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Supplementary Information for

LIN28B/*let-7* control the ability of neonatal murine auditory supporting cells to generate hair cells through mTOR signaling

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Supplemental figures

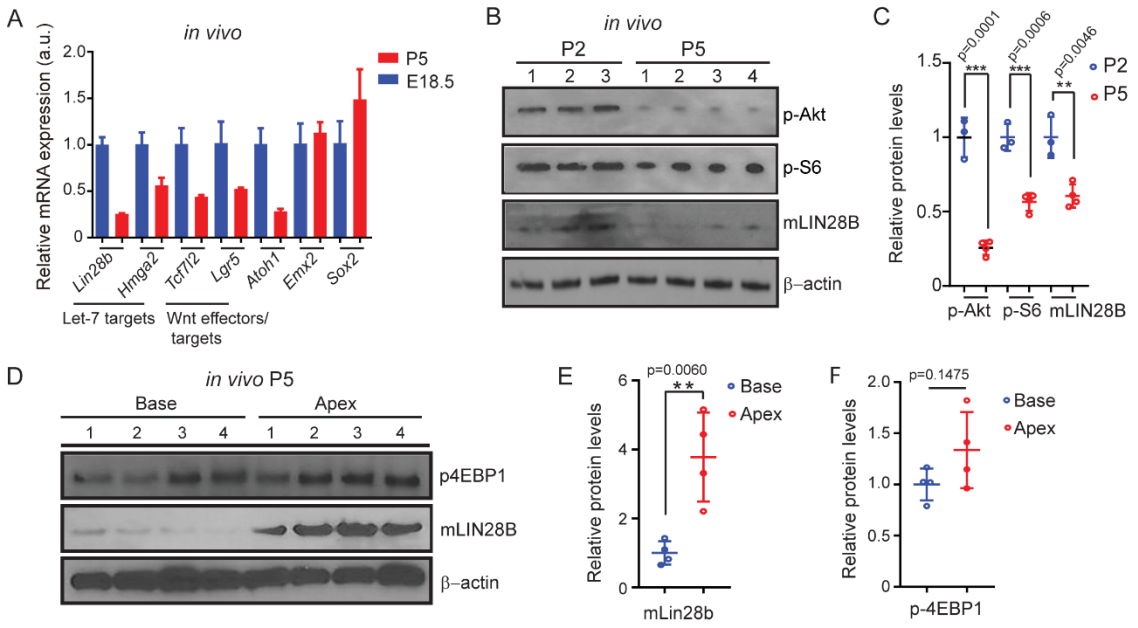

Fig. S1. LIN28B expression positively correlates with mTOR activity in the maturing cochlea. *(A)* RT-PCR analyzing mRNA abundance of *let-7* targets (*Lin28b*, *Hmga2*), Wnt signaling effectors/targets (*Tcf7l2, Lgr5*) and *Atoh1*, *Emx2*, *Sox2* in cochlear sensory epithelia obtained from wild type mice stages E18.5 (blue) and P5 (red) (graphed are mean ± SD, technical replicate, from one representative experiment, three independent experiments). *(B)* Immunoblots for p-Akt, p-S6, murine (m) LIN28B and β-actin (loading control) using protein lysates of acutely isolated cochlear sensory epithelia from wild type mice stages P2 (n=3) and P5 (n=4). *(C)* Normalized p-Akt, p-S6 and murine (m) LIN28B protein expression in *(B)* (n=3 for P2 and n=4 for P5, from one representative experiment, two independent experiments). *(D)* Immunoblots for p-4EBP1, murine (m) LIN28B and β-actin (loading control) using protein lysates of acutely isolated sensory epithelia obtained from the cochlear apex and base of wild type mice stage P5. *(E-F)* Normalized LIN28B protein levels in *(D)* (n=4). *(F)* Normalized p-4EBP1 protein levels in *(D)* (n=4, from one representative experiment, two independent experiments). Graphed are individual data points and mean ± SD in (*C*), (*E*) and (*F*). N represents number of animals analyzed per group. Two-tailed, unpaired Student's *t* test was used to calculate *P* values. Abbreviation: a.u., arbitrary unit.

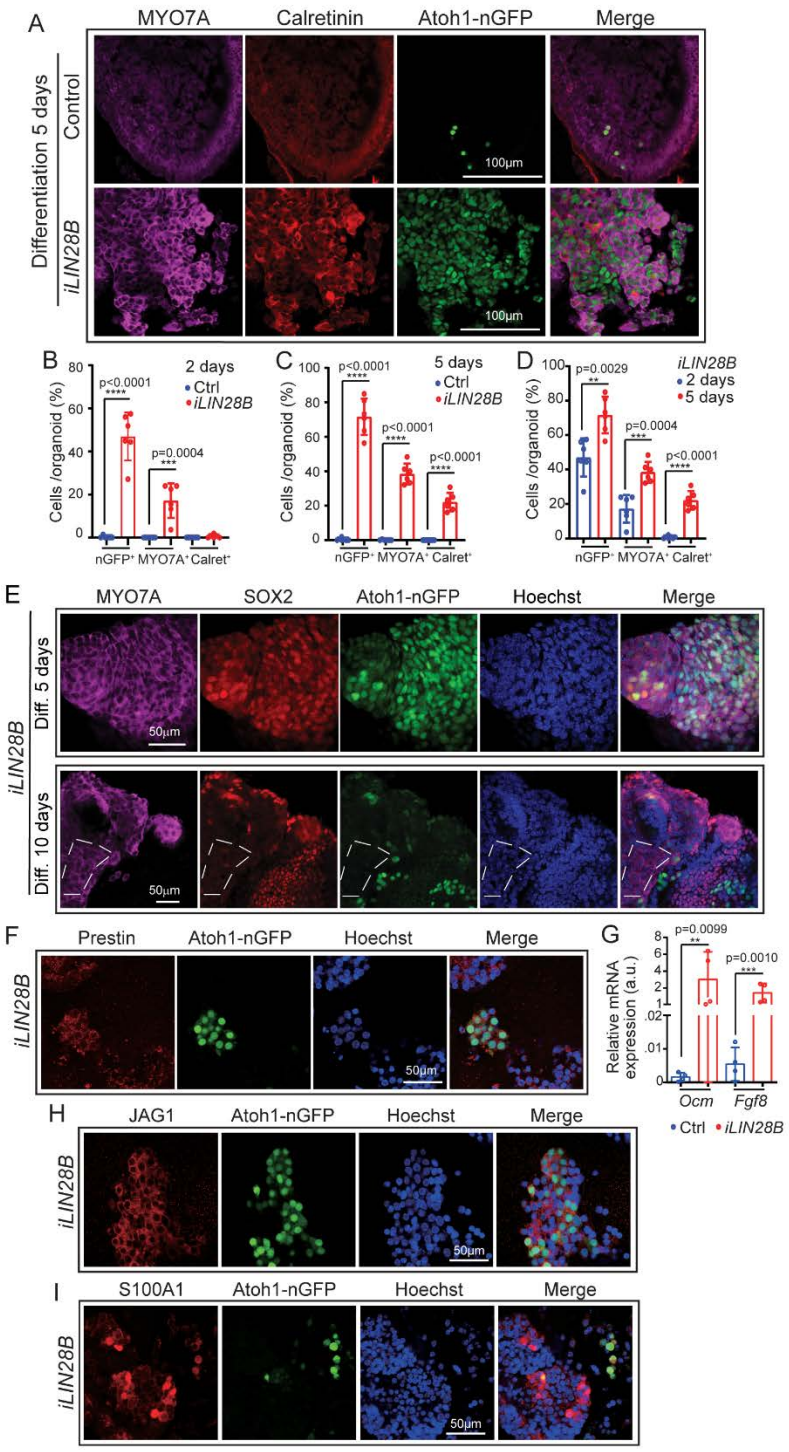

Fig. S2. Newly formed hair cells in LIN28B overexpressing organoids express inner and outer hair cell markers. Cochlear organoid cultures were established from stage P5 *Atoh1-nGFP iLIN28B* transgenic mice and *Atoh1-nGFP* control littermates that lacked the *LIN28B* transgene and were cultured as described in Fig. 2*A*. *(A)* Confocal images of control and LIN28B overexpressing organoids after two and five days of differentiation. Atoh1-GFP (green) and MYO7A (magenta) marks newly formed hair cells. Calretinin (red) marks presumptive inner hair cells. *(B-D)* Percentage of Atoh1-nGFP, MYO7A and calretinin positive cells per organoid in control (Ctrl) and

LIN28B overexpressing (*iLIN28B*) organoid cultures after two days (*B* and *D*), five days (*C* and *D*) of differentiation (graphed are average values for each animal and their mean ± SD, n=6 animals per group, two independent experiments). (*E*) Confocal images of LIN28B overexpressing organoids after five and ten days of differentiation. Immature hair cells are identified by their co-expression of Atoh1-GFP (green) and MYO7A (magenta) and SOX2 (red). White dashed lines encircle a group of more mature hair cells that express MYO7A but lack SOX2 and Atoh1-GFP expression. Hoechst (blue) labels cell nuclei. (*F*) Confocal images of LIN28B overexpressing organoids after seven days of differentiation. Atoh1-nGFP (green) and prestin (red) co-expression marks presumptive outer hair cells, Hoechst (blue) labels cell nuclei. (*G*) RT-PCR of inner (*Fgf8*) and outer (oncomodulin, *Ocm*) hair cell-specific gene expression in control and LIN28B overexpressing (*iLIN28B*) organoids after seven days of differentiation (mean ± SD, n=4 animals per group, two independent experiments). *(H and I)* LIN28B overexpressing organoids after seven days of differentiation contain Atoh1-nGFP^{+} hair cells (*H* and *I*, Atoh1-nGFP, green) and Atoh1-nGFP^{-} JAGGED1^{+} (*H*, JAG1, red) and Atoh1-nGFP^{-} S100A1^{+} (*I,* S100A1, red) supporting cells. Note that Atoh1-nGFP^{+} S100A1^{+} cells represent inner hair cells. Two-tailed, unpaired Student's *t* test was used to calculate *P* values.

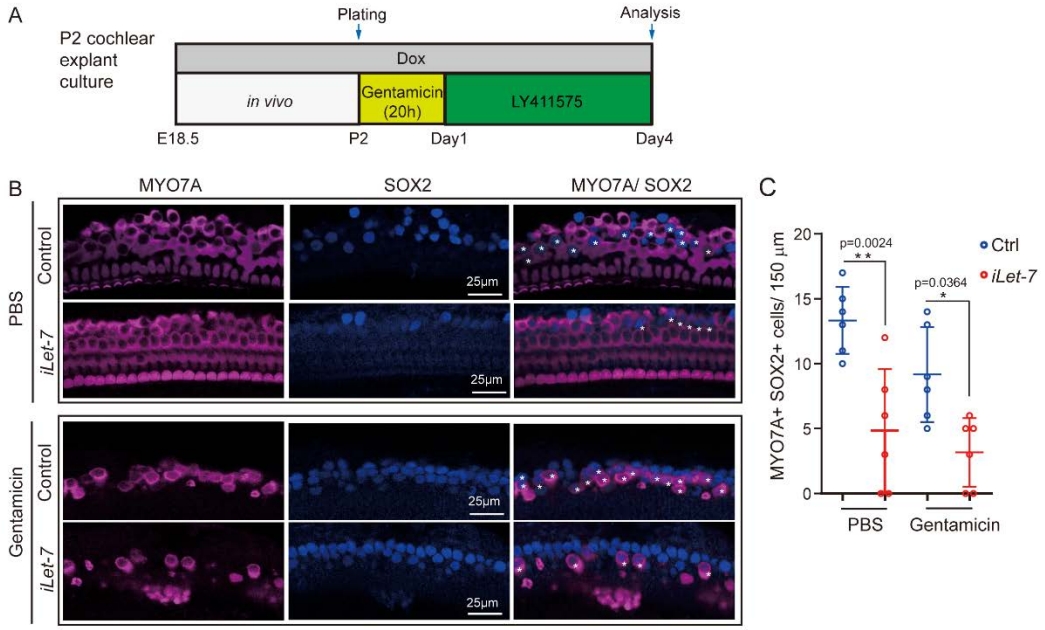

Fig. S3. *Let-7* overexpression inhibits hair cell regeneration in early postnatal cochlear explants. *(A)* Experimental strategy. Cochlear explant cultures were established from P2 *iLet-7* transgenic mice and littermates that lacked *let-7g* transgene (control). To ablate hair cells, one cochlea from each animal received gentamicin (100 µg/mL), while the other cochlea received PBS (vehicle control) for 20 hours. To induce supporting cell-to-hair cell conversion, cultures were treated with Notch inhibitor LY411575 for three days starting at day one. *(B)* Shown are representative confocal images of mid-apical turn of control and *let-7g* overexpressing (*iLet-7*) cochlear explants immuno-stained for MYO7A (magenta) and SOX2 (blue). Note that new hair cells express MYO7A and SOX2, whereas pre-existing hair cells only express MYO7A. *(C)* Quantification of newly formed hair cells (MYO7A^{+} SOX2^{+}) in control (blue) and *let-7g* overexpressing cochlear explants (red) in *(B).* Graphed are individual data points, representing the average value per animal, and mean ± SD, n=6 animals per group, two independent experiments. Two-way ANOVA with Tukey's correction was used to calculate *P* values.

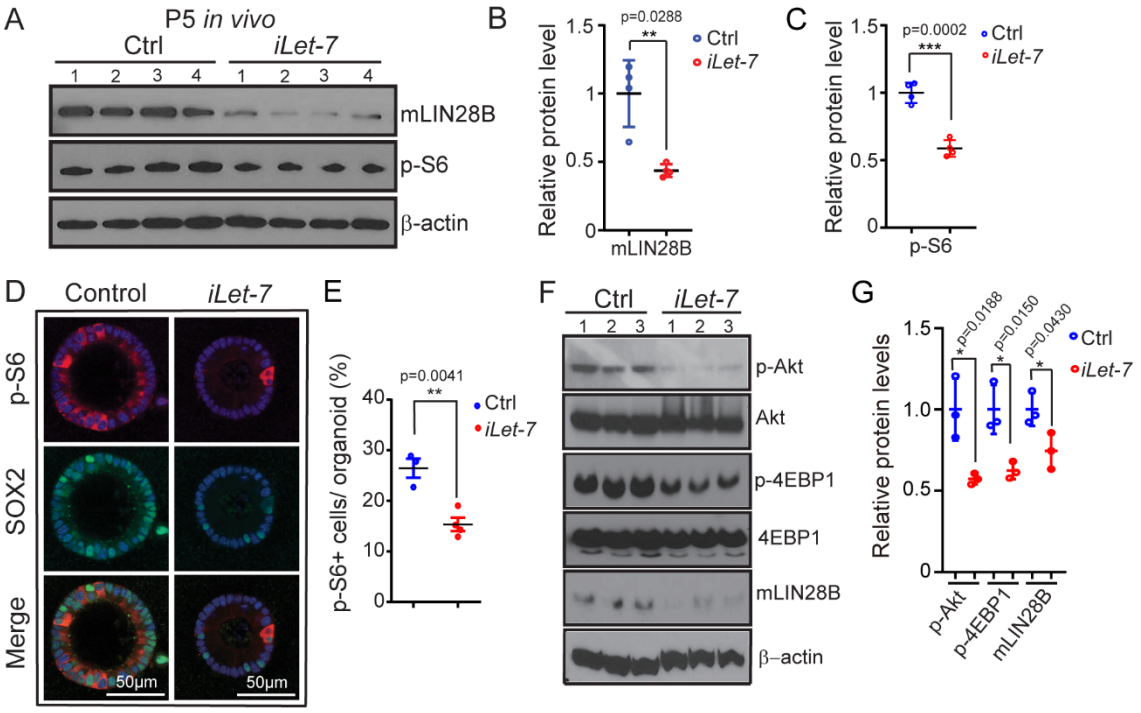

Fig. S4. *Let-7g* negatively regulates mTOR signaling in early postnatal cochlear epithelial cells. (*A-C*) *Let-7g* overexpression attenuates mTOR signaling in cochlear epithelial cells *in vivo*. *iLet-7* transgenic mice and control littermates that lacked *let-7g* transgene received dox starting at E18.5 until tissue harvest at P5. *(A)* Immunoblots for p-S6, endogenous murine (m) LIN28B and β-actin using protein lysates of acutely isolated control (ctrl) and *let-7g* (*iLet-7*) overexpressing cochlear sensory epithelia. (*B-C*) Normalized murine (m) LIN28B and p-S6 protein expression in (*A*) (n=4, from one representative experiment, three independent experiments). (*D-G*) *Let-7g* overexpression attenuates mTOR signaling in cochlear organoids. Cochlear organoid cultures were established from stage P2 *iLet-7* transgenic mice and control littermates. Dox was present throughout the 10-day long expansion phase. (*D*) Confocal images of control and *let-7g* overexpressing (*iLet-7*) organoids co-stained for p-S6 (red) and SOX2 (green). Nuclei were counterstained with Hoechst (blue). (*E*) Percentage of p-S6^{+} cells per organoid shown in (*D*) (n=3 for control and n=4 for *iLet-7* group, from one experiment). (*F*) Immunoblots for p-Akt, Akt, p-4EBP1, 4EBP1, murine (m) LIN28B and β-actin using protein lysates from control and *iLet-7* transgenic organoids. (*G*) Normalized p-Akt, p-4EBP1 and mLIN28B protein expression in (*F*) (n=3, from one representative experiment, three independent experiments). Graphed are individual data points and mean ± SD. Note that the individual data points in (*E*) represent the average value per animal. N represents number of animals analyzed per group. Two-tailed, unpaired Student's *t* test was used to calculate *P* values.

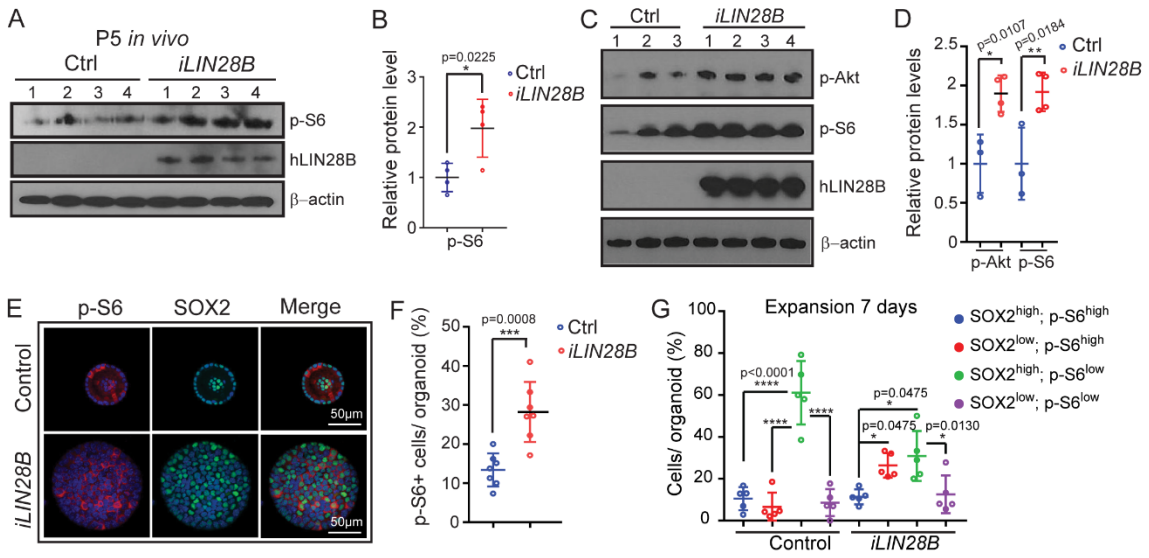

Fig. S5. LIN28B positively regulates mTOR signaling in early postnatal cochlear epithelial cells. (*A-B*) LIN28B overexpression enhances mTOR signaling in cochlear epithelial cells *in vivo*. *iLIN28B* transgenic mice and control littermates received dox starting at E18.5 until tissue harvest at P5. *(A)* Immunoblots for p-S6, human (h) LIN28B and β-actin using cochlear epithelial protein lysates from stage P5 control and LIN28B overexpressing mice. *(B)* Normalized p-S6 protein levels in (*A*) (n=4, from one representative experiment, three independent experiments). (*C-G*) LIN28B overexpression increases mTOR activity in cochlear organoids. Cochlear organoid cultures were established from stage P5 *iLIN28B* transgenic mice and control littermates. Organoid cultures were maintained as outlined in Fig. 2*A*. (*C*) Immunoblots for p-Akt, p-S6, human (h) LIN28B and β-actin using protein lysates of control and *iLIN28B* organoids. (*D*) Normalized p-Akt and p-S6 protein levels in (*C*) (n=3 for control and n=4 for *iLIN28B*, from one representative experiment, three independent experiments). (*E*) Confocal images of P5 control and P5 *iLIN28B* transgenic organoids after nine days of expansion. Organoids were immuno-stained for mTOR target p-S6 (red) and supporting cell/pro-sensory cell marker SOX2 (green). Nuclei were counterstained with Hoechst (blue). (*F*) Percentage of p-S6^{+} cells per organoid shown in (*E*) (n=7, two independent experiments). (*G*) Percentage of p-S6^{+} cells (p-S6-high) that express SOX2 at high or low level in (*E*) (n=5, two independent experiments). Graphed are individual date points and the mean ± SD. Note that the individual data points in (*F*) and (*G*) represent the average value per animal. N represents number of animals analyzed per group. Two-tailed, unpaired Student's *t* test was used to calculate *P* values.

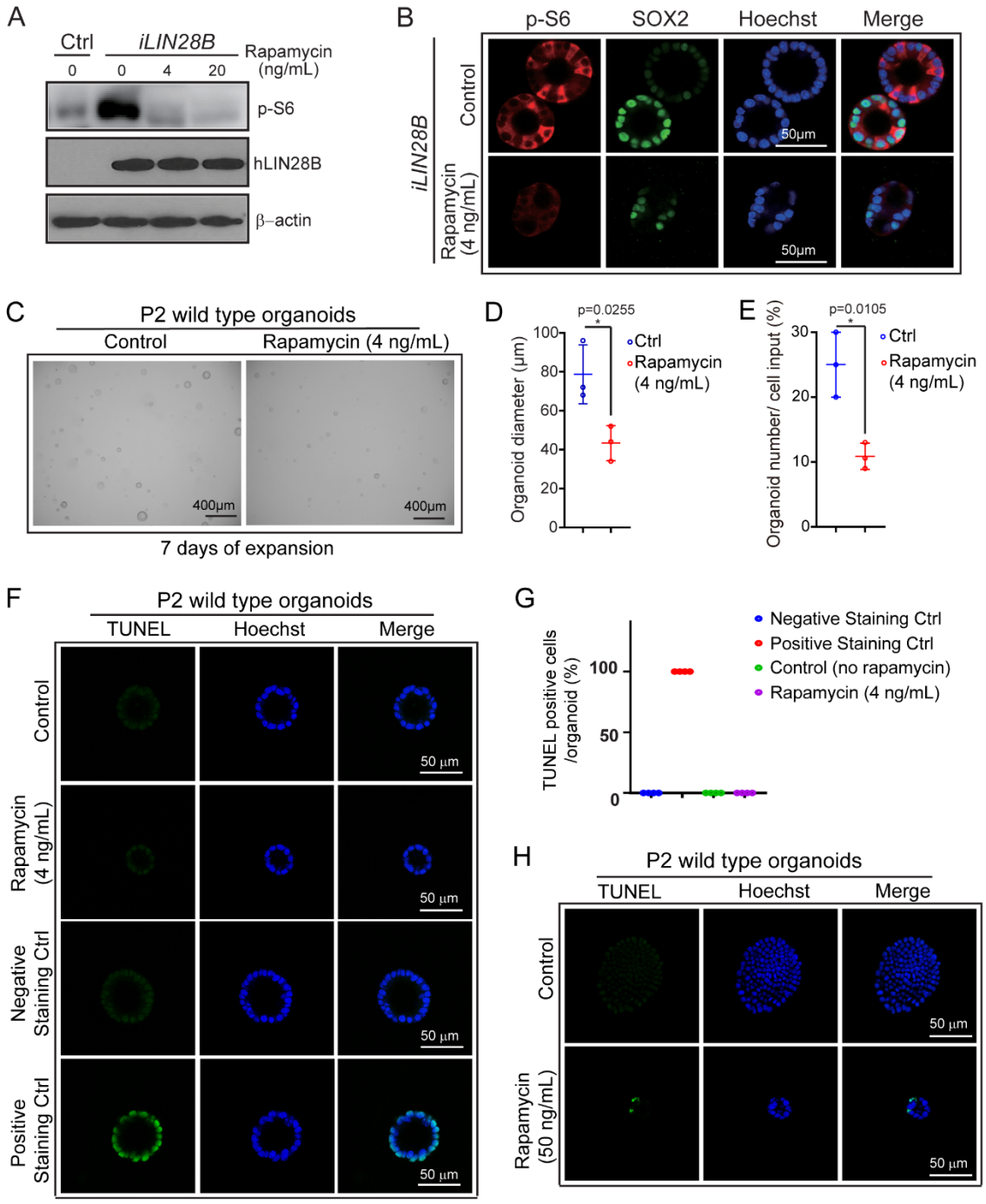

Fig.S6. Low dosage of rapamycin inhibits cochlear organoid growth without inducing cell death. (*A-B*) 4 ng/mL of rapamycin is effective in inhibiting mTOR activity in LIN28B overexpressing cochlear organoids. (*A*) Immunoblot of p-S6 and human (h) LIN28B in protein lysates collected from untreated (0 ng/mL of rapamycin) control (Ctrl) and LIN28B overexpressing (*iLIN28B*) organoids and LIN28B overexpressing organoids that were treated for 5 hours with 4 ng/mL or 20 ng/mL of rapamycin. Note that rapamycin treatment has no effect on LIN28B transgene expression. (*B*) Confocal images of LIN28B overexpressing organoids treated with 4 ng/mL rapamycin or vehicle control (DMSO) for seven days. P-S6 (red) immuno-staining marks cells with high mTOR activity, SOX2 (green) marks supporting cells/pro-sensory cells. Hoechst (blue) marks cell nuclei. (*C-H*)

Cochlear organoid cultures were established from P2 wild type mice and cultured using expansion conditions (see Fig.1 *A*). (*C-G*) Cochlear organoids received 4 ng/mL rapamycin or vehicle control (DMSO) at day one. Organoid growth (*C-E*) and cell death using TUNEL staining (*F-G*) was analyzed six days later. (*C*) Bright field of cochlear organoids treated with 4 ng/mL rapamycin or vehicle control (DMSO). (*D*) Organoid diameter and (*E)* organoid forming efficiency in (*C*) (n=4 animals per group, two independent experiments). (*F*) Confocal images of TUNEL (green) and Hoechst (blue) stained control (DMSO) and rapamycin (4 ng/mL) treated P2 wild type organoids, including negative and positive controls for TUNEL staining. (*G*) Percentage of TUNEL^{+} cells in (*F*) (n=4 animals per group, two independent experiments). (*H*) Cochlear organoids were cultured with 50 ng/mL of rapamycin or vehicle control (DMSO) starting at day three and cell death using TUNEL staining was analyzed four days later. Shown are representative confocal images of TUNEL (green) and Hoechst (blue) stained organoids. Graphed are individual data points and mean ± SD. Individual data points in (*D*), (*E*) and (*G*) represent average value per animal. Two-tailed, unpaired Student's *t* test was used to calculate *P* values.

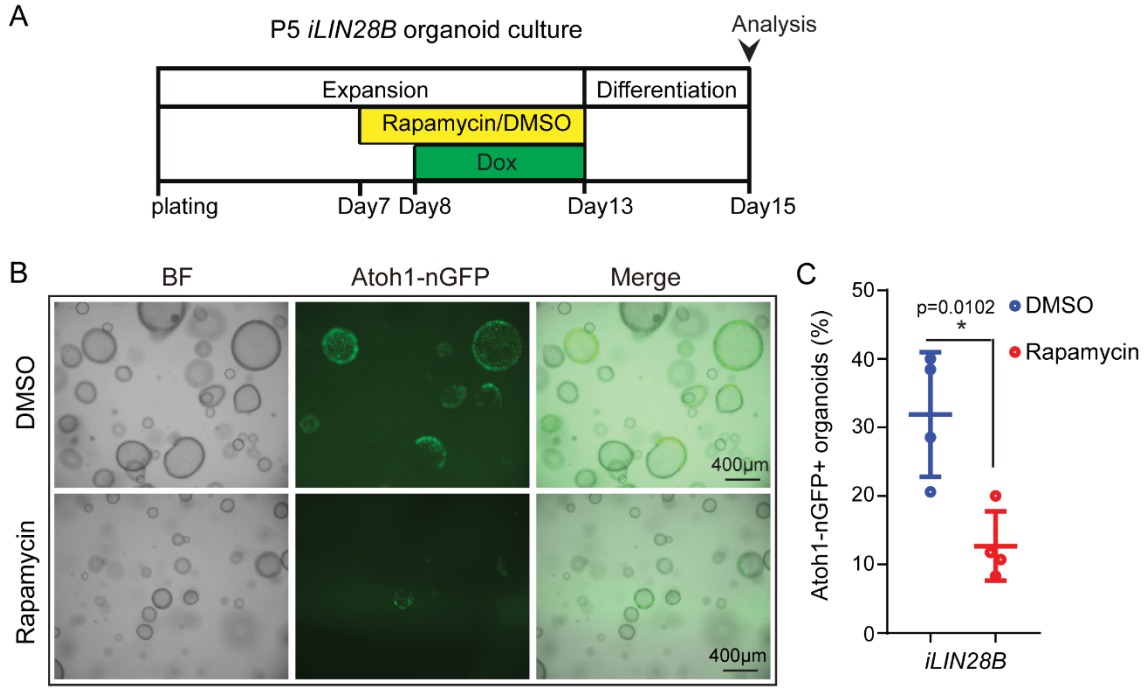

Fig.S7. Rapamycin pre-treatment inhibits *Atoh1* induction in LIN28B overexpressing organoids. (*A*) Experimental strategy. Organoid cultures were established from stage P5 *Atoh1-nGFP iLIN28B* transgenic mice and maintained as outlined in Fig. 2*A*. Rapamycin (4 ng/mL) or vehicle control (DMSO) was added to the culture media at seven days of expansion. One day later, dox was added to induce LIN28B expression. (*B*) BF and green fluorescent (Atoh1-nGFP) images of LIN28B overexpressing organoids treated with DMSO or rapamycin after two days of differentiation. (*C*) Percentage of Atoh1-nGFP^{+} organoids in (*B*) (graphed are individual data points and mean ± SD, n=4 animals per group, two independent experiments). Two-tailed, unpaired Student's *t* test was used to calculate *P* values.

Methods

Table S1. List of genotyping primers.

Mouse line	Genotyping primers	Product size
Atoh1-GFP, p27-GFP	EGFP1: CGA AGG CTA CGT CCA GGA GCG CAC EGFP2: GCA CGG GGC CGT CGC CGA TGG GGG TGT	TG= 300bp
R26-M2rtTA	MTR: GCG AAG AGT TTG TCC TCA ACC F: AAA GTC GCT CTG AGT TGT TAT WTR: GGA GCG GGA GAA ATG GAT ATG	WT=650bp MT=340bp
Col1a-TRE- let-7S21L, Col1a-TRE-LIN28B	ColA: GCA CAG CAT TGC GGA CAT GC ColB: CCC TCC ATG TGT GAC CAA GG ColC: GCA GAA GCG CGG CCG TCT GG	WT=300bp TG=450bp
UBC-CreERT2	MT-F: GAC GTC ACC CGT TCT GTT G MT-R: AGG CAA ATT TTG GTG TAC GG WT-F: CTA GGC CAC AGA ATT GAA AGA TCT WT-R: GTA GGT GGA AAT TCT AGC ATC ATC C	WT=324bp TG=475bp
Lin28a floxed	Flox-F: TCC AAC CAG CAG TTT GCA G Flox-R: GCA GCT GGT AAG AAG AAA CCT G	WT=356bp Flox=500bp
Lin28b floxed	Flox-F: AAC GCA CAT TGC AAA TAC CC Flox-R: TTC ATC TGG CTC CTT TCT CG	WT=221bp Flox=338bp

Table S2. List of antibodies used for immunostaining.

Reagent type	Designation	Source	Identifiers	Additional information
primary antibody	anti-Calretinin, mouse monoclonal	Sigma-Aldrich	Cat.# MAB1568	1:500 dilution
primary antibody	anti-JAG1 goat polyclonal	Santa Cruz	Cat.# sc-6011	1:500 dilution
primary antibody	myosin VIIa rabbit polyclonal	Proteus Biosciences	Cat.# 25-6790	1:500 dilution
primary antibody	prestin goat polyclonal	Santa Cruz	Cat.# sc-22692	1:500 dilution
primary antibody	S100A1 Rabbit polyclonal	Abcam	Cat.# ab11428	1:500 dilution

primary antibody	SOX2 goat polyclonal	Santa Cruz	Cat.# sc-17320	1:500 dilution
primary antibody	p-S6 (Ser240/244) Rabbit monoclonal	Cell Signaling	Cat.# 5364	1:500 dilution
secondary antibody	donkey anti-rabbit IgG (H+L) Alexa Fluor 546	ThermoFisher	Cat.# A10040	1:1000 dilution
secondary antibody	donkey anti-rabbit IgG (H+L) Alexa Fluor 647	ThermoFisher	Cat.# A-31573	1:1000 dilution
secondary antibody	donkey anti-mouse IgG (H+L) Alexa Fluor 546	ThermoFisher	Cat.# A-10036	1:1000 dilution
secondary antibody	donkey anti-goat IgG (H+L) Alexa Fluor 488	ThermoFisher	Cat.# A-11055	1:1000 dilution
secondary antibody	donkey anti-goat IgG (H+L) Alexa Fluor 546	ThermoFisher	Cat.# A-11056	1:1000 dilution

Table S3. List of qPCR primers.

Gene	**Forward Primer**	**Reveres Primer**
Ano1	TTC CCT CTG GCT CCA CTC TTC	GGC ATC CAG GCG GAT CT
Atoh1	ATG CAC GGG CTG AAC CA	TCG TTG TTG AAG GAC GGG ATA
Cybrd1	AGA CTG CCA TGG ACC TGG AA	CCG GCA TGG ATG GAT TTC
Emx2	GAA TCC GCT TTG GCT TTC TG	GAC ACA AGT CCC GAG AGT TTC C
F2rl1	CGG ACC GAG AAC CTT GCA CCG	GTG AGG ATG GAC GCA GAG AACT
Fat3	CAC AGC CCT TGA ATA CAG TGA	TGC CTT TGC ATC TCC TTC CT
Fgf8	ATC AAC GCC ATG GCA GAA G	AGT ATC GGT CTC CAC AAT GAG CTT
Fst	GAA AAC CTA CCG CAA CGA ATG	TCC GGC TGC TCT TTG CAT
Hmga2	CAG AAG AAA GCA GAG ACC ATT	TTG TTG TGG CCA TTT CCT AGG T
Isl1	CGG AGA GAC ATG ATG GTG GTT	AGG GCG GCT GGT AAC TTT G
Lgr5	CCC CAA TGC GTT TTC TAC GT	GAA GGA CGA CAG GAG ATT GGA T
Lin28a	TCC AAA GGA GAC AGG TGC TAC A	TTG CAT TCC TTG GCA TGA TG
Lin28b	CAT GGC ACT GGC CAC TGT AA	ATC ATG GAG ATG AAT CCG AAT CC
Myo7a	CCC CCT CTG AGA AGT TCG TTA A	TGT GTC CGA GTT CCG TTG AC
Ocm	ACC AGA GTG GAT ACC TGG ATG	CGT CGC TCT GGA ACC TCT GT

Pou4f3	GCA CCA TCT GCA GGT TCG A	CCG GCT TGA GAG CGA TCA T
S100a1	TGG ATG TCC AGA AGG ATG CA	CCG TTT TCA TCC AGT TCC TTC A
Sox2	CCA GCG CAT GGA CAG CTA	GCT GCT CCT GCA TCA TGC T
Tcf7l2	AAA CCC TCA AGG ATG CTC GTT	CCA CCG GTA CTT TGT TCG AAA
Trim71	ATC GGG AGT GTG AGC TGT TG	GGC GTG AAC ATA ATG CGG TC
Rpl19	GGT CTG GTT GGA TCC CAA	TGC CCG GGA ATG GAC AGT CA

Table S4. List of antibodies used for immunoblotting.

Reagent type	Designation	Source	Identifiers	Additional information
primary antibody	Akt rabbit monoclonal	Cell Signaling	Cat.# 4691	1:1000 dilution
primary antibody	Akt (Ser473) rabbit monoclonal	Cell Signaling	Cat.# 4060	1:1000 dilution
primary antibody	p-4E-BP1(Thr37/46) rabbit monoclonal	Cell Signaling	Cat.# 2855	1:2000 dilution
primary antibody	4E-BP1 rabbit monoclonal	Cell Signaling	Cat.# 9644	1:2000 dilution
primary antibody	human LIN28B rabbit polyclonal	Cell Signaling	Cat.# 4196	1:2000 dilution
primary antibody	mouse LIN28B rabbit polyclonal	Cell Signaling	Cat.# 5422	1:500 dilution
primary antibody	p-S6(Ser240/244) rabbit monoclonal	Cell Signaling	Cat.# 5364	1:1000 dilution
primary antibody	β-actin mouse monoclonal	Santa Cruz	Cat.# 47778	1:500 dilution