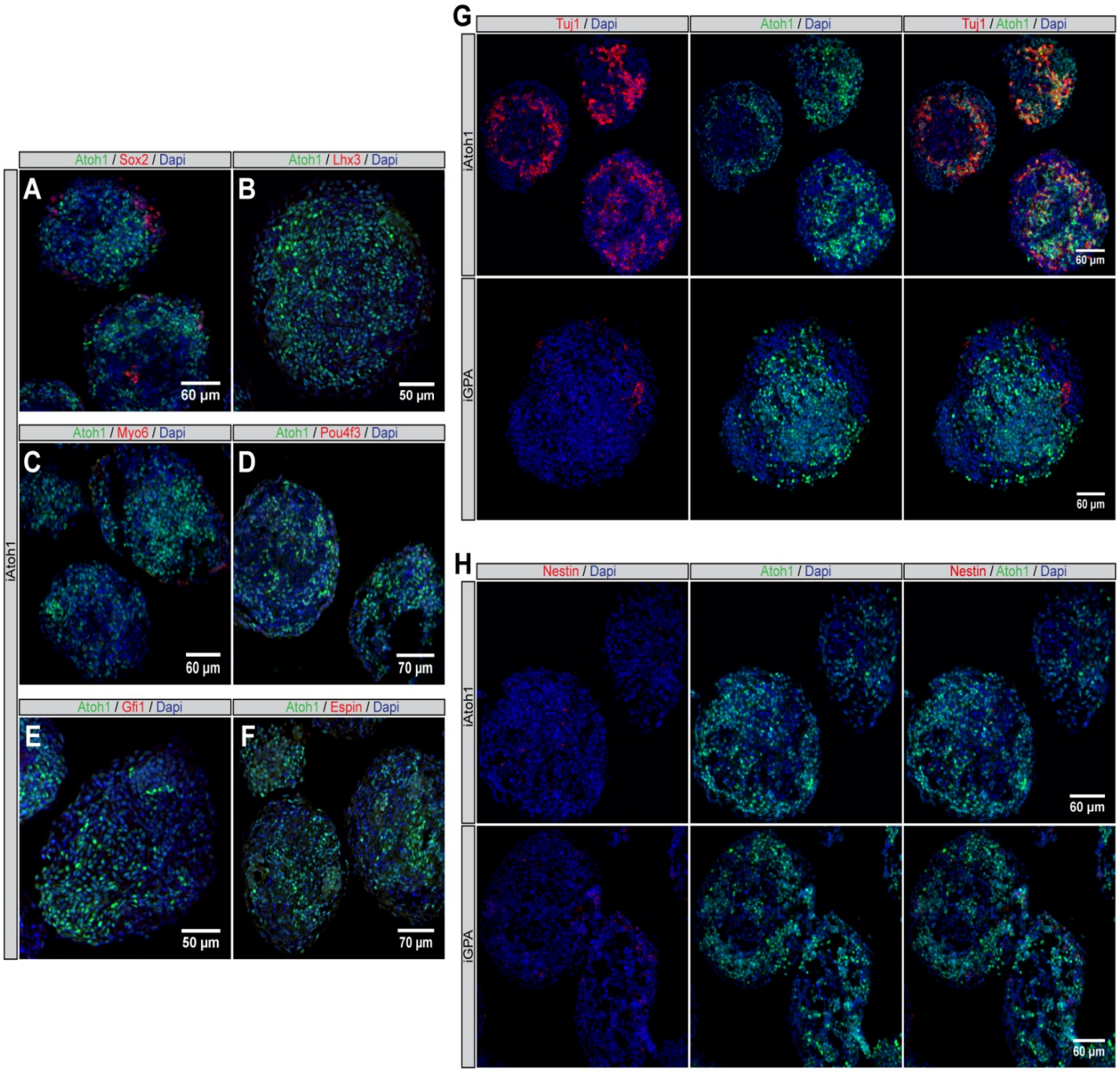


**Fig. S2. Forced expression of Atoh1 promotes neuronal differentiation but not hair cell fate.**

(A-F) Immunostaining for several HC specific markers performed in day 8 iAtoh1-derived EBs previously exposed to Dox for 4 days. Among induced Atoh1$^{+}$ cells, no significant up-regulation was observed for Sox2 (A), Lhx3 (B), Myo6 (C), Pou4f3 (D), Gfi1 (E) and Espin (F).

(G, H) Immunostaining for Tuj1 and Nestin in Day 8 EBs derived from iAtoh1 and iGPA mESCs, after 4 days of Dox induction. Tuj1+ neurons can only be detected in iAtoh1 EBs, not in iGPA EBs. Nestin is not induced in any of the cases.

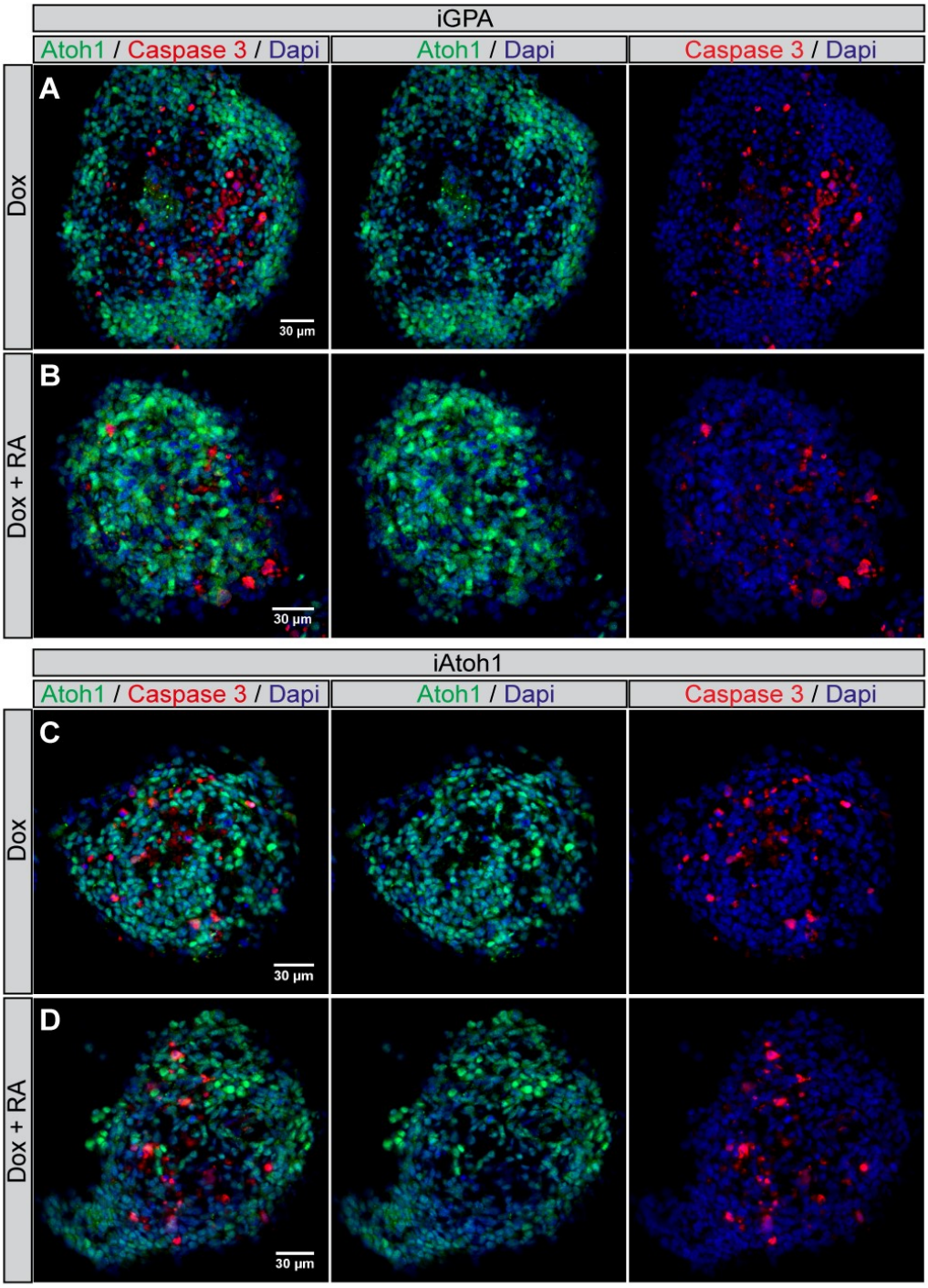


**Fig. S3. Cells overexpressing Atoh1 and GPA in Dox-induced EBs do not show differences in cell survival.**

Immunostaining analysis for activated Caspase 3 and Atoh1 performed in iGPA-derived EBs (A and B) and in iAtoh1-derived EBs (C and D) at day 8. Quantification of Atoh1+/aCas3+ cells in iAtoh1 (2,64 ± 1,4%) and iGPA (2,59 ± 1,2%) EBs revealed no significant difference between the two conditions.

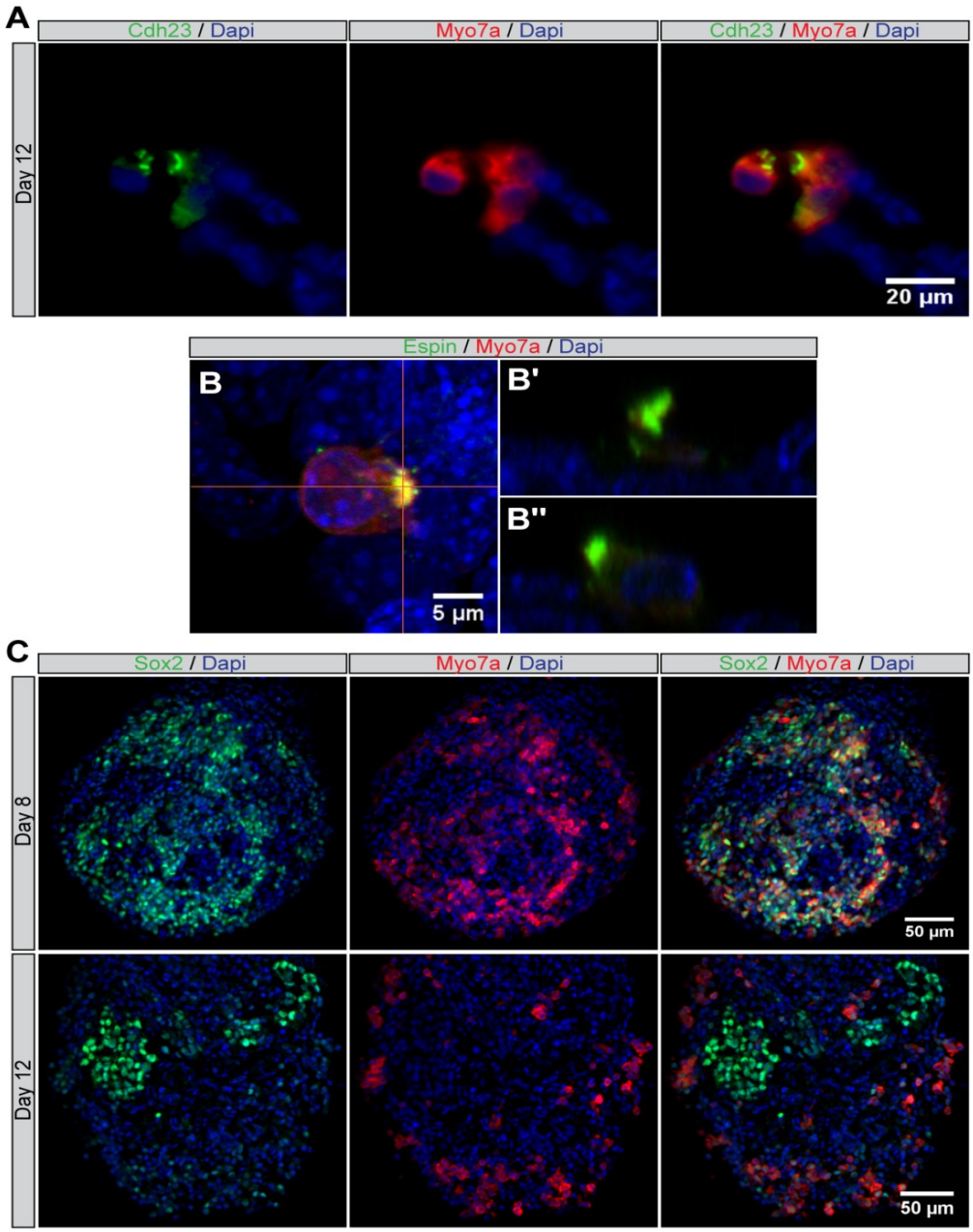


**Fig. S4. iHC progenitors can differentiate and develop hair bundle-like structures.**
(A) Immunostaining for Cdh23 and Myo7a in day 12 iGPA EBs exposed to Dox during 8 days.
(B) High magnification views of the hair bundle-like protrusions labelled with Myo7a and Espin in iHCs co-cultured with embryonic chicken utricle mesenchymal cells (protocol described in Figure 3E). Note that B' and B'' are orthogonal views showing Espin$^{+}$ structures oriented to the top of the culture dish.
(C) Immunostaining for Sox2 in iGPA-derived EBs reveals a decrease in its expression in Myo7a$^{+}$ cells from day 8 to day 12.

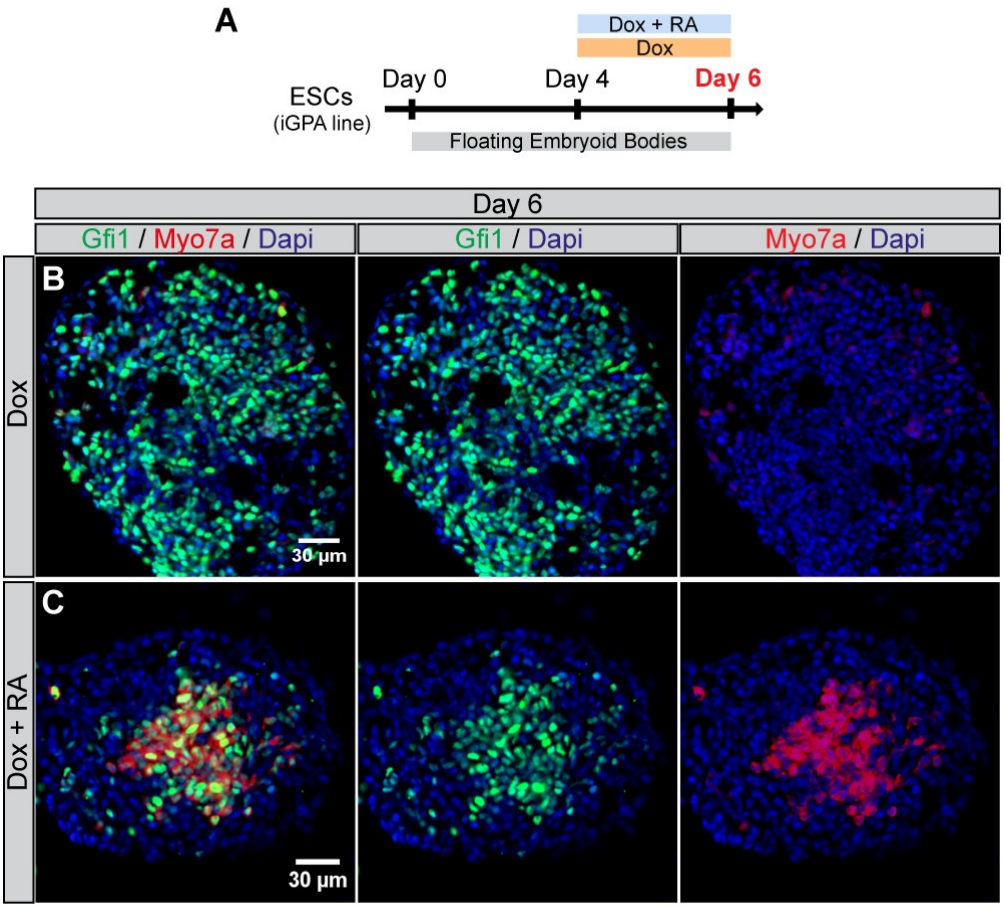


**Fig. S5. RA promotes early onset of HC differentiation.**

(A) Schematic diagram of the ES cell differentiation protocol through EB formation, including a 2 day period of Dox induction.

(B and C) Immunostaining analysis of Myo7a and Gfi1 expression performed in EBs harvested at day 6, showing strong up-regulation of Myo7a only in the Dox + RA condition (B) and not with Dox stimulation alone (C).

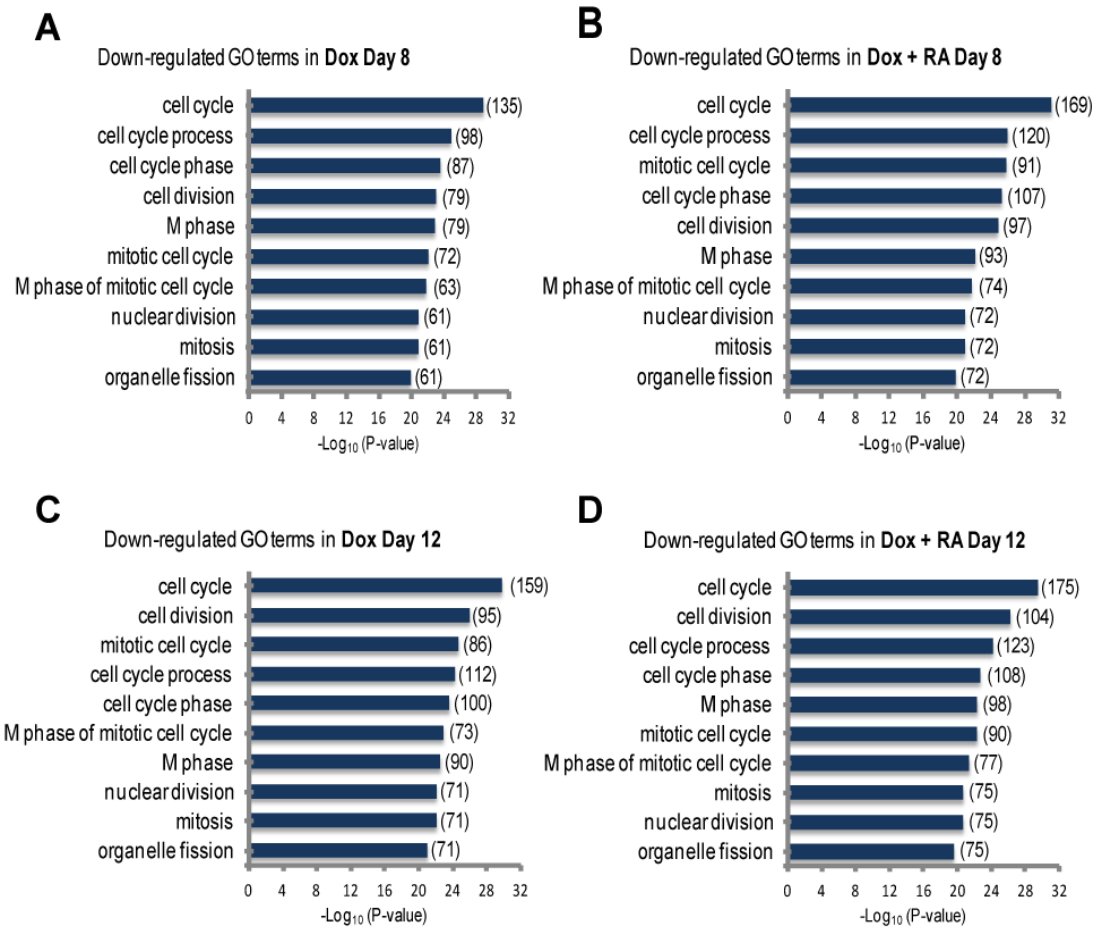


**Fig. S6. Analyses of significant down-regulated genes in iHC transcriptomes.**
(A-D) Gene ontology (GO) analysis using DAVID functional annotation tool for the significant down-regulated genes detected in the four different iHC groups (expression fold changes > 2, *P*-value < 0,01), when compared to untreated cells at day 8 (A and B) and at day 12 (C and D). The number of up-regulated genes included in each GO functional term is shown.

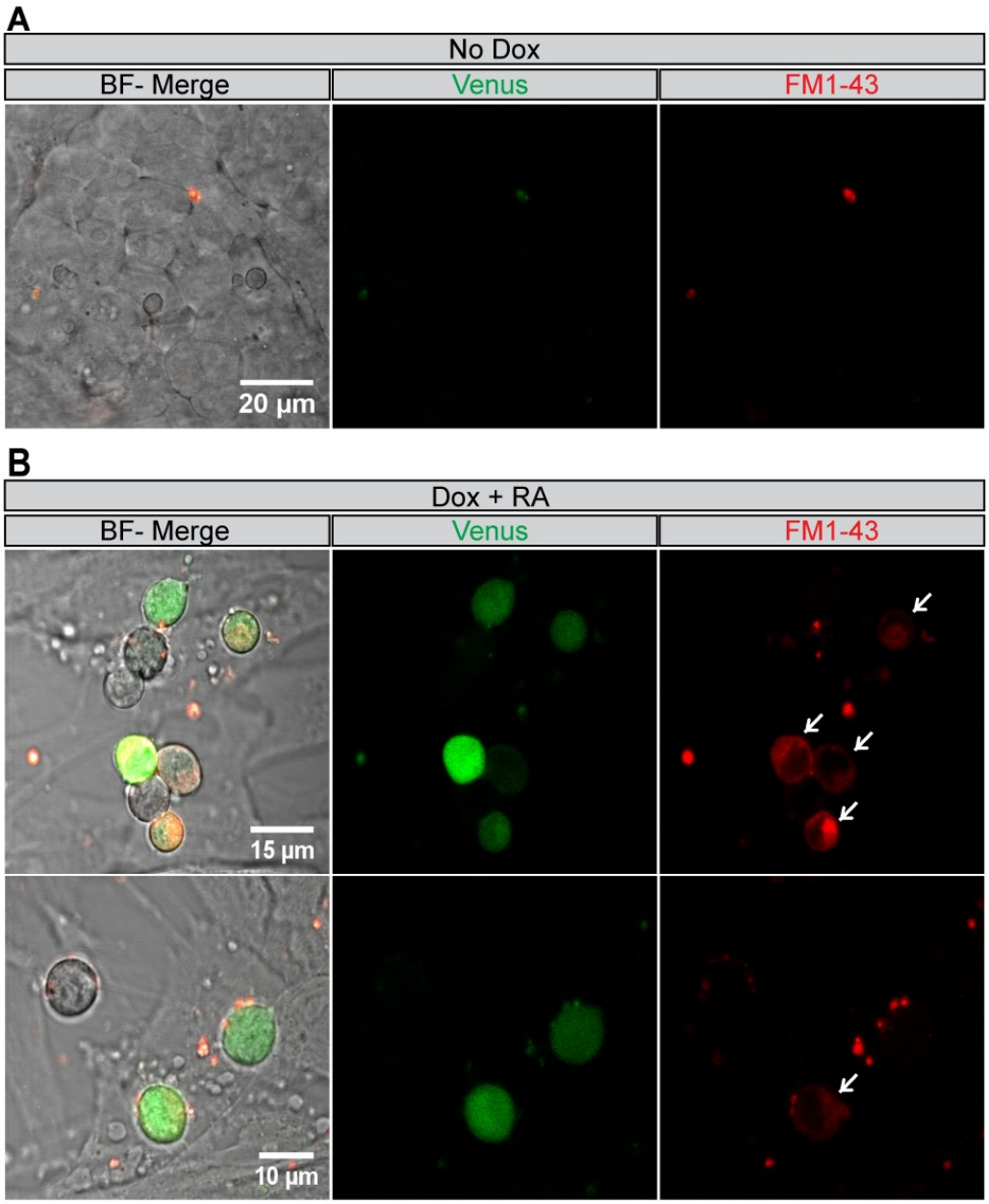


**Fig. S7. iHCs express mechanotransduction channels that enable specific and fast FM1-43 permeation.**

(A and B) The *in vitro* differentiation protocol (described in Figure 3E) for co-culture with embryonic chicken utricle mesenchymal cells was performed using the iGPA-Myo7a:mVenus reporter mESC line. At day 12, EB-derived cells grown in the presence or absence of Dox were incubated in 6µM FM1-43 for sixty seconds. Confocal bright field and fluorescence images in the "No Dox" condition shows absence of Venus and FM1-43 staining (A). In contrast, Dox treatment led to the appearance of Venus$^{+}$ iHCs that are permeable to FM1-43 dye (arrows) (B).

BF, Bright field; FM1-43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridiniumdibromide.

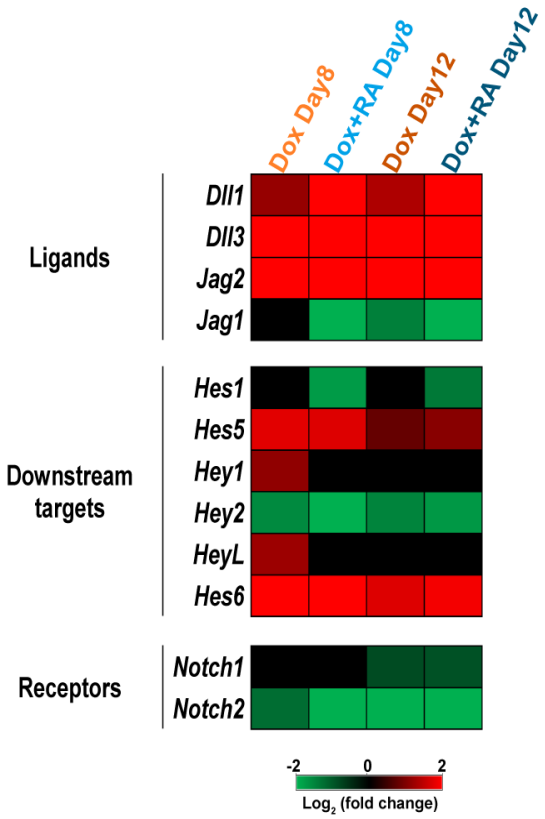


**Fig. S8. The Notch pathway is active during iHC differentiation.** Heat map depicting the relative fold changes in expression of various Notch pathway genes in the 4 iHCs populations at day 8 and at day 12. Only fold changes with a *P*<0,01 value are represented in this heat map.

**Table S1. iHC transcriptome profile.**

Worksheets: “Dox D8_UP-genes”, “Dox+RA D8_UP-genes”, “Dox D12_UP-genes” and “Dox+RA D12_UP-genes”. These contain all probe sets found to be significantly up-regulated (FC>2, *P*-value<0,01) in the four different iHCs groups, relative to uninduced cells.

Worksheets: “Dox D8_Down-genes”, Dox+RA D8_Down-genes”, “Dox D12_Down-genes” and “Dox+RA D12_Down-genes”. These contain all probe sets found to be significantly down-regulated (FC<-2, *P*-value<0,01) in the four different iHCs groups, relative to uninduced cells.

Worksheets: “D12>D8_Up-genes” and “RAD12>D8_Up-genes” contain all probe sets selected and analyzed by the criteria described in Figure 6F and G.

Worksheet “Deafness genes” contains a list of 88 genes and references used to generate the heat maps presented in Figure 6J and K.

Click here to Download Table S1

**Table S2. Primary antibodies used for immunocytochemistry, immunohistochemistry and flow cytometry analysis.**

| Antibody | Species | Source & Catalog Number | Dilution |
|---|---|---|---|
| Myo7a | Mouse | Hybridoma Bank (DSHB), 138-1 | 1:100 |
| Atoh1 | Guinea-Pig | Kind gift of Y. Sasai lab | 1:1000 |
| Pou4f3 | Rabbit | Sigma-Aldrich, HPA038215 | 1:50 |
| Espin | Rabbit | Kind gift of A. J. Hudspeth lab | 1:1000 |
| Cdh23 | Rabbit | Kind gift of Ulrich Mueller lab | 1:200 |
| Gfi1 | Guinea-Pig | Kind gift of Hugo Bellen lab | 1:2000 |
| Myo6 | Rabbit | Proteus Biosciences, 25-6791 | 1:800 |
| Lhx3 | Rabbit | Abcam, ab14555 | 1:200 |
| Nestin | Mouse | BD Pharmingen, 556309 | 1:20 |
| Sox2 | Rabbit | Millipore, AB5603 | 1:200 |
| Tuj1 | Mouse | Covance, MMS-435P | 1:500 |
| GFP | Rabbit | Abcam, ab290 | 1:400 |
| GFP | Chicken | Abcam, ab13970 | 1:1000 |
| Parvalbumin | Mouse | Sigmag-Aldrich, P3088 | 1:200 |
| HCS-1 | Mouse | Hybridoma Bank (DSHB), (Hair Cell Soma-1) | 1:100 |
| HCA | Mouse | Kind gift of Guy Richardson | 1:1000 |
| 3A10 | Mouse | Hybridoma Bank (DSHB) | 1:40 |
| Prox1 | Rabbit | Abcam, ab11941 | 1:200 |
| E-cadherin | Mouse | BD Transduction, 610181 | 1:100 |
| Cleaved Caspase-3 | Rabbit | Cell signaling, 9661 | 1:200 |
| Phalloidin-488 | Probe conjugated to Alexa 488 | Molecular Probes, A12379 | 1:100 |

## Supplementary Material and Methods

### Generation of iAtoh1 and iGPA ESC lines

Murine *Atoh1* ORF was amplified by PCR using the IMAGE clone IRAVp968D0993D as template. The PCR product was cloned into the Plox vector to produce the final Atoh1Plox vector that was used to generate the iAtoh1 mESC line. For the iGPA line, *Atoh1* ORF amplification was performed by adding a 2A peptide (2AP) sequence obtained from the *Thosea asigna* virus (TaV) of the *tetraviridae* virus family (Szymczak et al., 2004). This PCR product was cloned into the Plox plasmid, generating the 2APAtoh1Plox vector. Next, a similar 2AP sequence (designed from TaV) and the *Pou4f3* ORF were amplified from the IMAGE clone IRCKp5014M137Q and cloned into the 2APAtoh1Plox vector to produce the 2APPou4f32APAtoh1Plox vector. Finally, *Gfi1* ORF was amplified by PCR from the NM_010278 clone (kind gift from Dr. T. Möröy, University of Montreal) and cloned into the 2APPou4f32APAtoh1Plox vector to produce the final construct Gfi12APPou4f32APAtoh1Plox, herein named GPAPlox vector. To establish iAtoh1 and iGPA mESC lines, 20 µg of the respective Plox vector (Atoh1Plox and GPAPlox) and 20µg of a CRE expression plasmid (pTurbo-Cre) were electroporated into the Ainv15 ESC line (ATCC) (Kyba et al., 2002), and cells were selected using 350 µg/ml of G418,as previously described (Ting et al., 2005). Proper insertion of the Atoh1 and Gfi1-Pou4f3-Atoh1 cDNA was confirmed in G418-resistant colonies using PCR.

### Generation of iGPA-Myo7a:mVenus ESC line

A regulatory DNA sequence of the murine Myo7a locus (including a 2063 bp DNA fragment from the 5' region upstream of the ATG initiation codon, and a 1776 bp fragment encompassing the Myo7a exon1 and intron1) was amplified by PCR using the BAC RP23-109F24 as template. This Myo7a regulatory DNA sequence (Boeda et al., 2001) was cloned into a vector containing the cDNA for mVenus and a poly(A) signal sequence followed by a selection cassette, which includes a phospho-glycero-kinase (PGK) promoter driving transcription of a Blasticidin resistance gene. The final Myo7a-mVenusPGKBlas vector was digested with Acc65I for linearization and electroporated into the iGPA mESC line. Electroporated mESCs were grown in the presence of 3 µg/ml blasticidin and resistant colonies (around 20 to 30 individual clones) were picked and expanded for further analysis. Differentiation assays were performed for each individual clone to evaluate the capacity to drive *mVenus* expression specifically in the iMyo7a$^{+}$ population.

**mESC cell maintenance and differentiation**

All mESCs used in this study (Ainv15, iAtoh1, iGPA and iGPA-Myo7a:mVenus) were routinely grown at 37$^0$C in a 5% $CO_2$ incubator in *Dulbecco's* Modified Eagle's Medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS) (ES-qualified, Invitrogen), 2 ng/ml LIF and 1 mM 2-mercaptoethanol, on gelatin-coated (0.1%) Nunc dishes. Cells were passaged every other day, at constant plating density of 3×10$^4$ cells/cm$^2$.

For EB formation, mESCs were seeded on 60-mm bacterial-grade Petri dishes at 3×10$^4$ cells/cm$^2$, in DMEM medium in the absence of LIF. EBs formed within 24 hours, and medium was changed every 2 days. Supplementation with 2 µg/ml doxycycline (diluted in sterile PBS and filtered through a 0,2 µm filter unit) (Sigma-Aldrich), 1 µM retinoic acid (RA) (diluted in 0,01% DMSO) (Sigma-Aldrich) and 10 nM LY411575 (Lanz et al., 2004) (diluted in 0,01% DMSO) were initiated at day 4 and maintained until the required time point for analysis (day 8 or day 12). When required, 10 µM EdU (Molecular Probes) was added to cultures 30 min before fixation.

For co-cultures, 6 days old EBs untreated and treated with Dox + RA were dissociated using 0.25% trypsin-EDTA (Invitrogen) in PBS, and seeded (3.5x10$^5$ cells/cm$^2$) on top of inactivated embryonic chicken utricle periodic mesenchyme cells, in 4-well plates (Greiner). Co-cultures were grown using Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F-12, Invitrogen) supplemented with 1x B27 and N2 (Invitrogen), in the presence or absence of 2 µg/ml Dox plus 1 µM RA. Medium was changed every 2 days until analysis at day 12. Isolation and inactivation by Mitomycin C (2 µg/ml, Sigma-Aldrich) of embryonic chicken utricle periodic mesenchyme cells (E17-18) was performed as previously described (Oshima et al., 2010).

**Immunocytochemistry**

EBs (6, 8 and 12 days old) were fixed with 1% paraformaldehyde (PFA) during 15 min at room temperature (RT). After fixation, EBs were cryoprotected in 15% sucrose (Sigma-Aldrich) in PBS, embedded in a solution containing 7.5% gelatine (Sigma-Aldrich) and 15% sucrose in PBS, frozen and cryosectioned (8-10 µm). EB sections were immersed in PBS at 37$^o$C until gelatin was completely dissolved, and then processed for immunocytochemistry. Fixed cells in coverslips or de-gelatinized EBs sections were blocked with 10% FBS and 0.05% Tween in PBS for 1 hour, followed by incubation overnight (O/N) with primary antibodies (Table S2). Sections were washed 3 times in PBS followed by incubation for 1 hour at RT with AlexaFluor-conjugated secondary antibodies (Molecular Probes) and 0.15% DAPI (Sigma-Aldrich). Detection of EdU incorporation was performed according to the manufacturer's instructions, Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Molecular Probes).

Electroporated embryos were fixed with 4% PFA at 4°C O/N, cryoprotected in 30% sucrose,embedded in 7.5% gelatin:15% sucrose in PBS, frozen, and 12 µm cryostat sections were prepared. Permeabilization was performed using 0.1% Triton X-100 for 10 min, followed by blocking (10% FBS, 0.1% Tween) for 1 h at RT. Primary antibodies were incubated O/N at 4°C (Table S2). Sections were subsequently washed and incubated with appropriate Alexa Fluor-conjugated secondary antibodies (Molecular Probes) for 1 h and 0.15% DAPI.

**Imaging and cell counts**

Fluorescent and bright-field images of fixed sections and cells were captured with DM5000B microscope using a DC350F camera (Leica Wetzlar, Germany), or using Zeiss LSM510 META, Zeiss LSM 710 confocal microscopes. Living cells and EBs were photographed under an upright 2-photon Zeiss LSM 510 META, and an inverted microscope Leica DMIL with a DC200 camera. All digital images were formatted with Photoshop CS (Adobe, San Jose, CA) and ImageJ.

The number of Myo7a, Espin, Pou4f3 and Venus expressing cells was quantified as a proportion of total number of induced cells (eGFP/Venus$^+$, Atoh1$^+$ or 3 TF$^+$ cells), or total number of cells in EBs (Dapi). The number of positively labeled cells was quantified by counting 4 to 5 randomly selected fields corresponding to a minimum of 10,000 cells, counted as DAPI nuclei. Three independent experiments were counted per each condition using Photoshop CS Cell Counter software.

**Flow cytometry analysis**

For live cell analysis, EBs were dissociated and resuspended in 4% FBS in PBS. All cells were analyzed in LSR Fortessa *(*BD Biosciences). For intracellular staining, fixed cells (15 min, 1% PFA) were blocked and permeabilized for 1 hour with 0.25% saponin and 5% sheep serum in PBS, followed by incubation for 1 hour with primary antibodies for Myo7a and GFP (Table S2). Cells were washed 2 times in 0.25% saponin in PBS followed by incubation for 1 hour with AlexaFluor-conjugated secondary antibodies (Molecular Probes). Data were analyzed with FlowJo software (Tree Star).

**Scanning electron microscopy**

The co-cultures of EB-derived progenitors and mitotically inactivated chicken utricle mesenchyme treated with Dox + RA were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, with 3 mM $CaCl_2$, for 90 minutes at RT. After rinsing in buffer, cells were post-fixed in 1% $OsO_4$ in 0.1 M cacodylate buffer for 90 min. Samples were processed through the thiocarbohydrazide-$OsO_4$ repeated procedure (Davies and Forge, 1987) before dehydration in an ethanol series and critical point drying. Samples were mounted on scanning electron microscope stub and sputter-coated with

platinum. The cells were visualized in a JEOL 6700F cold field emission instrument operating (JEOL UK, Welwyn Garden City) operating at 3 or 5 kV.

**FM1-43 uptake assay**

Stock solutions of 3 mM FM1-43 [*N*-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide) (Molecular Probes) were dissolved in water. To assess iHC fast permeation to the FM1-43 dye, co-cultures of inactivated utricle mesenchyme and iGPA-Myo7a:mVenus-derived progenitors, untreated or treated with Dox + RA for 8 days, were washed twice with Hanks' Balanced salt solution (HBSS, Invitrogen) and exposed for 60 seconds to HBSS solution containing 6µM of FM1-43. Live cells were immediately washed three times in HBSS solution and observed on an upright confocal microscope (Zeiss LSM 510 Meta) using a 40X water immersion lens. Several images were captured from live cultures in random fields immediately after dye application.

**Real-time quantitative PCR**

Real-Time quantitative PCRs were carried out using iTaq Universal Sybr Green Supermix (Bio-Rad) on 7500 Real-Time and ViiA 7 Real-Time PCR systems (Applied Biosystems). Primers were designed using Primer3 program followed by BLAST searches to confirm their specificity. All quantitative PCR data were performed with at least 2 repeats. The PCR products were confirm by proper melting curves and agarose-gel electrophoresis. The relative amount of each transcript was normalized to the level of GAPDH. Relative expression levels in the various Dox-treated samples were referred to the levels of expression in control untreated (without Dox) which were arbitrarily set to 1. The primer pairs sequences (forward and reverse) used: Myo7a (5'-AATCACATCAGGTACAGCGAAGA-3', 5'-CGGGGAAGTAGACCTTGTGGA-3'), Cdh23 (5'-AACAGCACAGGCGTGGTGA-3', 5'-TGGCTGTGACTTGAAGGACTG-3'), Espin (5'-GGTCTCAGCCACTGCTCAATG-3', 5'-GAATGTCTCGTCTCCAGGCAG-3'), Lhx3 (5'-AACAACAGTAACGCCTTGCTT-3', 5'-CACACGGCATTCCAGAACAG-3'), Myo6 (5'-GAGAGGCGGATGAAACTTGAGA-3', 5'-CTTCGGAGTGCCATGTCACC-3'), Atoh1 (5'-ATGCACGGGCTGAACCA-3', 5'-TCGTTGTTGAAGGACGGGATA-3'), Pou4f3 (5'-GCAAGAACCCAAATTCTCCA-3', 5'-TAGATGATGCGGGTGGATCT-3'), Gfi1 (5'-AGGAACGCAGCTTTGACTGT-3', 5'-TGAGATCCACCTTCCTCTGG-3'), Sox2 (5'-ATGGACAGCTACGCGCAC-3', 5'-CGAGCCGTTCATGTAGGTCTG-3'), Nestin (5'-CTGGAACAGAGATTGGAAGGCCGCT-3', 5'-GGATCCTGTGTCTTCAGAAAGGCTGTCAC-3'), Tuj1 (5'-AAGGTAGCCGTGTGTGACATC-3', 5'-ACCAGGTCATTCATGTTGCTC-3'), GAPDH (5'-ATTCAACGGCACAGTCAAGG-3', 5'-TGGATGCAGGGATGATGTTC-3').

**Microarray sample preparation and data analysis**

Total RNA was extracted from untreated EBs at day 8 and day 12, and from FACS-sorted EBs at day 8 and 12 treated with Dox or Dox + RA. All cells were derived from the iGPA-Myo7a:mVenus ES line. Cell sorting of Venus$^{+}$ populations were done on a FACS Aria cell sorter (Becton Dickinson). RNA concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) Mouse Genome 2.1 ST Arrays Strip by using the Ambion WT Expression Kit (Life Technologies, CA, USA) and Affymetrix GeneChip WT Terminal Labeling Kit, according to the manufacturer's protocols. Briefly, 100 ng of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit, Affymetrix) was used in a reverse transcription reaction (Ambion WT Expression Kit) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate cRNA (Ambion WT Expression Kit). 15 µg of this cRNA was used for a second cycle of first-strand cDNA synthesis (Ambion WT Expression Kit). 5.5 µg of single stranded cDNA was fragmented and end-labeled (GeneChip WT Terminal Labeling Kit, Affymetrix). Size distribution of the fragmented and end-labeled cDNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay. 3.5 µg of end-labeled, fragmented cDNA was used in a 150 µl hybridization cocktail containing added hybridization controls (GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips, Affymetrix), of which 120 µl were hybridized on array strips for 20 h at 48°C. Standard post hybridization wash and double-stain protocols (GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips, Affymetrix) were used on an GeneAtlas system (Affymetrix), followed by scanning of the array strips.

The 16 scanned arrays were analyzed first with Expression Console software (Affymetrix) using RMA to obtain expression values and for quality control. Control probe sets were removed and log2 expression values of the remaining 33710 transcripts were imported into Chipster 2.4 (Kallio et al., 2011). Differential expression was determined by empirical Bayes two-group test (Smyth, 2004) with Benjamini-Hochberg multiple testing correction and a p-value cut-off of 0.01. Gene ontology (GO) analysis was performed by the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/).

**Chicken embryo *in ovo* electroporations**

Fertilized White Leghorn chicken (Gallus gallus) eggs were incubated at 38$^{0}$C and 30–80% humidity to HH12 stage (Hamburger and Hamilton, 1992). *In ovo* electroporation of the chicken otic cup was performed as described previously (Freeman et al., 2012). The vectors pCAGGS-T2TP (encodes a transposase controlled by a CAGGS promoter)

(Takahashi et al., 2008) and pT2K-CAGGS-rtTA-M2, (for constitutive expression of a tetracycline-on activator located between the left and right ends of *Tol2*) (Takahashi et al., 2008) were co-electroporated with either pT2K-TRE-B1-H2BeGFP (consists of a bidirectional tetracycline-responsive element (TRE) controlling transcription of both eGFP fused with histone 2B (H2B) and a empty transcriptional unit) (Takahashi et al., 2008) or pT2K-TRE-B1-H2BeGFP-GPA (herein named TRE:GPA-eGFP), which is a modified version of pT2K-TRE-B1-H2BeGFP in which the ORF of Gfi1-2AP-Pou4f3-2AP-Atoh1 was directionally cloned downstream of the empty transcriptional unit of the vector multiple cloning site. At HH24 embryos, doxycycline (30 µ g per embryo, Sigma-Aldrich) was administered *in ovo* and embryos were harvested 2 or 4 days later.

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