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Supplemental information

Dormant *Nfatc1* reporter-marked basal stem/progenitor cells contribute to mammary lobuloalveoli formation

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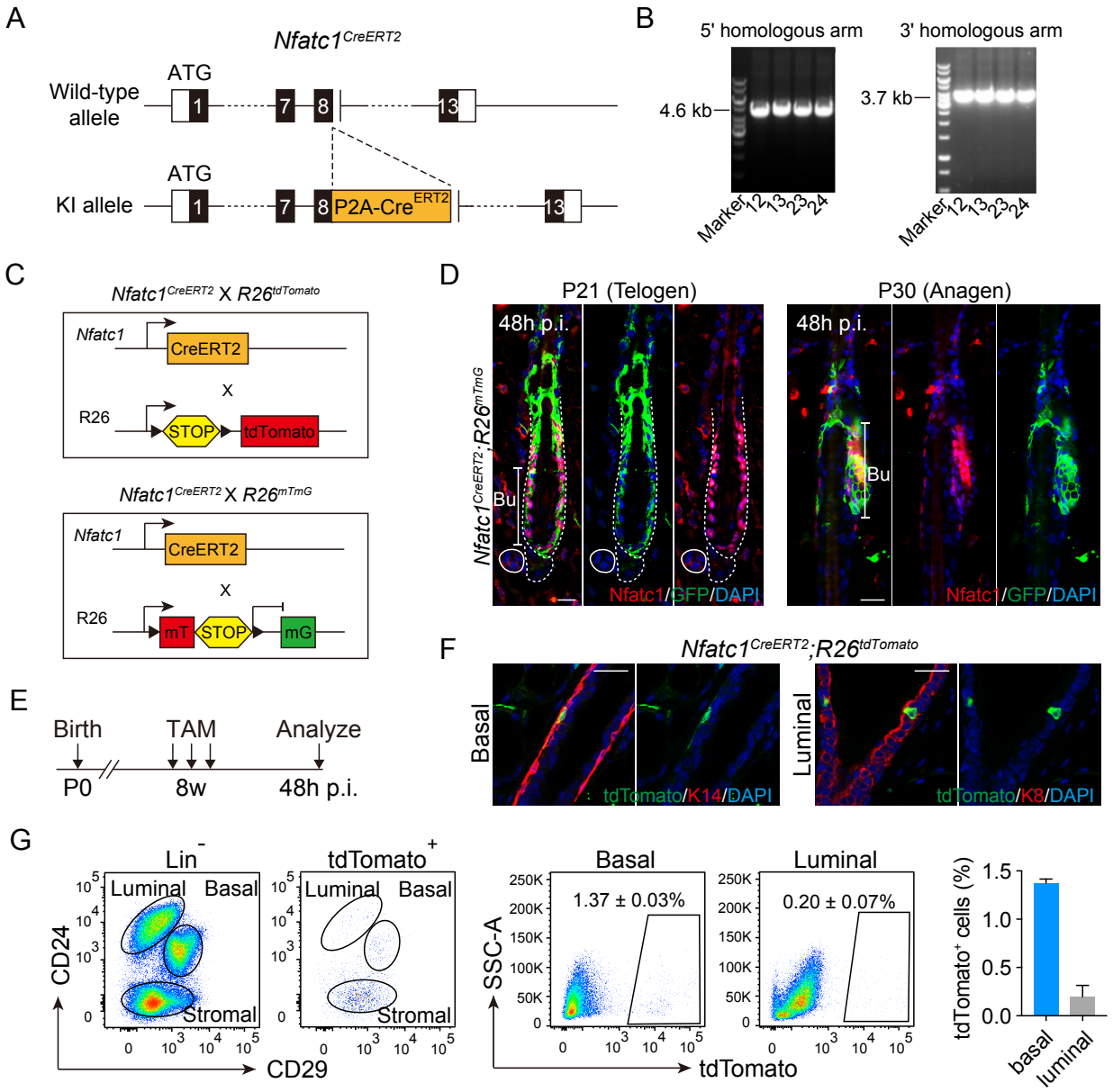

A
Nfatc1CreERT2
Wild-type allele
ATG
1
7
8
13
KI allele
P2A-CreERT2
B
5' homologous arm
3' homologous arm
4.6 kb
3.7 kb
Marker
12
13
23
24
C
Nfatc1CreERT2 X R26tdTomato
Nfatc1
CreERT2
X
R26
STOP
tdTomato
Nfatc1CreERT2 X R26mTmG
mT
mG
D
P21 (Telogen)
P30 (Anagen)
48h p.i.
Nfatc1CreERT2;R26mTmG
Bu
Nfatc1/GFP/DAPI
E
Birth
TAM
Analyze
P0
8w
48h p.i.
F
Nfatc1CreERT2;R26tdTomato
Basal
Luminal
tdTomato/K14/DAPI
tdTomato/K8/DAPI
G
Lin-
tdTomato+
Luminal
Basal
Stromal
CD24
CD29
SSC-A
tdTomato
1.37 ± 0.03%
0.20 ± 0.07%
tdTomato+ cells (%)
basal
luminal

Figure S1. Generation of *Nfatc1*^{CreERT2} knock-in mice. Related to Figure 1.
(A) Schematic representation of the *Nfatc1*^{CreERT2} targeting strategy.
(B) Identification of F0 *Nfatc1*^{CreERT2} mice by amplification of the 5' and 3' arms.
(C) Generation of *Nfatc1*^{CreERT2};*R26-mTmG* and *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice.
(D) Co-immunostaining assay of GFP (green) and Nfatc1 (red) in hair follicles from *Nfatc1*^{CreERT2};*R26*^{mTmG} mice at P21 (Telogen) and P30 (Anagen). The mice were administered single dose of TAM, and skin samples were collected 48 hours post-induction (p.i.). n = 3 mice for each timepoint. Bu indicates bulge. Scale bar, 25 μm.
(E) Experimental strategy for marking *Nfatc1*$^{+}$ cells using female *Nfatc1*^{CreERT2};*R26*^{mTmG} mice at the age of 8 weeks.
(F) Co-immunostaining assay of tdTomato (green) and K14 (red) in mammary glands from *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice at the age of 8 weeks. The mice were administered TAM 3 times, and the samples were collected 48 hours after the last induction. n = 3 mice. Scale bar, 50 μm.
(G) Flow cytometry for tdTomato^{+} cells from *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice at the age of 8 weeks. Another approach to analysis is also shown. Total tdTomato^{+} cells were applied to CD24 and CD29 gates, showing the distribution of tdTomato^{+} cells in the basal and luminal layers. Quantification analysis shows the percentages of tdTomato^{+} basal and luminal cells. n = 3 mice.

Data represent the mean value ± SD.

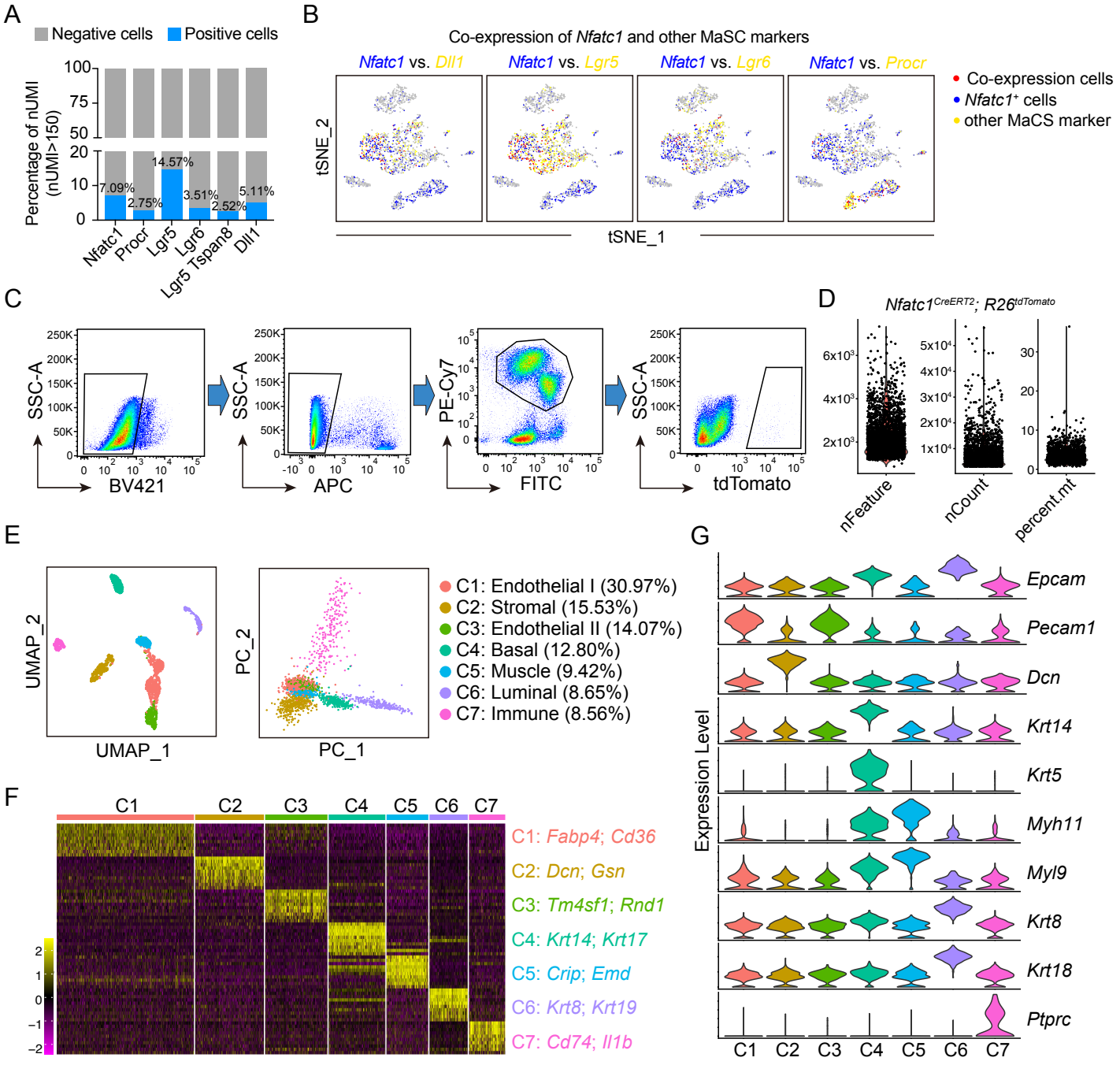

A
Negative cells
Positive cells
Percentage of nUMI (nUMI>150)
100
75
50
20
10
0
7.09%
2.75%
14.57%
3.51%
2.52%
5.11%
Nfatc1
Procr
Lgr5
Lgr6
Lgr5 Tspan8
Dll1
B
Co-expression of Nfatc1 and other MaSC markers
Nfatc1 vs. Dll1
Nfatc1 vs. Lgr5
Nfatc1 vs. Lgr6
Nfatc1 vs. Procr
tSNE_2
tSNE_1
Co-expression cells
Nfatc1+ cells
other MaCS marker
C
SSC-A
BV421
APC
PE-Cy7
FITC
tdTomato
D
Nfatc1CreERT2; R26tdTomato
nFeature
nCount
percent.mt
E
UMAP_2
UMAP_1
PC_2
PC_1
C1: Endothelial I (30.97%)
C2: Stromal (15.53%)
C3: Endothelial II (14.07%)
C4: Basal (12.80%)
C5: Muscle (9.42%)
C6: Luminal (8.65%)
C7: Immune (8.56%)
F
C1
C2
C3
C4
C5
C6
C7
C1: Fabp4; Cd36
C2: Dcn; Gsn
C3: Tm4sf1; Rnd1
C4: Krt14; Krt17
C5: Crip; Emd
C6: Krt8; Krt19
C7: Cd74; Il1b
G
Expression Level
Epcam
Pecam1
Dcn
Krt14
Krt5
Myh11
Myl9
Krt8
Krt18
Ptprc

Figure S2. *Nfatc1* reporter-marked stem/progenitor cells are dormant and distinct from other known MaSCs. Related to Figure 2.

(A) Quantification analysis showing the percentages of *Nfatc1*$^{+}$, *Procr*$^{+}$, *Lgr5*$^{+}$, *Lgr6*$^{+}$, double *Lgr5*$^{+}$*Tspan8*$^{+}$ and *Dll1*$^{+}$ basal cells among total basal cells. A nUMI count > 150 was used as a cutoff to remove low-expression cells.

(B) Feature plots showing the co-expression of Nfatc1 and other MaSC marker genes (*Dll1*, *Lgr5*, *Lgr6*, and *Procr*). The expression of *Nfatc1* is shown in blue, the expression of other MaSC marker genes is shown in yellow, and co-expression is shown in red.

(C) The FACS strategy for sorting tdTomato^{+} cells for single-cell RNA-seq. *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice were administered TAM 3 times, and the samples were collected 48 hours after the last induction.

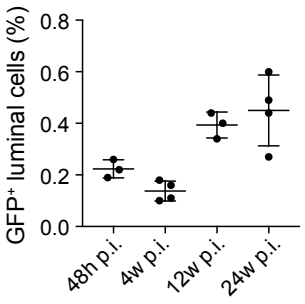
(D) Quality control for the scRNA-seq data of total tdTomato^{+} cells from *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice.

(E) UMAP and PCA plots reveal the cellular heterogeneity of the 2592 sorted tdTomato^{+} cells from *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice in (C).

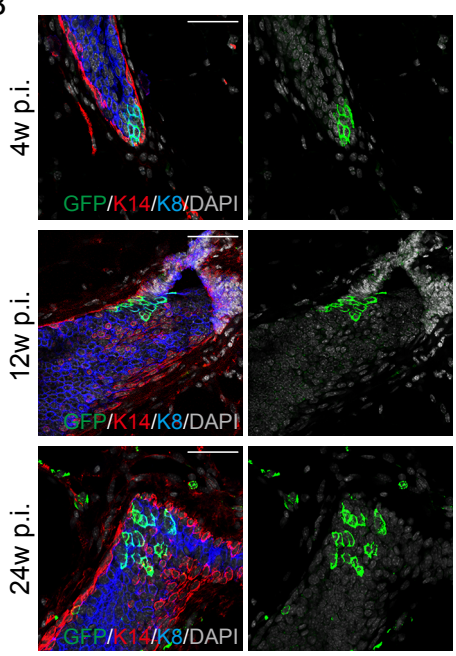
(F) Heatmap showing differentially expressed genes in each cluster in (E).

(G) Violin plots showing known signature genes in each cluster.

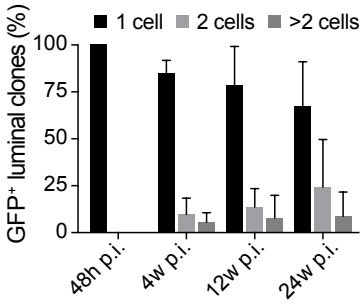
A



B



C



D

→ # of cells per clone

	1	2	3	>3	Total luminal clones
48h p.i.	52	0	0	0	52
4w p.i.	57	6	2	3	68
12w p.i.	46	15	5	11	77
24w p.i.	33	8	2	3	46

n = 3, 3, 4, 4

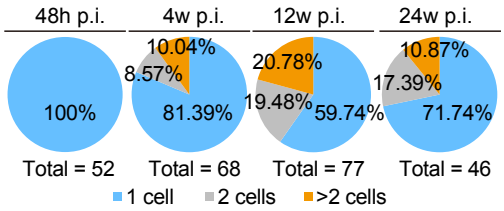


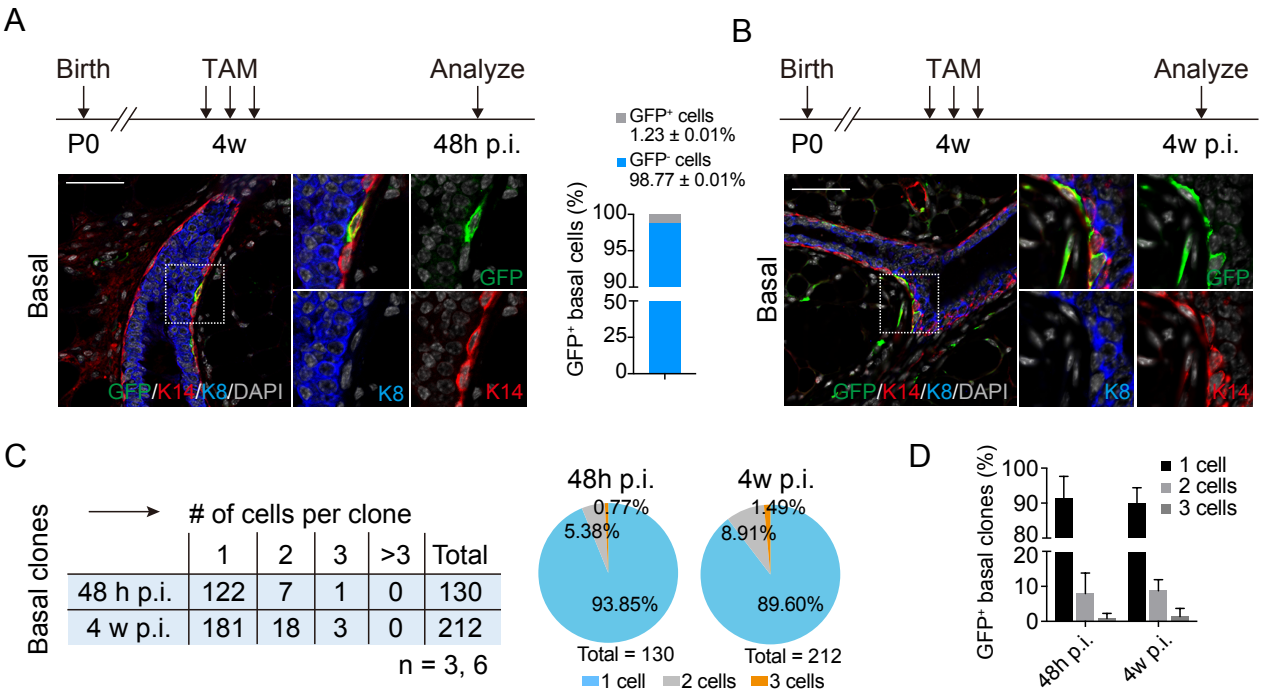
Figure S3. Contribution of *Nfatc1* reporter-marked luminal epithelial cells to the mammary epithelium during homeostasis. Related to Figure 3.

(A) Quantification of GFP^+ luminal cells among total luminal cells in $Nfatc1^{CreERT2}$;$R26^{mTmG}$ mice 48 hours and 4, 12, and 24 weeks post-induction. n = 3, 3, 4, and 4 mice, respectively.

(B) Co-immunostaining assay of GFP (green), K14 (red), and K8 (blue) in $Nfatc1^{CreERT2}$;$R26^{mTmG}$ mice 4, 12, and 24 weeks post-induction, showing the existence of luminal multicell GFP^+ clones. Scale bar, 50 μm.

(C and D) Quantification analysis showing the number and percentage of single-cell, two-cell and multicell luminal clones in $Nfatc1^{CreERT2}$;$R26^{mTmG}$ mice at the indicated timepoints (D). n = 3, 3, 4, 4 mice, respectively. Another statistical method is shown in (C). In total, 52, 68, 77 and 46 luminal clones were quantified, respectively.

Data represent the mean value ± SD.



C

Basal clones

# of cells per clone	1	2	3	>3	Total
48 h p.i.	122	7	1	0	130
4 w p.i.	181	18	3	0	212

n = 3, 6

D

E

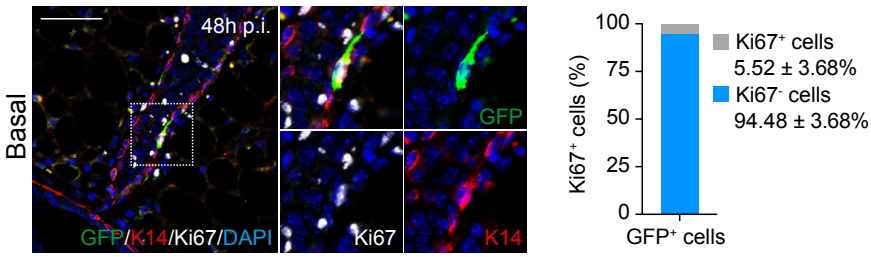


Figure S4. The contribution of *Nfatc1* reporter-marked epithelial cells to the homeostasis of the mammary epithelium during puberty. Related to Figure 3.

(A) Co-immunostaining assay of GFP (green), K14 (red), and K8 (blue) in the *Nfatc1*^{CreERT2};*R26*^{mTmG} mammary gland. The female mice were administered TAM 3 times at the age of 4 weeks, and the samples were collected 48 hours after the last induction. Quantification analysis showing the percentage of GFP^{+} basal cells among total basal cells at this timepoint. In total, 2018 basal epithelial cells from 3 mice were quantified.

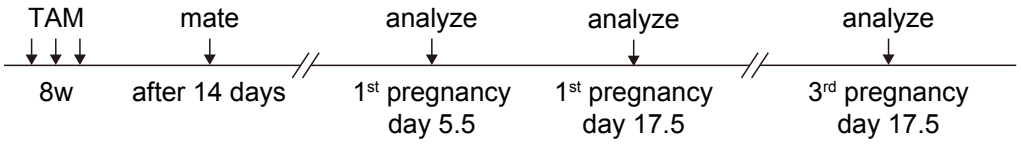
(B) Co-immunostaining assay of GFP (green), K14 (red), and K8 (blue) in the *Nfatc1*^{CreERT2};*R26*^{mTmG} mammary gland. The female mice were administered TAM 3 times at the age of 4 weeks, and the samples were collected 4 weeks after the last induction.

(C and D) Quantification analysis showing the number (left panel in (C)) and the percentage (right panel in (C)) of single-cell, two-cell and multicell basal clones in *Nfatc1*^{CreERT2};*R26*^{mTmG} mice at the indicated timepoints. Another statistical result is also shown in Panel (D). n = 3 and 6 mice 48 hours and 4 weeks after induction, respectively and a total of 130 and 212 basal clones were quantified, respectively.

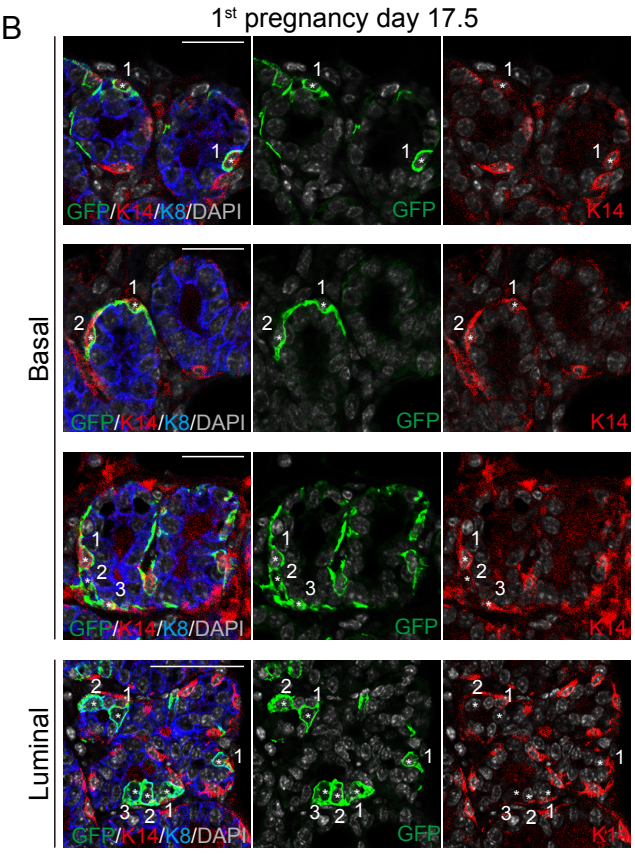
(E) Co-immunostaining assay of GFP (green), K14 (red) and Ki67 (white) in *Nfatc1*^{CreERT2};*R26*^{mTmG} mice. Scale bar, 50 μm. Quantification analysis shows the percentage of GFP^{+}Ki67^{+} basal cells per GFP^{+} basal cells. In total, 129 GFP^{+} cells from 3 mice were quantified.

Data represent the mean value ± SD.

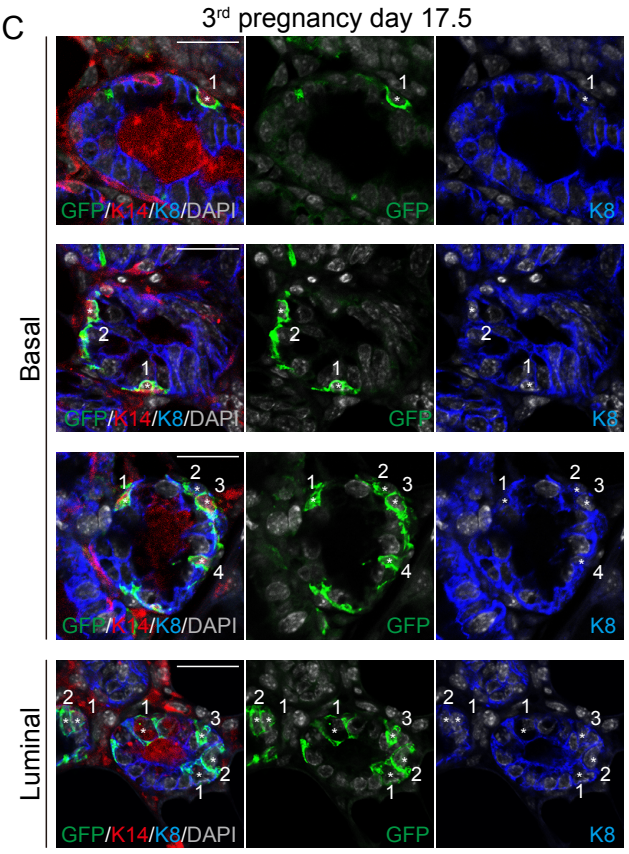
A



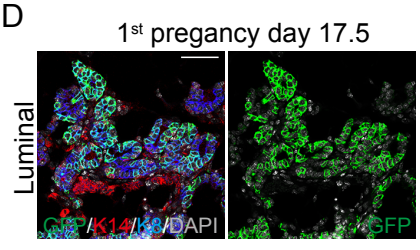
B



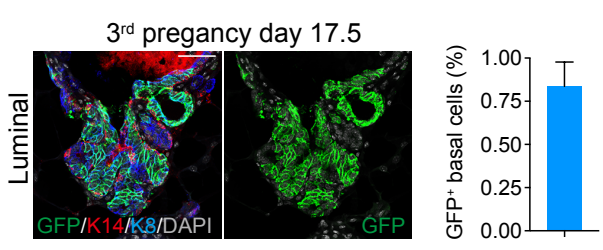
C



D



E



F

luminal clones → # of cells per clone

pergnancy cycle	1	2	3	4	>4	Total
1st day 5.5	46	12	8	0	0	66
1st day 17.5	49	24	12	4	26	115
3rd day 17.5	10	8	5	3	15	41

n = 3, 3, 3

G

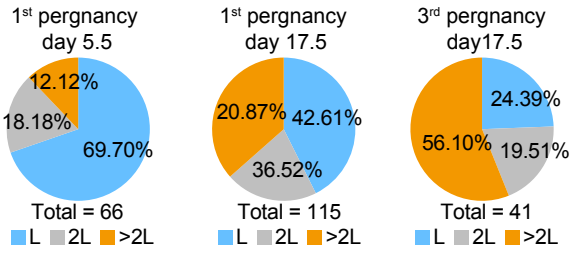


Figure S5. *Nfatc1* reporter-marked luminal cells contribute to lobuloalveolar development during pregnancy. Related to Figure 4.

(A) Strategy for the lineage tracing during pregnancy.

(B and C) Immunostaining analysis showing the identification standards for single-cell, two-cell and multicell GFP^{+} clones in the basal and luminal layers. *Nfatc1*^{CreERT2};*R26*^{mTmG} mice were analyzed on 1st pregnancy day 17.5 (B) and 3rd pregnancy day 17.5 (C).

(D and E) Co-immunostaining assay of GFP (green), K14 (red), and K8 (blue) in *Nfatc1*^{CreERT2};*R26*^{mTmG} mammary glands at 1st pregnancy day 17.5 (D) and 3rd pregnancy day 17.5 (E). Scale bar, 50 μm. Quantification analysis showing the percentage of GFP^{+} luminal cells among total luminal cells for *Nfatc1*^{CreERT2};*R26*^{mTmG} mice at 1st pregnancy day 17.5 (D) and 3rd pregnancy day 17.5 (E). n = 3 mice for each timepoint.

(F and G) Clonal analysis showing the number (F) and the percentage (G) of single-cell, two-cell and multicell luminal clones in *Nfatc1*^{CreERT2};*R26*^{mTmG} mice at 1st pregnancy day 5.5, 1st pregnancy day 17.5 and 3rd pregnancy day 17.5. n = 3 mice for each timepoint.

Data represent the mean value ± SD.

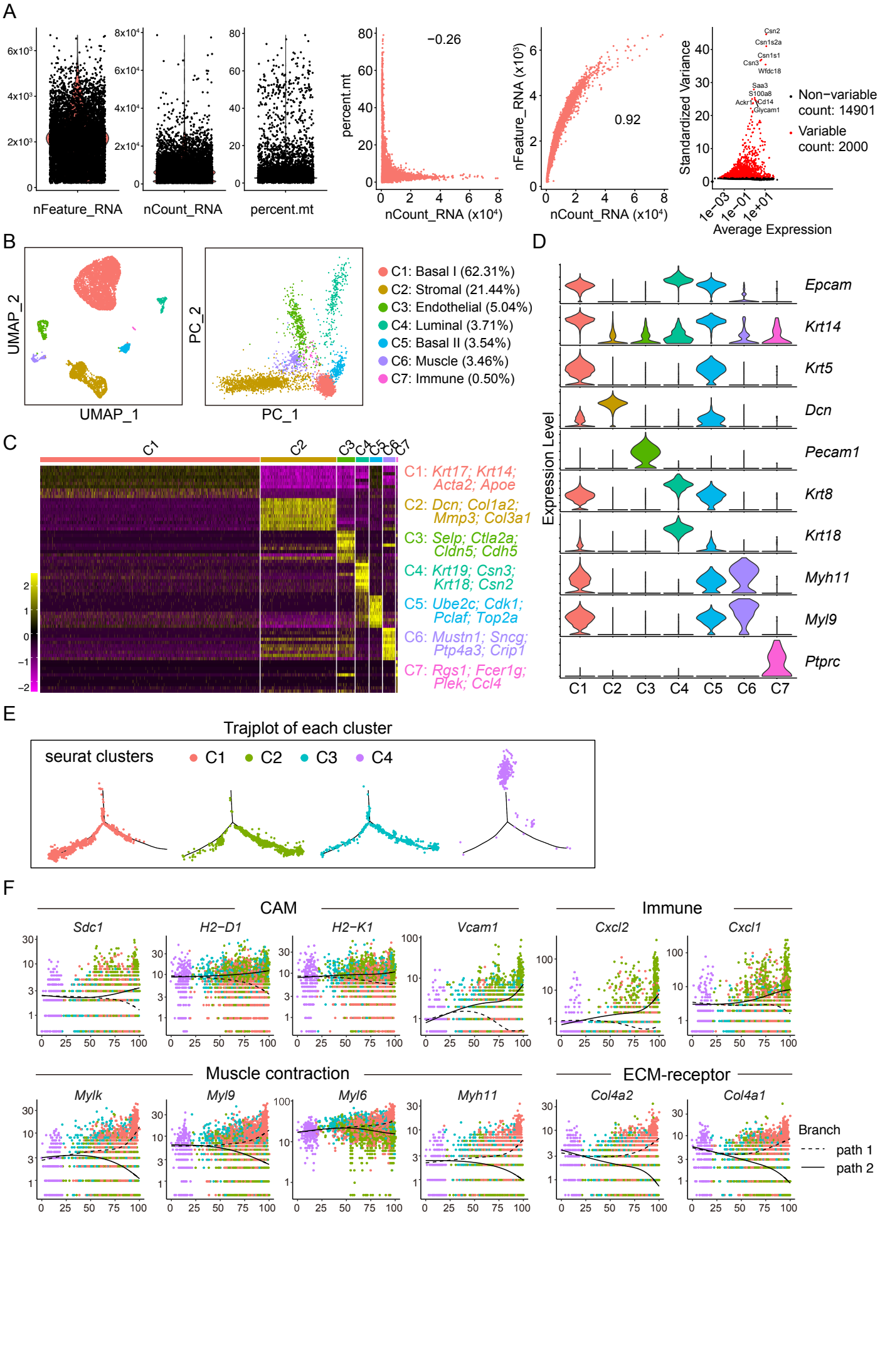

A
nFeature_RNA
nCount_RNA
percent.mt
−0.26
nCount_RNA (x10⁴)
0.92
nFeature_RNA (x10³)
Standardized Variance
Average Expression
Non−variable count: 14901
Variable count: 2000
B
UMAP_1
UMAP_2
PC_1
PC_2
C1: Basal I (62.31%)
C2: Stromal (21.44%)
C3: Endothelial (5.04%)
C4: Luminal (3.71%)
C5: Basal II (3.54%)
C6: Muscle (3.46%)
C7: Immune (0.50%)
C
C1: Krt17; Krt14; Acta2; Apoe
C2: Dcn; Col1a2; Mmp3; Col3a1
C3: Selp; Ctla2a; Cldn5; Cdh5
C4: Krt19; Csn3; Krt18; Csn2
C5: Ube2c; Cdk1; Pclaf; Top2a
C6: Mustn1; Sncg; Ptp4a3; Crip1
C7: Rgs1; Fcer1g; Plek; Ccl4
D
Expression Level
Epcam
Krt14
Krt5
Dcn
Pecam1
Krt8
Krt18
Myh11
Myl9
Ptprc
E
Trajplot of each cluster
seurat clusters
F
CAM
Immune
Muscle contraction
ECM-receptor
Sdc1
H2−D1
H2−K1
Vcam1
Cxcl2
Cxcl1
Mylk
Myl9
Myl6
Myh11
Col4a2
Col4a1
Branch
path 1
path 2

Figure S6. Nfatc1-lineage basal epithelial cells are heterogeneous during pregnancy. Related to Figure 5.

(A) Quality control for the scRNA-seq data of tdTomato^{+} cells sorted from *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice at 1st pregnancy day 14.5. The female mice were administered TAM 3 times at the age of 8 weeks and then bred until pregnancy. The samples were collected at 1st pregnancy day 14.5.

(B) UMAP and PCA plots reveal the cellular heterogeneity of the 6745 sorted tdTomato^{+} cells from *Nfatc1*^{CreERT2};*R26*^{tdTomato} mice on 1st pregnancy day 14.5.

(C) Heatmap showing differentially expressed genes in each cluster.

(D) Violin plots showing the signature genes for each cluster.

(E) Each cluster is shown in the order of the pseudotime trajectory.

(F) Some genes functioning in CAM, immune, muscle contraction and ECM receptors were distributed in distinct directions of the pseudotime trajectory.