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Supplemental Information

NEUROG1 Regulates CDK2 to Promote Proliferation in Otic Progenitors

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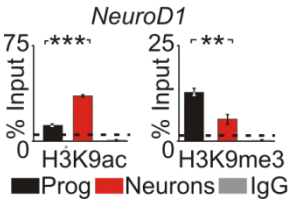
Fig.S1 H3K9ac and H3K9me3c hanges at the alternative *NeuroD1* promoter, related to Fig. 2
ChIP-qPCR of H3K9ac and H3K9me3 at the second *NeuroD1* TSS. (A) Enrichment of H3K9ac or H3K9me3 marks at the *Cdk2* promoter region in proliferating iMOP (n=3) and iMOP-derived neurons (n=3). Background levels were determined by ChIP-qPCR with non-specific rabbit IgG antibody (n=3). Error bars are depicted as ± SEM.

Fig.S2 NEUROG1 expression and binding, related to Fig. 3
(A) Western blot of NEUROG1 and ACTB in proliferating and differentiating PB-T-EGFP -dox and PB-T-*Neurog1* –dox cultures (n=3 for each condition). (B) Normalized NEUROG1 levels (n=3 for each condition). (C) Relative levels of NEUROG1 from PB-T-*Neurog1* cells cultured in 0, 0.5 and 1 µg/ml of dox (n=3 for each condition). (D) Enrichment of NEUROG1 at the *Pou5f1* promoter in PB-T-EGFP and PB-T-*Neurog1* cells cultured in 1 µg/ml dox (n=3 for each condition). Error bars are depicted as ± SEM.

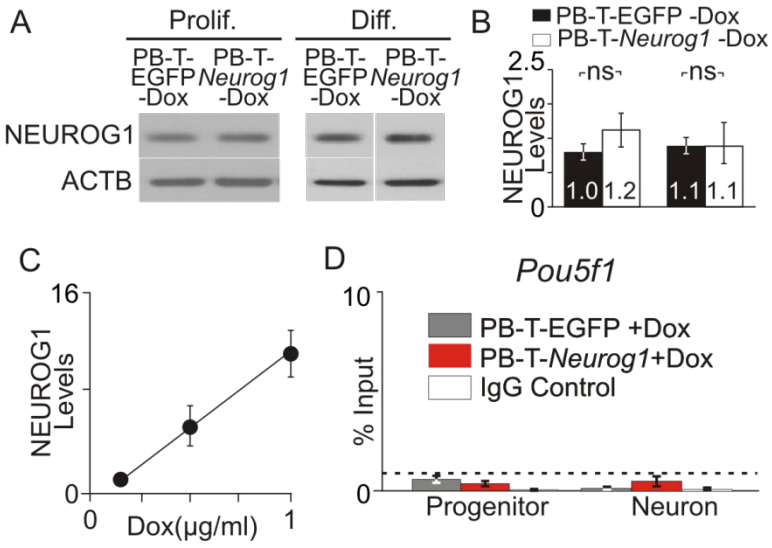
Fig.S3 Labeling iMOP cells with NucRed, related to Fig. 4
NucRed and Hoechst labeling of cells from (A) PB-T-EGFP –dox (n=3), (B) PB-T- EGFP +dox (n=3), (C) PB-T-*Neurog1* -dox (n=3) and (D) PB-T-*Neurog1* +dox (n=3) cultures. (E) Western blot of NEUROG1 and ACTB in proliferating PB-T-*Neurog1* –dox (n=3) and PB-T-*Neurog1* +dox (n=3) cultures. (F) Normalized NEUROG1 levels for PB-T-*Neurog1* –dox (n=3) and PB-T-*Neurog1* +dox (n=3) cultures.

Fig.S4 Cell counts and EdU labeling in iMOP cells cultured in CDK inhibitor, related to Fig. 6
(A) Relative percent of iMOP cells after treatment with 0-5 µM of K03861 (n=5 for each condition). (B) Relative percent of cells after treatment with 0, 0.5 and 1 µM of K03861 (n=5 for each condition). (C) Relative percent of iMOP cells after treatment with 0-1 µM of roscovitine (n=5 for each condition). (D) Relative percent of cells after treatment with 0, 0.1, 0.5 and 1 µM of roscovitine (n=5 for each condition). Hoechst and EdU labeled PB-T-*Neurog1* +dox cells in (E) control (n=3) and (F) 1 µM roscovitine (n=3) treated cells.

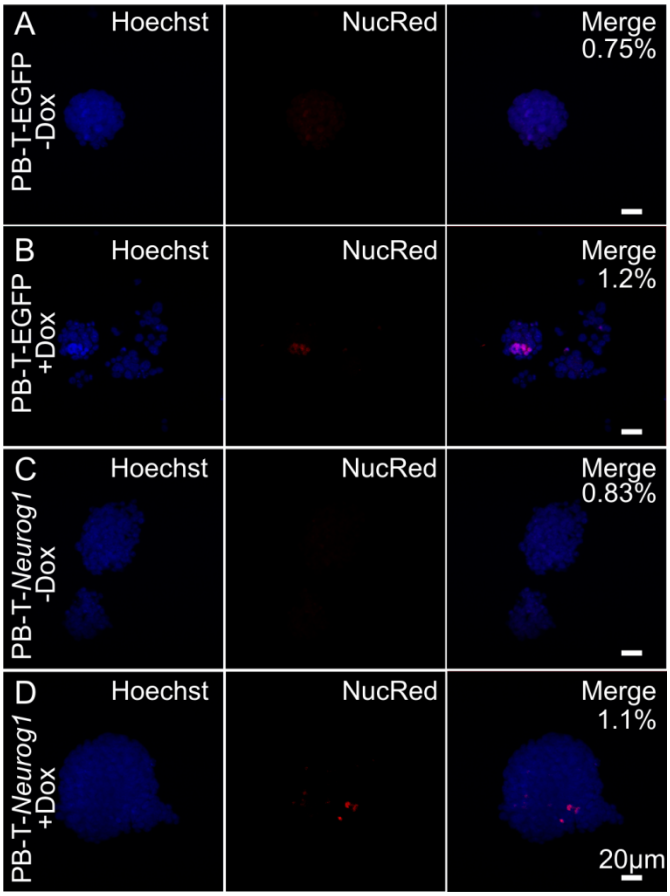
Supplemental Fig. 1
associated with Fig.2

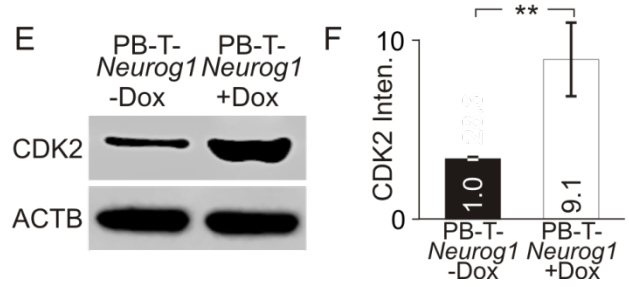


Supplemental Fig. 2
associated with Fig.3



Supplemental Fig.3
associated with Fig.4





Supplemental Fig.4
associated with Fig.6

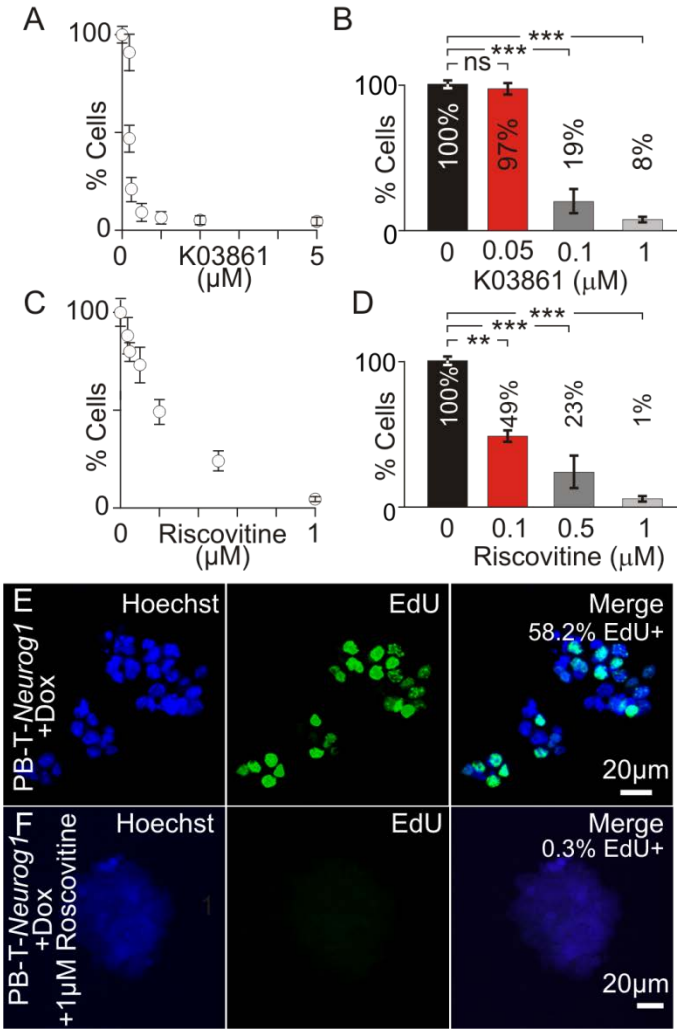


Table S1: Antibodies used for ChIP-seq, ChIP PCR, Western blot and immunostaining

Antibody	Company	Catalog #	Application	Amount
NEUROG1	EMD Millipore	AB15616	Western Blot	1:1000 dilution
CDK2	Santa Cruz	sc-6248	Western Blot	1:1000 dilution
ACTB	Santa Cruz	sc-1615	Western Blot	1:1000 dilution
NEUROD1	EMD Millipore	AB15580	Immunostaining	1:1000 dilution
TUBB3	BioLegend	801202	Immunostaining	1:1000 dilution
H3K4me3	Active Motif	39159	ChIP-seq	5 μg /IP
H3K27me3	Upstate	05-851	ChIP-seq	5 μg /IP
H3K9ac	Active Motif	39137	ChIP PCR	10ul/IP
H3K9me3	EMD Millipore	07-442	ChIP PCR	5 μg /IP

Table S2: Primers used for ChIP-qPCR

Primer	Forward primer sequence(5'-3')	Reverse primer sequence(5'-3')
Cdk2	CCTCTCCAATCTTCTCCACCTTT	AGCTCTCCTTGCGTTCCATC
NeuroD1	GATCTCATAACCCTGGAGCCT	AGCATCAGCAACTCGGCTAT
Pou5f1	GGATTGGGGAGGGAGAGGTGAAACCGT	TGGAAGCTTAGCCAGGTTCGAGGATCCAC
ActB	GTGGCTGCAAAGAGTCTACA	GGATCACTCAGAACGGACAC

Table S3: Primers used for qPCR

Primer	Forward primer sequence(5'-3')	Reverse primer sequence(5'-3')
Neurog1	CACCATGCCTGCCCCTTTGGAGACCT	CTAGTGGTATGGGATGAAACAGGGC
Cdk2	CCTGCTTATCAATGCAGAGGG	TGCGGGTCACCATTTCAGC
*NeuroD1*_1	ATGACCAAATCATACAGCGAGAG	TCTGCCTCGTGTTCCTCGT
*NeuroD1*_2	GGACTGGTAGGAGTAGGGATG	CTCAACCCTCGGACTTTCTTG
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table S4: Neurog1 shRNA Sequences

shRNA_1	CGTCGCGTCAAAGCCAACGAT
shRNA_2	TCTCGACTGCTCCAGCAGCAA
shRNA_3	CGGCCAGCGACACTGAGTCCT

Extended Experimental Procedures

Cell Culture for iMOP cells

iMOP cells were cultured as described (Jadali et al., 2016). For all experiments, iMOP cells were dissociated to initiate the experiment. For proliferating iMOP cells, cultures were analyzed 3 days after dissociation to allow cells to recover and start proliferating. For iMOP-derived neurons, cultures were maintained in neurobasal medium 7 days after dissociation to allow cultures to reach ~90% TUBB3 labeled cells before immunostaining or harvesting for molecular experiments. Cells were analyzed at these time points unless otherwise stated.

Generation of stable iMOP cell lines

iMOP doxycycline inducible stable cell lines were generated using the piggyBAC transposon system (Li et al., 2013). Clones were generated using Gateway technology. Entry clones containing EGFP and a bicistronic construct with *Neurog1* followed by an IRES EGFP were generated. Inserts from the entry clones were introduced into the PB-T expression vector. The expression vector contains a Tet response element (TRE) controlling the expression of target gene as well as a puromycin resistance gene for selection. To generate the stable cell line, the PB-T expression vector were co-transfected into cells with a plasmid encoding the reverse tetracycline transactivator (rtTA) containing a blasticidin resistance gene along with a plasmid overexpressing the piggyBAC transposase (PBase). The PB-T and TRE plasmids contain inverted terminal repeat sequences (ITRs) that are recognized by PBase. Transient expression of the PBase mediates stable integration of plasmid DNA from PB-T and TRE into the genome. After co-transfection of these three plasmids, step-wise selection with up to 10 μg /ml of puromycin and 5 μg /ml of blasticidin was used to select for iMOP cells that incorporated the PB-T and TRE elements. Proliferating iMOPs were cultured in doxycycline (dox) for 3 days and iMOP-derived neurons were cultured in dox for 7 days unless otherwise stated.

Western blot analysis

For quantitative Western blots, cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 10% glycerol) containing phosphatase inhibitor (Thermo

Scientific) and a mixture of protease inhibitors (Roche). Protein lysates (30 µg) were loaded and separated on 4-12% Bis Tris Novax NuPAGE gradient gels (Life Technologies), transferred to nitrocellulose membrane (GE Healthcare). The membrane was dried overnight and incubated in blocking buffer (1X PBS containing 5% nonfat dried milk) for 1 hr, followed by incubation with primary antibodies. Primary antibodies were detected using IRDye 800CW or IRDye 680RD conjugated secondary antibodies (LI-COR Biosciences). Fluorescence from the membrane was acquired using the Odyssey imaging system quantified using the Image Studio software.

ChIP-seq and ChIP-qPCR

ChIP-seq was accomplished as described (Kwan et al., 2015) using antibodies listed in Table S1. For H3K4me3, H3K27me3 and POLR2A, proliferating iMOP cells were used for ChIP-seq. Biological replicates for ChIP-seq was done and sequencing was performed on the Illumina Hi-seq 2000. ChIP PCR was done as described with minor modifications (Dahl and Collas, 2008a, b). Individual ChIP was performed using ~2 million cells using the established protocol. Self-renewing iMOP progenitors and iMOP-derived neurons were fixed with 1% formaldehyde in 1X PBS for 8 min and quenched with 125mM glycine for 5 min at RT. Cells were then rinsed 2 times in ice-cold PBS containing complete protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and collected. Cells were pelleted and either stored at -80°C until use or immediately processed. Cell pellets were lysed in 20 cell pellet volumes (1 ml) of L1 buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10 % Glycerol, 0.5 % NP-40, 0.25 % Triton X-100, complete protease inhibitor cocktail) for 10 min at 4 °C. Nuclei were then pelleted by centrifugation at 3000 rpm for 10 min at 4°C. The isolated nuclei were rinsed with 20 cell pellet volumes of L2 buffer (200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, complete protease inhibitor cocktail) for 10 min at RT and re-pelleted. Nuclei were resuspended in 4 cell pellet volumes of L3 buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0 and complete protease inhibitor cocktail) and sonicated using Bioruptor Pico. Sonication was performed for 24 cycles with of 30s on and 30s off to produce DNA fragments around 500 bp. Insoluble material was removed by centrifugation at 20,000g for 10 min at 4°C.

The final volume of the resulting nuclear lysate was adjusted to 1 mL with L3 buffer containing 0.3 M NaCl, 1 % Triton X-100, and 0.1% Na deoxycholate. For input DNA control, the DNA was obtained from 50 µl of nuclear lysate. For each immunoprecipitation, 100 ul of nuclear lysate was used. Antibodies were incubated with 10 µl Dynabead Protein A for 2 hrs, before addition of lystates for an overnight incubation at 4°C. For the negative control, IgG control antibodies were added to the lysate. Dynabeads were washed 3-6 times with RIPA buffer depending on the antibody used. To de-crosslinking and elute DNA from the Dynabeads, samples were incubated at 68°C for 4 hrs, treated with Proteinase K for 2 hrs at 42°C. DNA was purified using the Axygen AxyPrep Magnetic Bead Purification Kit according to the manufacturer's protocol. qPCR of ChIP DNA was accomplished using primer pairs that amplify the promoter region in the specified genes. Primer pairs are listed in Table S2. Results of qPCR after ChIP were compared to input DNA and displayed as a percentage relative to input DNA. To determine the background levels for the ChIP-qPCR data, a baseline threshold using values obtained from qPCR performed on a non-specific IgG immunoprecipitate and used as controls.

Bioinformatic Analysis

Sequence reads from fastq files were aligned to the mouse genome (mm9) using TopHat. Conversion of file formats including bam, gff bed and bedraph files were done using Bedtools. Unique regions represented from two biological replicates were merged to identify H3K4me3, H3K27me3 and POLR2A ChIP-seq reads. To determine enrichment at promoters, H3K4me3, H3K27me3 and POLR2A ChIP-seq binding to +/- 1kb around the transcription start site (TSS) was determined. Pairwise comparison of was done for H3K4me3 with POLR2A, H3K27me3 with POLR2A and H3K4me3 with H3K27me3. Only sequences that intersect with more than 1 bp were considered overlapping. The intersection between H3K4me3, H3K27me3 and POLR2A was used to define genes with bivalently marked promoters. To assign biological function to the cis-regulatory regions identified by ChIP-seq, gene ontology was performed using WebGestalt (Zhang et al., 2005). To confirm enrichment at genomic regions, MACS2 was used for peak calling. To visualize enrichment of reads at the promoters of different genes, bedgraph files were generated and IGV was used for visualization.

Use of shRNA, siRNA and small molecule

Three different shRNA designed to target murine *Neurog1* transcript in the pLKO.1 containing puromycin resistance cassettes were purchased from (Sigma) and tested for their ability to knockdown *Neurog1* transcript. shRNA sequences against *Neurog1* are listed in Table S4. shRNA_1 showed the most robust *Neurog1* knockdown and was subsequently used. To generate the blasticidin resistant lentiviral vector pLKO.1 *Neurog1* shRNA, the double-stranded hairpin oligonucleotide 5'-TCTCGACTGCTCCAGCAGCAA-3' was placed into the multiple cloning site of pLKO.1Blast. pLKO.1-blast-Scrambled containing the following sequence was used as a control*:* 5'-CCTAAGGTTAAGTCGCCCTCGCTC-3' (Addgene #1864). Viral vectors as well as packaging plasmids were transfected into 293FT cell line by the calcium phosphate co-precipitation method. Supernatant was collected 48 and 64 hours post-transfection and combined. Virus was concentrated by precipitating virus using PEG 6000 (Kutner et al., 2009). iMOP cells were infected at a multiplicity of infection between 5-10. Twenty-four hours after infection, cells were selected with 10 µg/ml blasticidin. For *Cdk2* transcript knockdown, scrambled FITC conjugated siRNA and *Cdk2* siRNA were purchased (Santa Cruz). siRNAs were transfected into iMOP cells every 24 hours for 3 consecutive days using the jetPRIME siRNA transfection reagent (Polyplus Transfection). CDK2 inhibitors ricovitine (Sigma) and K03861 (Selleckchem) were resuspended according to manufacturers. Inhibitors were used to culture cells for 3 days at the specified concentrations.

Statistical Analysis

All error bars shown in data are expressed as +/- standard error of the mean obtained from independent experiments unless otherwise stated. The numbers (n) of independent experiments are listed in the figure legend. Technical triplicates were included in each qPCR experiment. An unpaired two-tailed Student's t-test was used to determine statistical significance and associated with a p value. For all figures, p values are defined as * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.