Supplemental Experimental Procedures

Animals

CD-1 (Charles Rivers Labs) was the wild type strain; it was always used for developmental analysis because there can be strain differences in developmental timing. Other strains used were: Cre recombinase knock-in alleles *Ascl1CreERT2* (Kim et al., 2011) (abbreviated here *"$Ascl1^{CreER}$"), Shh^{Cre}* (Jackson Labs B6.Cg-$^{Shh\ tm1(EGFP/cre)Cjt}$/J) (Harfe et al., 2004), and *$FoxA2^{mcm}$* (Park et al., 2007) (Jackson Labs *$Foxa2^{tm2.1(cre/Esr1*)Moon}$/J*, *"$FoxA2^{CreER}$"); Cre*-expressing transgenic line *Wnt1-Cre* (Danielian et al., 1998) and Cre-dependent reporters *$Rosa26^{mTmG}$* , *$Rosa26^{ZsGreen}$*, and *$Rosa26^{Rainbow}$ ("$Rosa26^{Rbw}$")* (Madisen et al., 2010; Muzumdar et al., 2007; Rinkevich et al., 2011).

Immunohistochemistry and histology

Embryos and lungs from timed pregnancies, with noon of the day of vaginal plug detection designated E0.5, were dissected and fixed in 4% paraformaldehyde (PFA) for 0.5-2 hr. Immunostains of whole mount lungs were performed as described (Metzger et al., 2008) with the modifications noted below. Fixed lungs were dehydrated serially into 100% methanol and stored at -20°C. Lungs were rehydrated and incubated with blocking solution (5% goat serum, 0.5% Triton X-100 in PBS) and then with primary antibodies diluted in blocking solution for 48-72 hours at 4°C. On the next day, lungs were washed with blocking solution, followed by incubation with fluorescent-conjugated secondary antibodies overnight at 4°C. For signal amplification, a biotin-conjugated antibody was used with ABC Elite reagent (Vector) followed by fluorescein (FITC) or Cy3 Tyramide Reagent (Perkin Elmer). Lungs were dehydrated and placed in benzyl alcohol:benzyl benzoate (1:2) solution (BABB) prior to imaging by either laser scanning confocal fluorescence microscopy (Leica SP2 or Zeiss LSM 780) or optical projection tomography (OPT;Bioptonics 3001).

For immunostaining of lung sections, lungs were dissected and fixed in 4% PFA or Zamboni's fixative (American MasterTech Scientific, #FXZAMLT) for 0.5-2 hours and cryoprotected in 30% sucrose overnight at 4°C, frozen in optical cutting temperature compound (OCT, Tissue Tek) and stored at -80°C. Frozen tissue blocks were sectioned (20-50 μm) and the sections washed in 0.1% Tween-20 in PBS (PBST) and then incubated with blocking solution (5% goat serum, 0.3% Triton X-100 in PBS) for 1-5 hrs, and washed in PBST. The washed sections were then incubated with primary antibodies overnight at 4°C, washed with PBST, and then incubated with secondary antibodies at room temperature for 45 minutes, followed by staining with DAPI to mark nuclei.

Primary antibodies were: anti-Ascl1 (mouse, BD Pharmingen, 1:500), monoclonal anti-alpha smooth muscle actin (SMA)-Cy3, Sigma C6198, 1:250), anti-CGRP (rabbit, BioMol, 1:500 - 1:1000; guinea pig, Europroxima, 1:500-1:1000), anti-cleaved caspase3 (rabbit, Cell-signaling, 1:200), anti-E-cadherin (rat, Invitrogen, 1:500; rabbit, Cell-signaling, 1:250), anti-integrin β1 (rat, Millipore, 1:200),

anti-laminin γ1 (rat, Millipore, 1:500), anti-NCAM (rabbit, Millipore, 1:500), anti-PGP9.5 (rabbit, Dako, 1:500), anti-ZO-1 (rabbit, Invitrogen, 1:200), and anti-Snail (rabbit, 1:500). The anti-Snail antiserum was raised against a peptide (RMSLLHKHQESGSSGGPR) with 95% sequence identity to the N-terminus of mouse Snail1, 68% to Snail 2, and 63% to Snail 3 (Rukstalis and Habener, 2007). Secondary antibodies were directly conjugated to Alexa -488, -555, or 633 (Invitrogen), or to Alexa 647 (donkey anti-rat, Jackson ImmunoResearch) and all used at 1:250 dilution.

Mapping locations of pulmonary NE cells

In addition to left lobe lateral branches (L.L1-L.L4 and L.D1-L.D4) (see Experimental Procedures), left lobe branches L.M1, L.M2, L.M3, L.M4, L.L1.A1, L.L1.A2, L.L1.V1, L.L1.V2 were analyzed in whole mount E18 CD-1 lungs co-stained for anti-alpha smooth muscle actin (bronchial branches) and CGRP (NE cells). The 3D bronchial branch lineage was easy to visualize in whole-mounts, however only positions of large NE clusters (NEBs) could be reliably scored at this resolution. Right lungs were similarly prepared and analyzed in the right caudal lobe (R.Cd.L1-4, R.Cd.D1-3, R.Cd.M1-3) and also at secondary branches in the accessory lobe, although this analysis was less systematic. In addition, E18 lungs from *Ascl1*$^{CreER/+}$*; Rosa26*$^{mTmG/+}$ mice injected with tamoxifen at E14 to label NE cells were sectioned (entire left lobes, 40-50 μm sections), immunostained for E-cadherin, and analyzed by confocal microscopy to identify NEBs at locations L.L1-4. All positions and mapping strategies gave similar results.

Lineage labeling and clonal analysis of NE cell proliferation

Lineage labeling to investigate the number of progenitors that contribute to NEBs was done using *Shh*$^{Cre/+}$;*Rosa26*$^{Rbw/+}$ embryos to label all epithelial cells with one of three fluorescent proteins early in lung development. Lungs were harvested at E18, fixed with 4% PFA as described above, cryosectioned (35 μm sections) and immunostained with rabbit anti-CGRP and secondary goat anti-rabbit Alexa633. For each cluster or NEB, cell number and fluorescent colors were scored by confocal microscopy.

For clonal analysis, individual NE progenitors were sparsely labeled by intraperitoneal tamoxifen injection of *Ascl1*$^{CreER/+}$*;Rosa26*$^{Rbw/+}$ mice soon after Ascl1 expression initiates (E11.5; 4 mg tamoxifen) or a day later (E12.5; 3 or 4 mg tamoxifen). Embryos were harvested at E17.5, and lungs were dissected, embedded, and sectioned (35 μm) as above. Sections were immunostained for CGRP to identify NEBs, and the number of NE cells and expression of Rainbow reporters was determined by confocal fluorescence microscopy.

EdU incorporation and phospho-histone analysis of NE cell proliferation

Wild-type CD-1 timed pregnant females at embryonic stages E13.5, E14.5, E15.5, and E16.5 were injected intraperitoneally with 300 ug of the synthetic deoxyribonucleotide analogue EdU (5-ethynyl-2'-deoxyuridine; Invitrogen C10337). Two hours later, embryonic lungs were dissected, fixed,

and embedded, and the frozen blocks stored at -80°C until sectioning. Sections (20-30 μm thick) were immunostained for Ascl1 and detected with goat anti-mouse secondary antibody (A555). EdU signal was detected using click chemistry (A488, Invitrogen/Life Tech) according to manufacture's instructions, and nuclei were counterstained with DAPI (1 μg/ml). Specific bronchi were visualized by confocal microscopy and NE (Ascl1-positive) and non-NE (Ascl1-negative bronchial epithelial cells) were scored for EdU-positive nuclei. Lung sections stained for Ascl1 and cell proliferation marker phosphorylated histone 3 (anti-phospho-Histone 3-Ser10, rabbit, Millipore, 1:250) gave similar results, although the EdU protocol labeled more cells.

Activated caspase-3 immunostaining of NE cell death

Wild type CD-1 lungs were harvested during the period of NE clustering and local clearing of solitary NE progenitors (E14.5, E15.5, and E16.5), and fixed, embedded, sectioned, and immunostained for Ascl1 (secondary anti-mouse IgG_1 A555) and for activated Caspase-3 (rabbit anti-cleaved Caspase-3; secondary anti-rabbit A488) to detect dying cells, and nuclei were counterstained with DAPI. Confocal z-stacks were acquired along the bronchial epithelium, and all NE (Ascl1-positive) and non-NE cells (Ascl1-negative, DAPI-positive epithelial cells) within each z-stack (~30 μm thick) were scored for expression of cleaved Caspase-3. Scoring was done both at proximal and more distal positions to include regions with NE progenitors at different stages of development.

Classifying NE cell developmental intermediates

Intermediates were divided into 5 different structural classes, as follows: Class 1: Typical columnar or pseudostratified epithelial morphology without cellular protrusions or extensions and indistinguishable from neighboring non-NE bronchiolar epithelial cells. Class 2: Cells with multiple cellular extensions originating from apical or lateral plasma membrane and up to 30 μm in length. Class 3: Cells with irregular shapes (twisted, bent) and not aligned with neighboring epithelial cells, and no contact, or contact by just a thin projection, with laminin-positive basement membrane. Class 4: Cells with similar orientation as neighboring airway epithelial cells but with basal thin extensions swirling toward basement membrane with similar extensions from neighboring NE cell intermediates and converging at a common point along basement membrane. Class 5: Cells with normal apicobasal orientation and in cluster with other NE cells and fully contacting basement membrane. The only intermediates outside these five structural classes were ones oriented horizontally along the basement membrane (FigS3C, cell 16h) and rare "spiky" cells (FigS3C, cell 20b).

Live imaging of NE cells in slice cultures

Ascl1$^{CreER/+}$*;Rosa26*$^{ZsGreen/mTmG}$ mice were induced with 3 mg tamoxifen by oral gavage at E13 to label pulmonary NE cell intermediates with ZsGreen and membrane GFP. All other cells express TdTomato, which allows direct visualization of the epithelium and easy distinction of NE cells (ZsGreen

and mGFP-positive cells within the epithelium) from Ascl1-positive neurons (ZsGreen and mGFP-positive cells outside the epithelium). Lungs were harvested at E15 and left lobes and right caudal lobes were separated and embedded in 3% agarose and then sectioned with Compresstome (VF 200) to generate lung slices (175 μm thick). All dissection steps prior to placement in culturing chamber were performed in cold (4° C) PBS. Individual lung slices were transferred to an 8-well coverglass chamber (Lab-Tek #70378-82) and a drop of Matrigel (Corning #354230) was placed on top of the sample and incubated at 37°C for 10 minutes to solidify. Culture media (DMEM +F12 and 10% fetal bovine serum) + penicillin-streptomycin (final concentration 100 μg/ml) was added and slices were returned to 37°C for 2-3 hours to allow cultures to establish. Time lapse imaging (image acquisition every 20 minutes for over 60 hours) was performed using a Zeiss 780 confocal equipped with an environmental chamber to maintain the culture at 37°C and 5% CO_2. Under these culture conditions, lung slices showed minimal cell death after 48 hours as assayed by SYTO10 staining (Dead Red kit #L7013, Invitrogen/Life Tech).

Marker expression analysis of NE intermediates

NE developmental intermediates in the lungs of $Ascl1^{CreER/+}$;$Rosa26^{ZsGreen/+}$ mice were labeled with ZsGreen and DAPI as described above and co-stained for E-cadherin and a NE, adhesion, or apical basal marker as indicated in the figures. Thinner sections (35 μm) were used for optimal immunostaining, which was especially important for detecting and analyzing ZO-1. For the all pre-NE specification and some of the differentiation stage samples, when all NE progenitors are of a single class, lungs from wild type CD-1 mice were used and immunostained for Ascl1 or CGRP to detect NE cells. NCAM staining was only detected in NE cells after cluster formation (class 5 intermediates).

Table S1. Multiple Lineage Contributions to Pulmonary NE Clusters, Related to Figure 3.

NE cluster[1]	No. of NE cells[2]	Cherry cells[2]	Orange cells[2]	Cerulean cells[2]
1	5	2	2	1
2	7	2	3	2
3	7	2	3	2
4	8	3	2	3
5	10	3	5	2
6	14	3	6	5
7	15	5	5	5
8	19	3	10	6
9	21	5	10	6
10	27	11	9	7
11	28	12	6	10
12	38	12	16	10

[1] NE clusters in *Shh*$^{Cre/+}$;*Rosa26*$^{Rbw/+}$ mice with airway epithelial progenitors stochastically labeled early in lung development with one of three fluorescent proteins (Cherry, Orange, or Cerulean), harvested at E18, and co-stained with CGRP to identify NE cell clusters.

[2] Number and color of cells in cluster determined by high-resolution confocal z-stacks through cluster. All miniclusters (clusters 1-4) and NEBs (clusters 5-12) examined were composed of cells of all three colors; similar results obtained for >100 additional clusters analyzed by conventional fluorescence microscopy.

Table S2. Clonal Analysis of NE Cell Progenitors, Related to Figure 3

Table S2A. Very sparse labeling and full analysis of NE clusters

NE cluster[1]	No. of cells[2]	Ch cells[2]	Or cells[2]	Ce cells[2]
1	8	0	1	0
2	10	0	1	0
3	12	0	0	0
4	20	0	0	0
5	20	0	0	0
6	21	0	0	0
7	25	0	0	0

[1] NE progenitors labeled by injection of $Ascl1^{CreER/+}$; $Rosa26^{Rbw/+}$ mice with 4 mg tamoxifen (E11.5). Lungs harvested at E17.5, and stained for CGRP (NE cells). Few NE progenitors express Ascl1 at induction, and only two labeled airway epithelial cells (yellow) were identified in entire lung. Cluster 1 is a mini-cluster and 2-7 are NEBs. Cluster 2 is shown in Figure 3B.
[2] Number and color (Ch, Cherry; Or, Orange; Ce, Cerulean) of NE cells in cluster.

Table S2B. Sparse labeling and partial analysis of NE clusters

NE cluster[1]	No. of cells[2]	Ch cells[2]	Or cells[2]	Ce cells[2]
1	1	0	0	0
2	2	0	0	0
3	2	0	0	0
4	2	0	0	0
5	2	1	0	0
6	2	0	0	0
7	2	0	0	0
8	3	0	0	0
9	3	0	0	0
10	3	0	0	0
11	3	0	0	0
12	4	0	0	0
13	4	0	0	0
14	4	0	0	0
15	4	0	0	0
16	4	1	1	0
17	5	0	0	0
18	5	0	0	0
19	5	0	0	0
20	5	0	0	0
21	5	0	0	0
22	5	0	1	1
23	5	0	0	0
24	5	0	0	0
25	5	0	0	0
26	5	0	0	0
27	6	0	0	0
28	6	0	0	0
29	6	0	0	0
30	6	0	0	0
31	6	0	0	1
32	6	0	0	0
33	6	0	0	1
34	6	0	1	0
35	6	0	1	0
36	7	0	0	0
37	7	1	0	0
38	7	0	0	0
39	7	0	0	0
40	7	0	0	0
41	7	0	1	0
42	8	0	0	0
43	8	0	0	0
44	8	0	1	1
45	8	0	0	1
46	8	0	0	0
47	16	0	0	0
Total	247	3	6	5

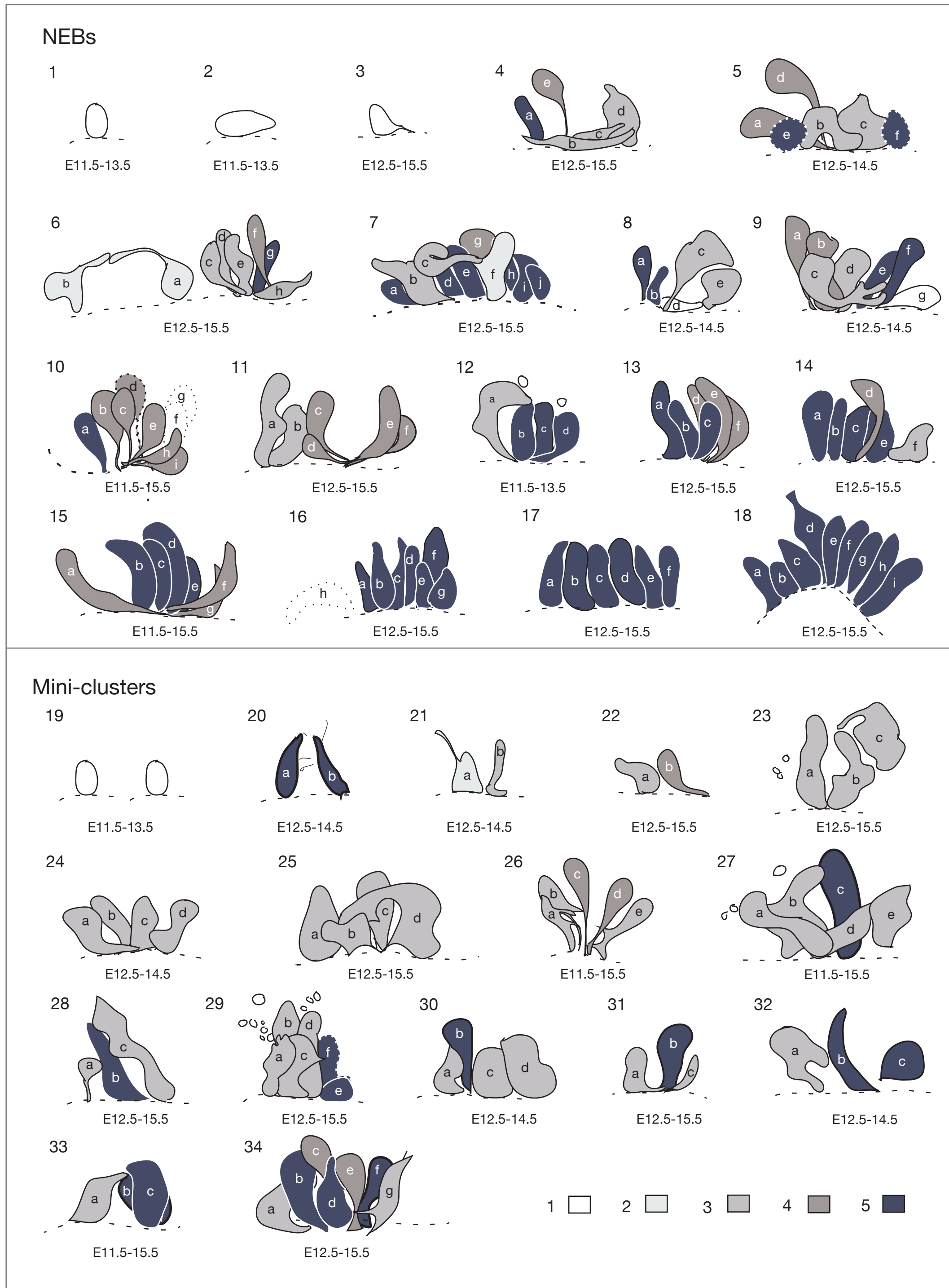
[1] NE progenitors were labeled as in Table S2A except 3 mg tamoxifen was injected at E12.5, when more NE progenitors express Ascl1. All NE clusters along left primary bronchus were scored.
[2] Number and color of NE cells in cluster as in Table S2A except only a single focal plane was examined per cluster. Miniclusters and NEBs were not distinguished, although clusters 42-47 are most likely NEBs. No clusters contained 2 cells of same color. Calculated labeling efficiency: Cherry (3 of 247 scored cells, 1.2%), Orange (2.4%), and Cerulean (2%), combined (5.6%).

Table S2C. Less sparse labeling and partial analysis of NE clusters

NE cluster[1]	No. of cells[2]	Ch cells[2]	Or cells[2]	Ce cells[2]
1	1	0	0	0
2	1	0	0	0
3	1	0	1	0
4	1	0	1	0
5	1	0	0	0
6	1	0	0	0
7	2	0	0	0
8	2	0	0	0
9	2	0	0	0
10	2	0	0	0
11	2	0	0	0
12	2	0	0	0
13	2	0	0	0
14	2	0	0	0
15	2	0	0	0
16	2	0	0	0
17	2	0	0	0
18	2	0	0	0
19	2	1	0	0
20	2	1	0	0
21	2	0	1	0
22	2	0	1	0
23	2	0	1	0
24	2	0	1	0
25	2	0	1	0
26	2	0	1	0
27	3	0	0	1
28	3	2	0	0
29	3	0	1	2
30	3	0	0	0
31	3	0	0	1
32	3	0	0	0
33	3	0	0	0
34	3	0	0	0
35	3	0	1	0
36	3	1	0	0
37	3	0	0	0
38	3	0	0	0
39	3	0	0	0
40	3	0	0	0
41	4	0	2	0
42	4	0	0	0
43	4	0	1	1
44	4	0	1	0
45	4	0	1	0
46	4	0	0	0
47	4	0	0	1
48	4	0	0	0
49	4	2	1	0
50	4	0	1	0
51	4	0	1	0
52	4	0	0	0
53	4	0	1	0
54	5	0	1	0
55	7	0	1	0
Total	152	7	21	6

[1] NE progenitors labeled as in Table S2B except with 4 mg tamoxifen.
[2] Number and color of NE cells determined as in Table S2B. Labeling efficiency: Cherry (4.6%), Orange (13.8%), Cerulean (3.9%), combined (22.3%); the expected number of clusters with more than one cell of same color (magenta) due to independent recombination events is 4, the same as the number observed.

Table S3. Schematics of NE Cell Intermediates Scored in Developing NEBs[1], Related to Figure 5



[1]NEBs (1-18) and mini-clusters (19-34) pulse-labeled, harvested, analyzed, and schematized as in Figures S5 and S6 with pulse (tamoxifen induction day) and harvest day indicated below each schematic.

Supplemental References

Danielian, P.S., Muccino, D., Rowitch, D.H., Michael, S.K., and McMahon, A.P. (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr. Biol. *8*, 1323–1326.

Harfe, B.D., Scherz, P.J., Nissim, S., Tian, H., McMahon, A.P., and Tabin, C.J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell *118*, 517–528.

Kim, E.J., Ables, J.L., Dickel, L.K., Eisch, A.J., and Johnson, J.E. (2011). Ascl1 (Mash1) defines cells with long-term neurogenic potential in subgranular and subventricular zones in adult mouse brain. PLoS ONE *6*, e18472.

Madisen, L., Zwingman, T.A., Sunkin, S.M., Oh, S.W., Zariwala, H.A., Gu, H., Ng, L.L., Palmiter, R.D., Hawrylycz, M.J., Jones, A.R., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci *13*, 133–140.

Metzger, R.J., Klein, O.D., Martin, G.R., and Krasnow, M.A. (2008). The branching programme of mouse lung development. Nature *453*, 745–750.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis *45*, 593–605.

Park, E.J., Sun, X., Nichol, P., Saijoh, Y., Martin, J.F., and Moon, A.M. (2007). System for tamoxifen-inducible expression of cre-recombinase from the Foxa2 locus in mice. Dev. Dyn. *237*, 447–453.

Rinkevich, Y., Lindau, P., Ueno, H., Longaker, M.T., and Weissman, I.L. (2011). Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. Nature *476*, 409–413.

Rukstalis, J.M., and Habener, J.F. (2007). Snail2, a mediator of epithelial-mesenchymal transitions, expressed in progenitor cells of the developing endocrine pancreas. Gene Expression Patterns *7*, 471–479.