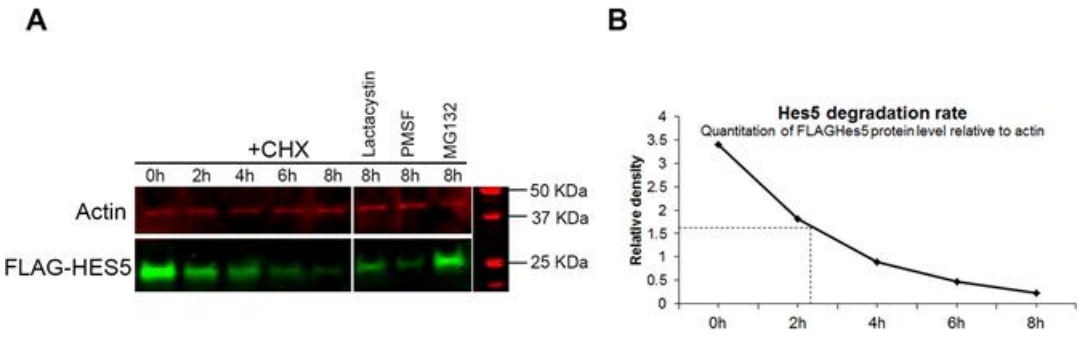

Fig. S1, related to Fig. 1. HES5 protein stability *in vitro*. 293 cells were transfected with CMV-FLAG-Hes5 plasmid. After 24h cells were treated with cycloheximide (CHX) alone, or cycloheximide and proteasome inhibitors, and collected at the indicated time points. Whole-cell extracts were made according to (Gallagher, 2007) and western blotted with anti-FLAG antibody (A). The intensity of the signals relative to actin was measured using Image J (NIH) (B). Values shown in B are average of two independent experiments. HES5 is degraded with a half-life of 2.5 hours. Proteasome pathway inhibitors MG132 or Lactacystin increased HES5 stability, while the protease inhibitor PMSF had no effect.

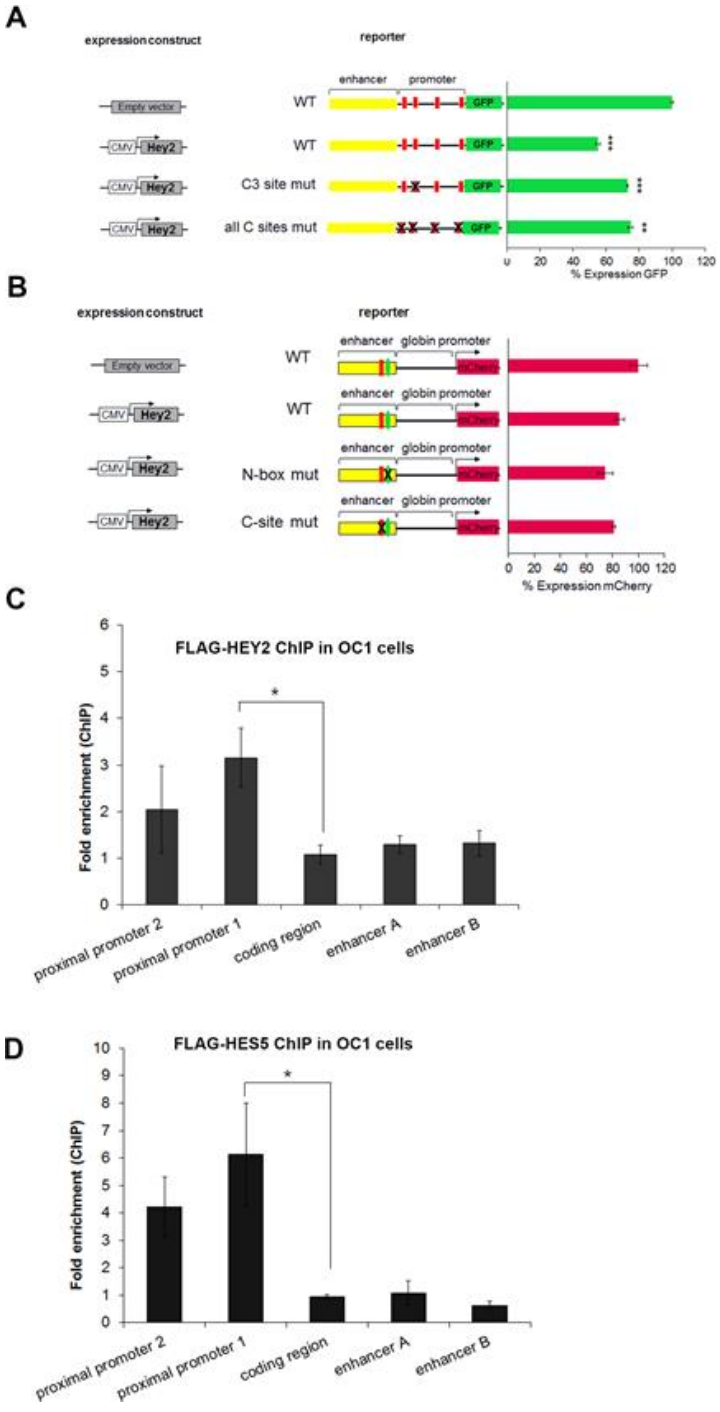

Fig. S2, related to Fig. 2. HEY2 is capable of repressing Atoh1 transcription through both the promoter and enhancer regions. Quantification of flow cytometry analysis in 293 cells transfected with the indicated plasmids. **(A)** The promoter C-sites were mutated as described in Fig. 2. Hey2 repression of Atoh1 promoter was dependent on the conserved C3 site. Mutation of additional C-sites

only slightly increased the percentage of GFP^+ cells, suggesting that part of HEY2-mediated repression of Atoh1 is likely independent of the C-sites in the promoter region. Values are mean ± SEM, n = 3; [***] P < 0.0005, [**] P < 0.005, Student's t-test. **(B)** The C-site and N-box in the Atoh1 3' enhancer sequence were mutated by site directed mutagenesis (Methods). Hey2's weak inhibitory effect (as seen in A) is not dependent on the N-box or C-site within the Atoh1 enhancer. Values are mean ± SEM, n = 3. **(C and D)** HEY2 binds to Atoh1 promoter *in vitro* similar to HES5. ChIP-qPCR result in OC1 (organ of Corti 1) cells that were transfected with CMV-FLAG-Hey2 (C), CMV-FLAG-Hes5 (D) plasmid or CMV-GFP plasmid (control) for 48 hours. Chromatin immunoprecipitation was performed with anti-FLAG antibody. The result is reported as fold enrichment (Hey2 or Hes5 transfected % input/GFP transfected % input). Shown are the values as mean ± SEM for three independent replicates. [*] $p < 0.05$, Student's *t*-test.

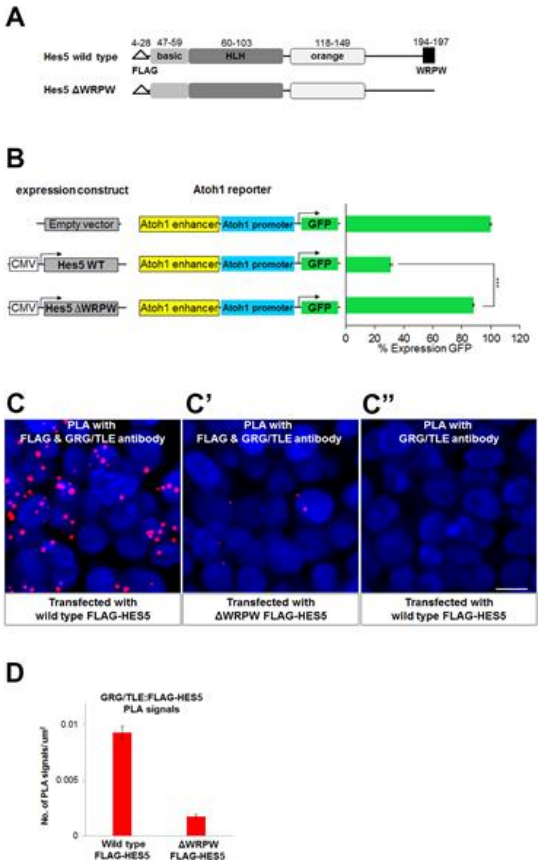

Fig. S3, related to Fig. 5. The HES5 WRPW motif is required for the repression of the *Atoh1* promoter and for interaction with GRG/TLE. (A) Schematic representation of mouse HES5 protein showing the amino acid sequence and different domains. Four amino acids of the C-terminal WRPW motif were deleted in Hes5 ΔWRPW**. (B)** Quantification of flow cytometry analysis of 293 cells transfected with the indicated plasmids reported as a percentage of cells expressing GFP relative to RFP control (not shown, empty vector control set to 100%). When Hes5 C-terminal motif WRPW was deleted, HES5 inhibitory effect on Atoh1 reporter was significantly reduced. Values are mean ± SEM, n = 3. [***] $p < 0.0005$. **(C)** Proximity ligation assay showing requirement for the WRPW motif for interaction with endogenous GRG/TLE. Confocal images of 293 cells transfected with FLAG-Hes5 wild type or WRPW-deleted expression plasmids (red dots indicate interaction). DAPI (blue). Scale bar = 20 µm. **(D)** Quantification of PLA signals shown in (C).

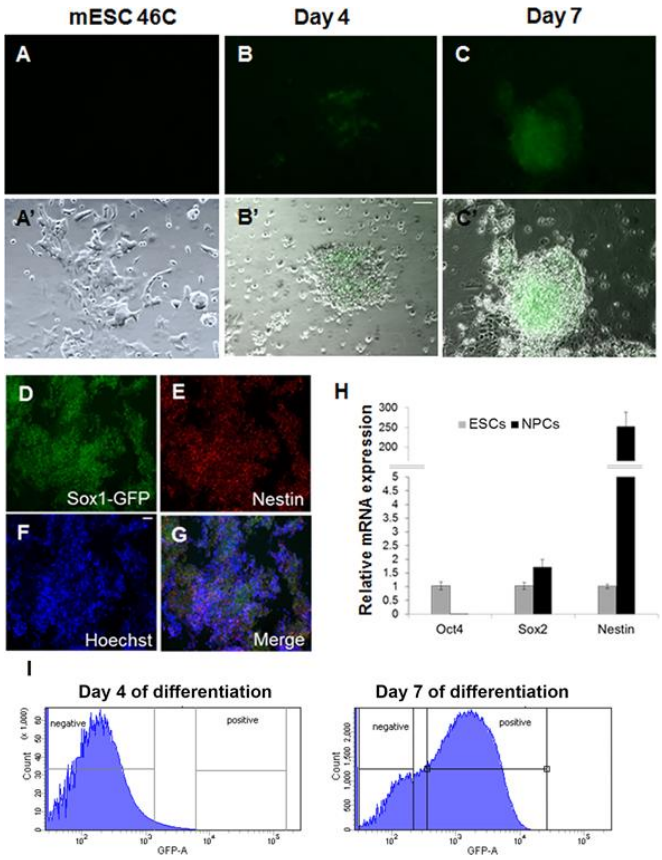

Fig. S4, related to Fig. 5. Sox1-GFP knock-in mouse embryonic stem cell (mESC) line 46C expresses GFP and Nestin upon neural differentiation. ESCs (line 46C) were cultured and differentiated to neural progenitor cells (NPCs) following a monolayer cell culture protocol (Ying and Smith, 2003; Ying et al., 2003). The expression of GFP allows monitoring of the differentiation procedure and also FACS-purification to obtain a highly purified population of NPCs. **(A-C)** Fluorescent and **(A'-C')** brightfield images of differentiation of mES cells. mESCs were cultured without (A and A') and with differentiation media for 4 days (B and B'), or 7 days (C and C'). (**D-H**) ESCs in differentiation media downregulate Oct4 and upregulate Nestin by day 7 as shown by immunostaining (D-G), and real time quantitative PCR (H). **(I)** The fluorescence activated cell sorting (FACS) analysis of 46C ES cells on day 4 and 7 of differentiation. As the cell differentiate, the percentage of Sox1-GFP poisitive cell increases. Scale bars = 50 µm.

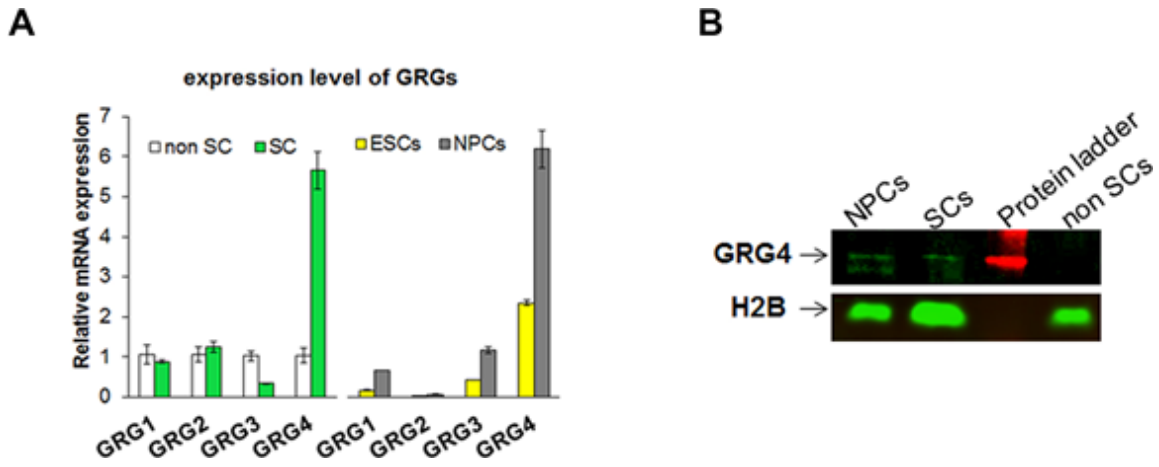

Fig. S5, related to Fig. 5. (A) GRG4 is the predominant family member expressed in supporting cells and neural progenitor cells (NPCs). qPCR analysis of FACS-purified supporting cells ($p27^+$ SC), non-supporting cells ($p27^-$ non SC), 46C ES cells and NPCs. n = 4. Values are mean ± SEM. **(B)** GRG4 is expressed in NPCs and supporting cells. Immunoblotting of GRG4 with 25,000 FACS-purified NPCs, purified $p27^+$ supporting cells (SCs) and $p27^-$ non-supporting cells (non SCs) at P1. Histone H2B was used as control.

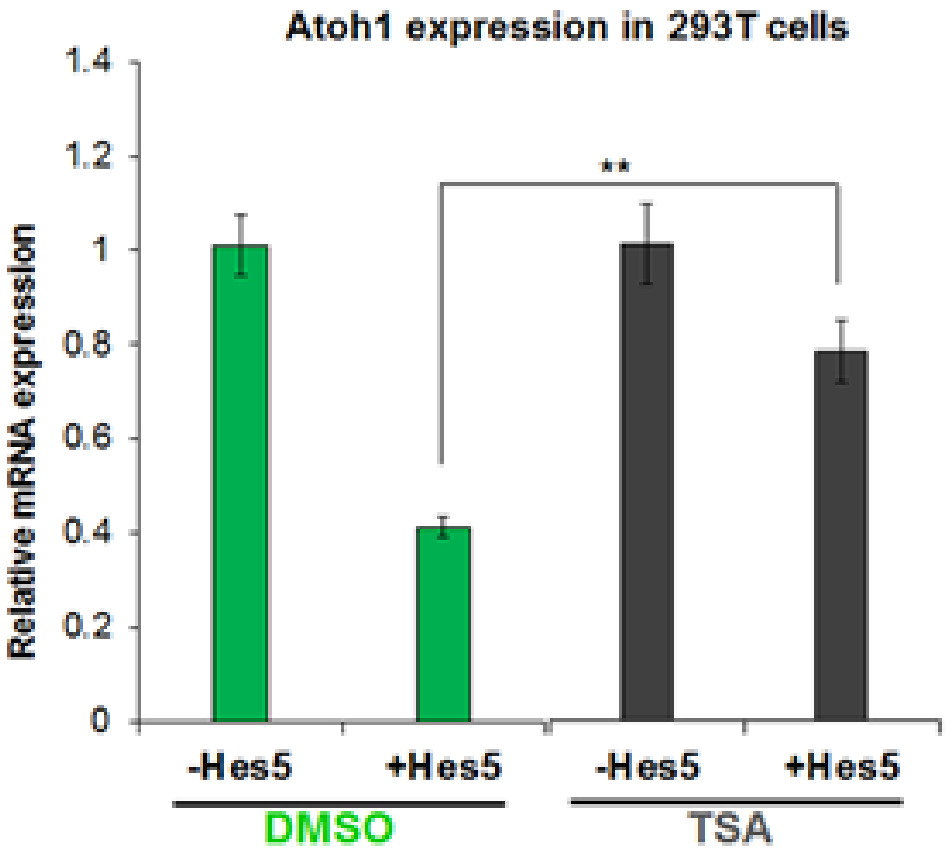

Fig. S6, related to Fig. 6. HES5 cannot repress Atoh1 expression in the absence of HDAC activity. Hes5 overexpression in 293 cells in the absence and presence of HDAC inhibitor trichostatin A (TSA) for 15h. Values are mean ± SEM, n = 3; [**] P< 0.001, Student's *t*-test.

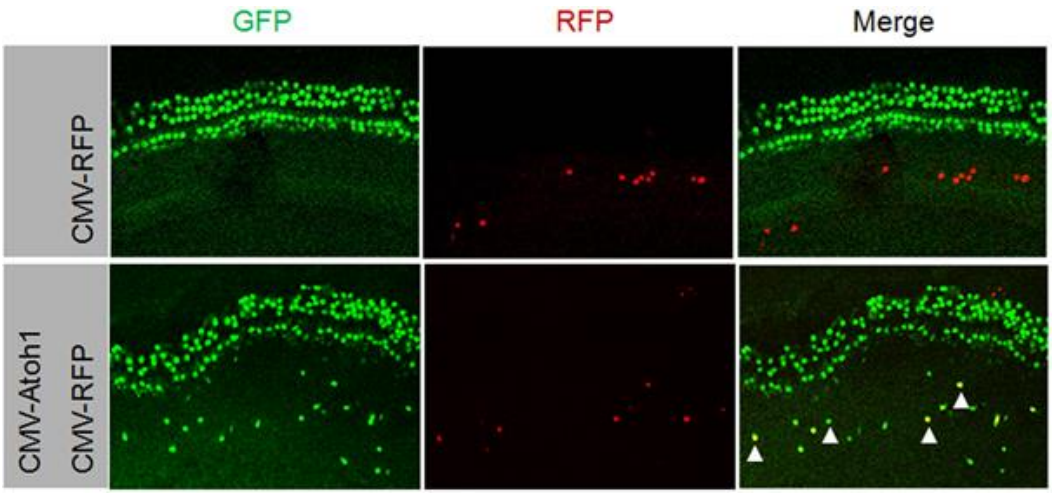

Fig. S7, related to Fig. 7. The Atoh1 enhancer acts as an autoregulatory element in response to ectopic expression of Atoh1. A CMV-RFP expression plasmid with or without an Atoh1 expression plasmid was electroporated into GER (greater epithelial ridge, epithelial cells lying medial to the organ of Corti) of Atoh1 enhancer/β-globin promoter/GFP transgenic mouse at P1. Overexpression of Atoh1 in this area, where Atoh1 is not normally expressed, induced the Atoh1 enhancer activity in the transgene and resulted in the appearance of GFP-positive cells in the GER. This confirms the autoregulatory activity of the Atoh1 enhancer (Helms et al., 2000), and suggests the presence of a positive feedback loop in the organ of Corti.

Supplementary Materials and Methods

Embryonic stem cell culture and differentiation to neural progenitor cells (NPCs)

46C ES cells were cultured on gelatin-coated tissue culture plates in GMEM (Invitrogen) supplemented with 15% Embryonic Stem (ES) Screened FBS (HyClone), 1 mM sodium pyruvate (Invitrogen), non-essential amino acid (Invitrogen), 0.1% 2-Mercaptoethanol (Invitrogen) and 10 ng/ml leukemia inhibitory factor (LIF) (Millipore). For neural differentiation, 46C cells were cultured on gelatin-coated 6-well plates at a density of $1\times10^4/cm^2$ (10^5 cells per well). Culture medium was DMEM/F12 combined 1:1 with Neurobasal medium (Invitrogen) supplemented with 1% B27 (Invitrogen), 0.5% N2 (Invitrogen), 25 µg/ml bovine serum albumin (Sigma). Medium was changed every other day for six days. On day six the medium was changed to DMEM/F12 combined 10:1 with Neurobasal medium supplemented with 0.1% B27, 0.5% N2 and 20 ng/ml FGF and EGF (R&D).

Plasmids and reporter constructs

Hes5 expression plasmids:

The pCS2-CMV-FLAG-Hes5 expression plasmid included 1.4 kb Hes5 coding region (generously provided by Verdon Taylor (Basak and Taylor, 2007), University of Basel, Switzerland) tagged by 5' 3X FLAG epitope inserted downstream of CMV enhancer/promoter in pCS2. Cloning details upon request.

Atoh1 reporter plasmids:

The Atoh1 enhancer/β-globin promoter/GFP plasmid, in which the mouse Atoh1 enhancer (GenBank: AF218258.1) and the human β-globin basal promoter drive the expression of GFP, was a generous gift from Jane Johnson, University of Texas (Lumpkin et al., 2003). The mCherry reporter (Fig. 2) was constructed by cloning a histone H2B-mCherry fusion into the Atoh1 enhancer/β-globin

promoter/GFP plasmid to replace the GFP coding region. Atoh1 enhancer/Atoh1 promoter/GFP reporter was constructed using a 226 bp fragment upstream of the *Atoh1* transcription start site, and 177 bp of *Atoh1* 5'-UTR to replace the β-globin promoter sequence in Atoh1 enhancer/β-globin promoter/GFP. For the Atoh1 enhancer/Atoh1 promoter/tdTomato reporter, tdTomato sequence was PCR amplified from FUtdTW (a gift from Connie Cepko (Rompani and Cepko, 2008), Addgene plasmid # 22478) and inserted in the Atoh1 enhancer/Atoh1 promoter/GFP reporter to replace the GFP sequence. Expression of the reporters carrying the Atoh1 autoregulatory enhancer element (Fig. S7) was observed in 293 cells, likely as a result of low level *Atoh1* expression as previously reported (Neves et al., 2012).

Hey2 expression plasmid (CMV-FLAG-Hey2) was generously provided by Manfred Gessler (University of Wuerzburg, Germany).

Flow cytometric analysis of reporter assays

48h post-transfection of 293 cells were suspended in DMEM/10% FBS, passed through a 40 µm cell strainer and analyzed using BD FACSAria II cytometer with lasers at 488- and 561-nm wavelengths and 100 µm nozzle. Cells were initially gated (P1) using forward scatter (FSC-A) and side scatter (SSC-A). Two sequential gates were used to exclude the cellular debris and clumps (P2: SSC-H vs. SSC-W, P3: FSC-H vs. FSC-W). Untransfected and single-transfected (GFP, mCherry or RFP) cells were used to determine the gates for the positive and negative populations, as well as to set up the compensation. For each sample about $1x10^5$ cells in gate P3 were analyzed. BD FACSDiva software was used to operate the system and analyze the cell populations. Two-tailed Student's t-test was used to determine the statistical significance.

Western blotting

Proteins extracted from transfected 293 cells were separated under reducing conditions on 12% NuPage Novex Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Millipore) using the XCell II Blot Module (life technologies). Membranes were blocked using Odyssey blocking buffer (LI-COR Biosciences) and probed with the following antibodies: anti-FLAG (1:1000, Sigma F7425); anti-actin (1:2000, Sigma A3853); anti-TLE4 (1:1000, Novus NB100-92363); anti-histone H2B (1:2000, Millipore 07-371); IRDye 800CW Goat anti-Rabbit IgG (1:20000); and IRDye 680RD Goat anti-Mouse IgG (1:10000) (LI-COR).

Proximity ligation assay

To visualize GRG/TLE:FLAG-HES5 interaction, proximity ligation assay (Soderberg et al., 2006) was performed using the Duolink kit (Sigma-Aldrich) in transfected 293 cells according to manufacturer's manual with the following antibodies: anti-FLAG (1:500, Sigma F7425); anti-pan GRG/TLE (1:100, Santa Cruz sc-13373 X).

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP experiments were done according to (Dahl and Collas, 2008). FACS-purified NPCs were fixed with 1% formaldehyde for 10 minutes and quenched by 125 mM Glycine for 5 minutes at room temperature. Cross-linked cells were centrifuged at 470g for 10 minutes at 4°C, washed twice with ice-cold PBS, lysed with 50 µl lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% (wt/vol) SDS, PMSF and protease inhibitor cocktail for 8 minutes on ice. RIPA buffer (10 mM Tris–HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 0.1% (wt/vol) Na-deoxycholate, protease inhibitor cocktail and PMSF) was added before sonication. Chromatin was sonicated to an average size of 200-300bp with High Intensity Ultrasonic Processor (50 Watt Model) on ice for 4 X 30s, with 30s intervals. After sonication, samples were centrifuged at 12000g for 10 minutes

at 4°C and 1% of the supernatant chromatin was set aside as input. The remaining chromatin was added to antibody-Dynabeads protein G complexes (4 µg FLAG antibody (Sigma F1804) or GRG/TLE antibody (Santa Cruz sc-13373 X) and 25 µl protein G beads (Invitrogen) were preincubated on a rotator for 2h at 4°C). The tubes containing the chromatin-antibody-protein G and the input tubes were placed on a rotor for 16h at 4°C. The beads were then captured in magnetic rack, washed with RIPA and TE. The washed beads were reverse cross-linked with elution buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% (wt/vol) SDS, 50 µg/ml proteinase K) at 1300 rpm for 4h at 68°C. ChIP and input DNA was purified by phenol–chloroform–isoamyalcohol extraction and ethanol precipitation and dissolved in TE. qPCR reactions were done in triplicate. H3K9ac ChIP with about 25,000 supporting cells (from p27/GFP mouse line) per experiment was done as above with minor differences. FACS-purified supporting cells were cross-linked for 8 minutes and sonicated for 8 X 30s, with 30s intervals. 2.4 µg H3k9ac antibody (Active Motif 39137) and 10 µl Dynabeads Protein A (Invitrogen) per sample were used. ChIP-qPCR primers are listed in table 2.

Table S1. Primers used in real time quantitative PCR (qPCR).

Gene	Forward (5'→3')	Reverse (5'→3')
Atoh1	GAGTGGGCTGAGGTAAAAGAGT	GGTCGGTGCTATCCAGGAG
Hes5	GCACCAGCCCAACTCCAA	GGCGAAGGCTTTGCTGTGT
GFP	CTGCTGCCCGACAACCA	TGTGATCGCGCTTCTCGTT
Gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
Hey1	CACTGCAGGAGGGAAAGGTTAT	CCCCAAACTCCGATAGTCCAT
Oct4	ACATCGCCAATCAGCTTGG	AGAACCATACTCGAACCACATCC
Nestin	GCTGGAACAGAGATTGGAAGG	CCAGGATCTGAGCGATCTGAC
Sox2	CTGTTTTTTCATCCCAATTGCA	GGAGATCTGGCGGAGAATA
GRG1	GACAGCCTAAGAGGCACAGAT	GGTCCTCGTTAGACACATCCA
GRG2	TGAGGACCAACCGTCAGAG	GCTGGACTGTCTGTGAGGT
GRG3	TGGATGTCTCTAATGAGGACCC	TTCAGACCACGGGCTTTGTC
GRG4	ATTGCAGCTCGCTATGACAGT	GAGGAGTCGTGTCTTGTCCAG
hAtoh1	TTGTCCGAGCTGCTACAAACG	GAGAAGCGAGTCCGGCAAC
hGAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

Table S2. Primers used in ChIP-qPCR. Numbers refer to the 5' end of the forward primer.

Primer	Forward (5'→3')	Reverse (5'→3')	Amplicon size (bp)
Atoh1-proximal promoter 2 (-354)	CCCTCACTCAGGTCGCCTG	CGTGCGAGGAGCCAATCA	205
Atoh1-proximal promoter 1 (-87)	GGGGAGCCGGGGGAGATACAC	ACCAGGTCGCGTGCAACGAAG	93
Atoh1-distal promoter (-1130)	ACAGAGCGGGACAGGTGGGT	CCTCGGGAGGCCCCGGTTTA	86
Atoh1-coding region (+1156)	ACATCTCCCAGATCCCACAG	GGGCATTTGGTTGTCTCAGT	119
Atoh1 enhancer-B (+5346)	AGAGCGGCTGACAATAGAGG	GTGCGCTCACCAGCTGAC	93
Atoh1 enhancer-A (+4264)	CACACCCCATTAACAAGCTG	GTCTGGCATATGGGGAATGA	112
Gapdh (+32)	GGGTTCCTATAAATACGGACTG	CTGGCACTGCACAAGAAGA	90

Table S3. Genotyping Primers.

Gene	Forward (5'→3')	Reverse (5'→3')
GFP	GTGAAGTTCGAGGGCGACAC	CGGACTGGGTGCTCAGGTAG
tdTomato	GTGACCGTGACCCAGGACTC	TGACGGCCATGTTGTTGTCCTC

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