

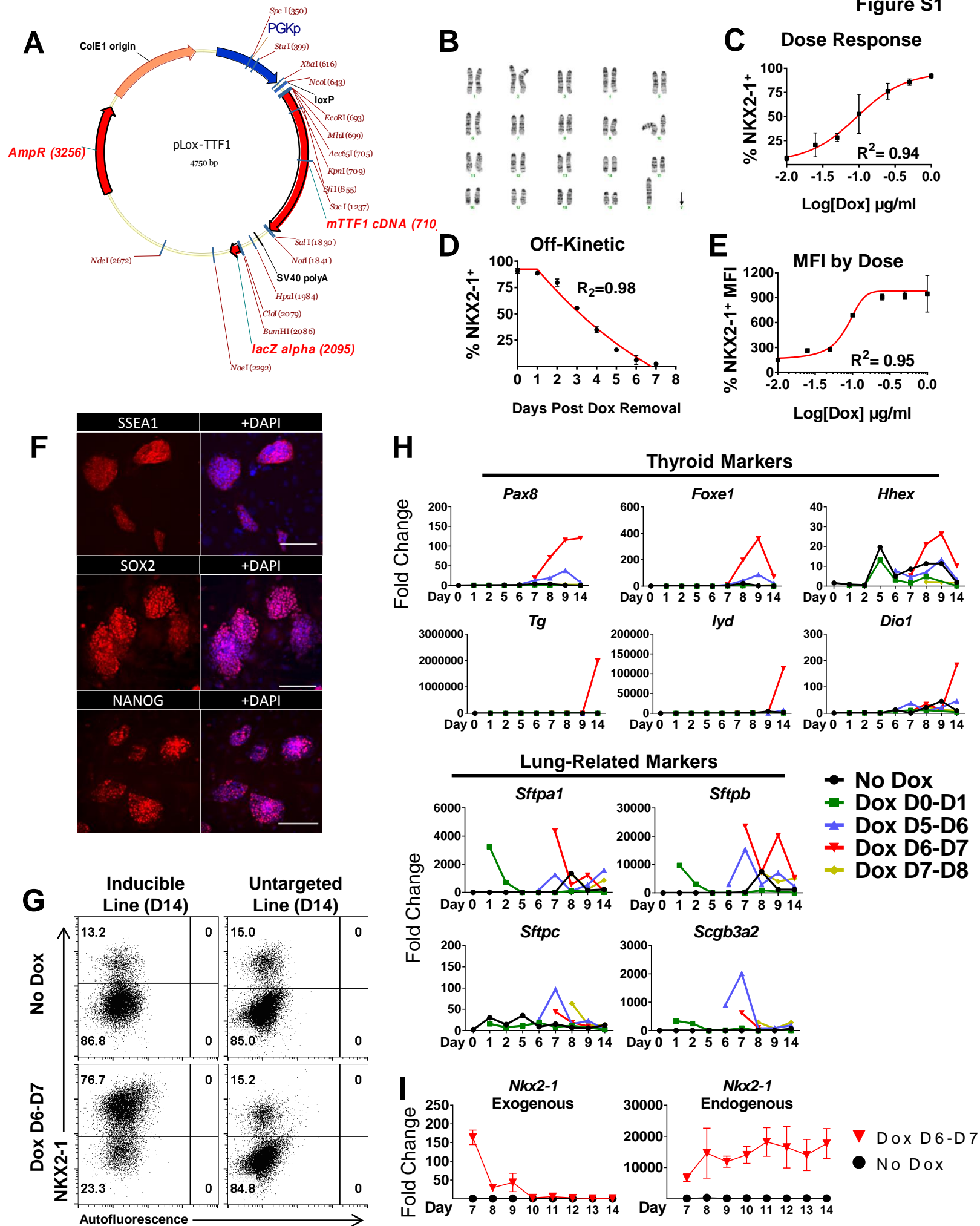
**Stem Cell Reports, Volume 8**

**Supplemental Information**

**Thyroid Progenitors Are Robustly Derived from Embryonic Stem Cells  
through Transient, Developmental Stage-Specific Overexpression of  
Nkx2-1**

**Keri Dame, Steven Cincotta, Alex H. Lang, Reeti M. Sanghrajka, Liye Zhang, Jinyoung Choi, Letty Kwok, Talitha Wilson, Maciej M. Kańduła, Stefano Monti, Anthony N. Hollenberg, Pankaj Mehta, Darrell N. Kotton, and Laertis Ikonomou**

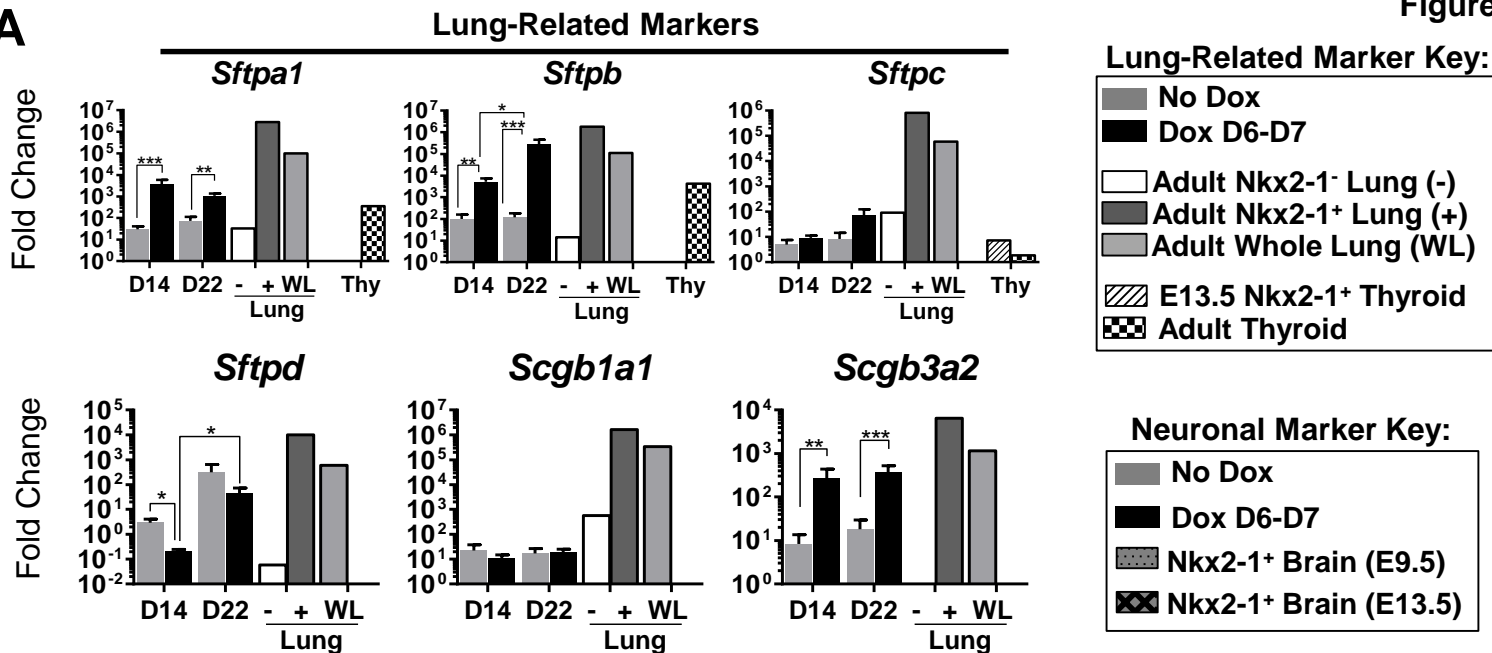
Figure S1



**Figure S1. (Related to Figure 1) Characterization of inducible line**

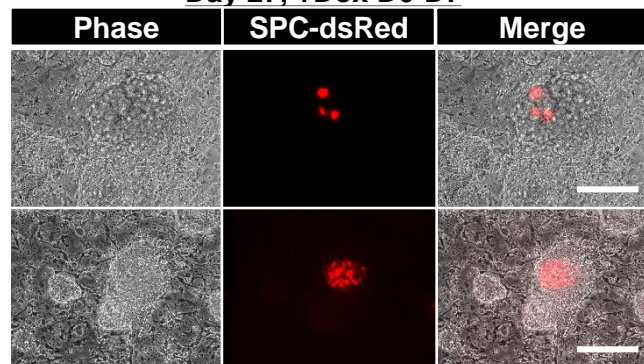
- (A) Plasmid map containing the Nkx2-1 transgene used to generate the iNkx2-1 line (Vector NTI graphic).
- (B) Karyotyping results for the iNkx2-1 line.
- (C) Dose response curve showing the percentage by flow cytometry of undifferentiated mES cells expressing NKX2-1 24 hr following a logarithmic gradient of Dox concentrations added to the media (n=2 independent experiments). Higher doses (>10  $\mu\text{g/ml}$ ) appeared to have a toxic effect on the cells (data not shown). A sigmoidal dose response, variable curve models the equation  $Y = 3.190 + \frac{95.79 - 3.190}{1 + 10^{\text{Log}(-1.028 - X)(-1.309)}}$
- (D) Off kinetics of NKX2-1 expression in the iNkx2-1 line by flow cytometry following the removal of Dox from the media of undifferentiated cells (n=3 independent experiments) showing the percentage of cells steadily decreases, with virtually no remaining NKX2-1<sup>+</sup> cells after one week. The trend line models a plateau, followed by one phase decay according to the equation  $Y = IF (x < 1, 92.45, -91.38 + (183.8)e^{-0.1211 \cdot (x-1)})$
- (E) Flow cytometry median fluorescence intensity (MFI) of the NKX2-1<sup>+</sup> population, following 24 hrs of treatment with increasing doses of Dox. The MFI of the NKX2-1<sup>+</sup> population was consistently high above 0.1  $\mu\text{g/ml}$  of Dox, indicating the effect of the Dox regulates the percentage of NKX2-1<sup>+</sup> cells and not the amount of expression per cell.
- (F) Immunostaining for markers of pluripotency in undifferentiated iNkx2-1 mES colonies. Scale bar represents 200  $\mu\text{m}$ .
- (G) D14 flow cytometry from parallel differentiations using the parent untargeted line and the targeted inducible line with and without Dox addition D6-D7.
- (H) Time course of thyroid and lung marker gene expression by RT-qPCR following individual 24-hr pulses of Dox treatments at specific stages of the directed differentiation protocol. Fold changes are calculated relative to D0 undifferentiated cells, error bars represent SD (n=3 independent experiments).
- (I) Time course of exogenous and endogenous Nkx2-1 expression following Dox treatment from D6-D7. Error bars represent SEM from three independent experiments

A



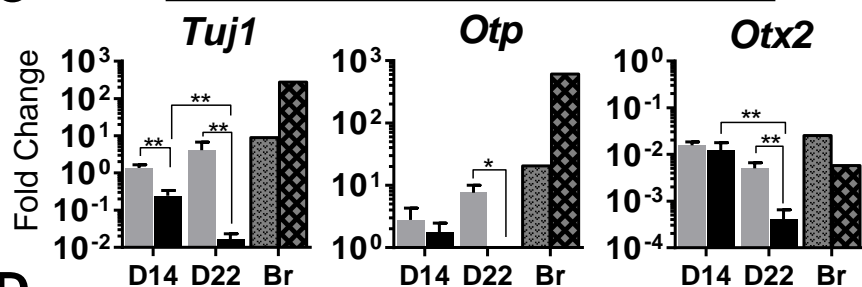
B

Day 27, +Dox D6-D7

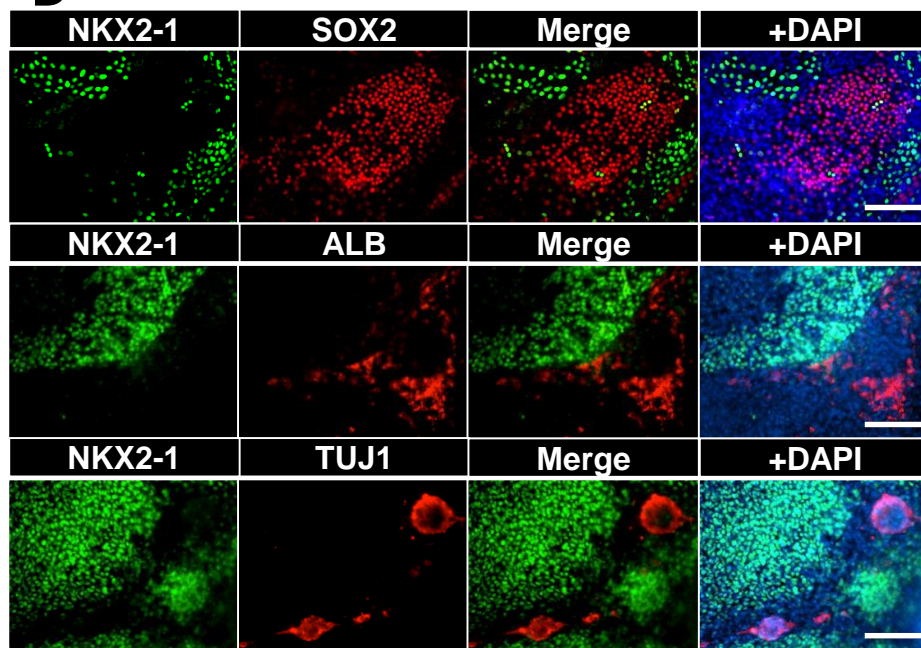


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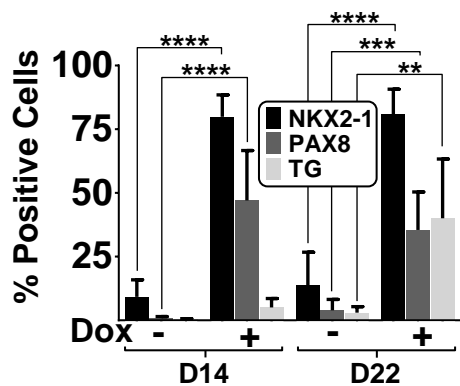
Neuronal Markers



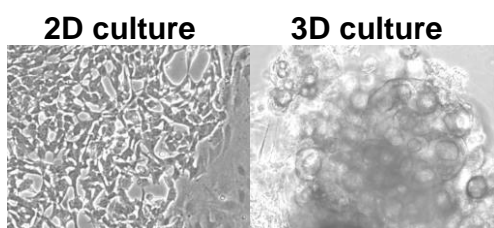
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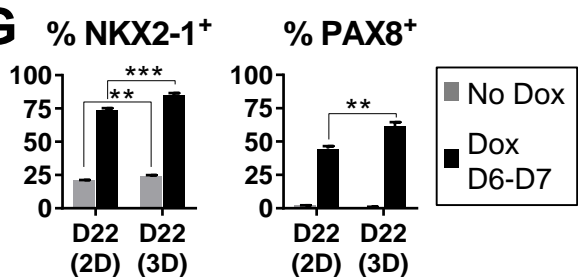
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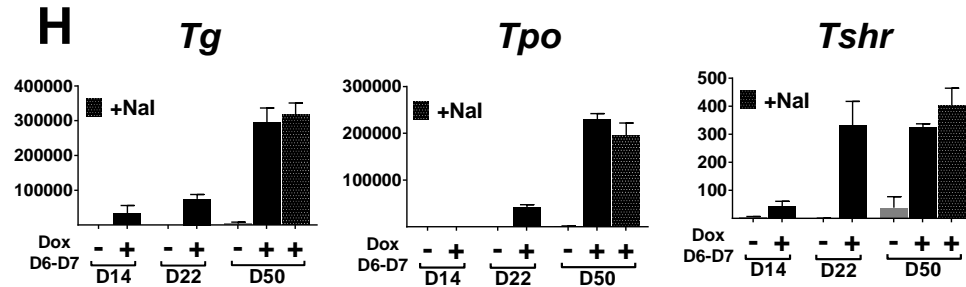
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G



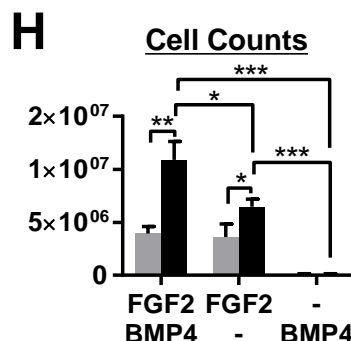
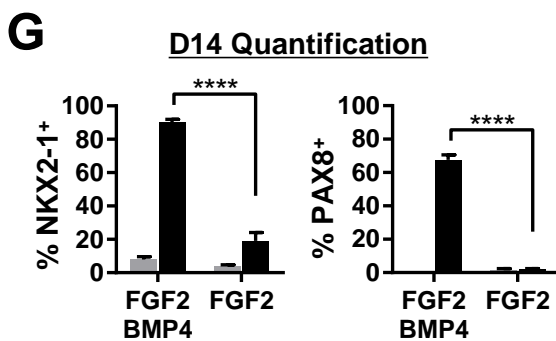
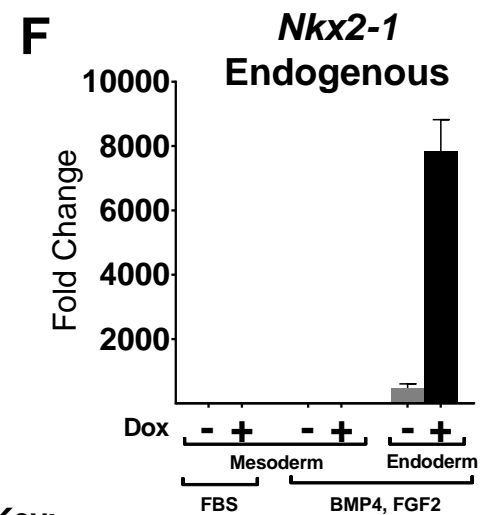
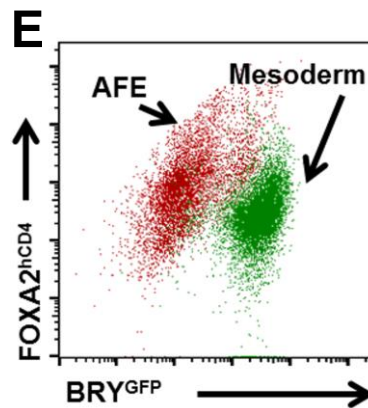
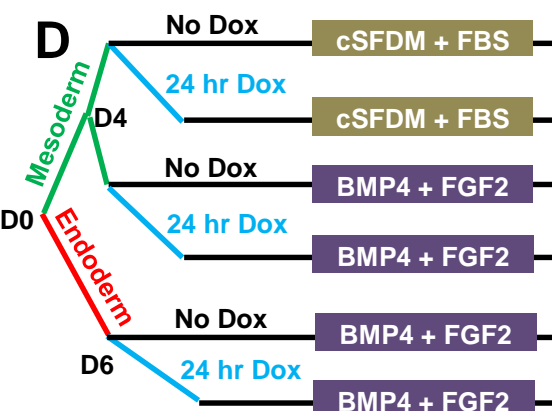
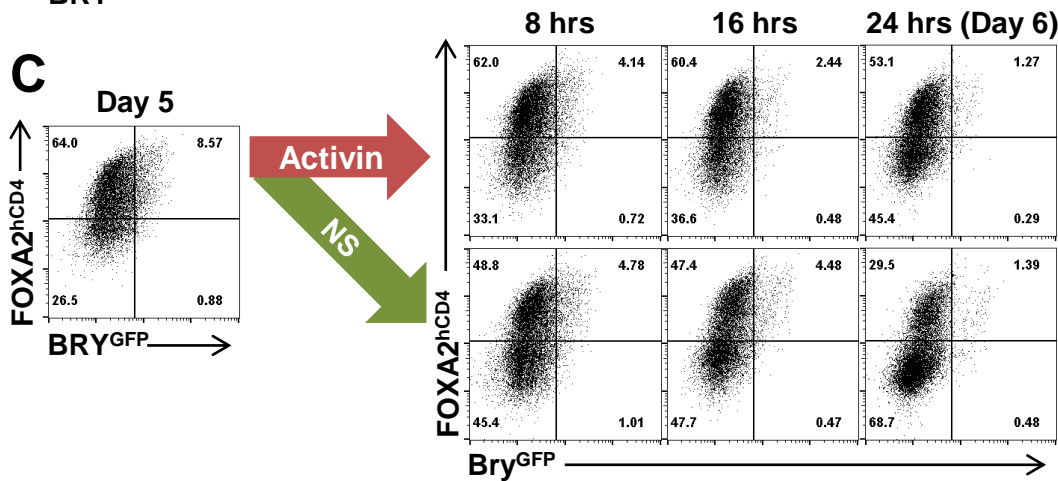
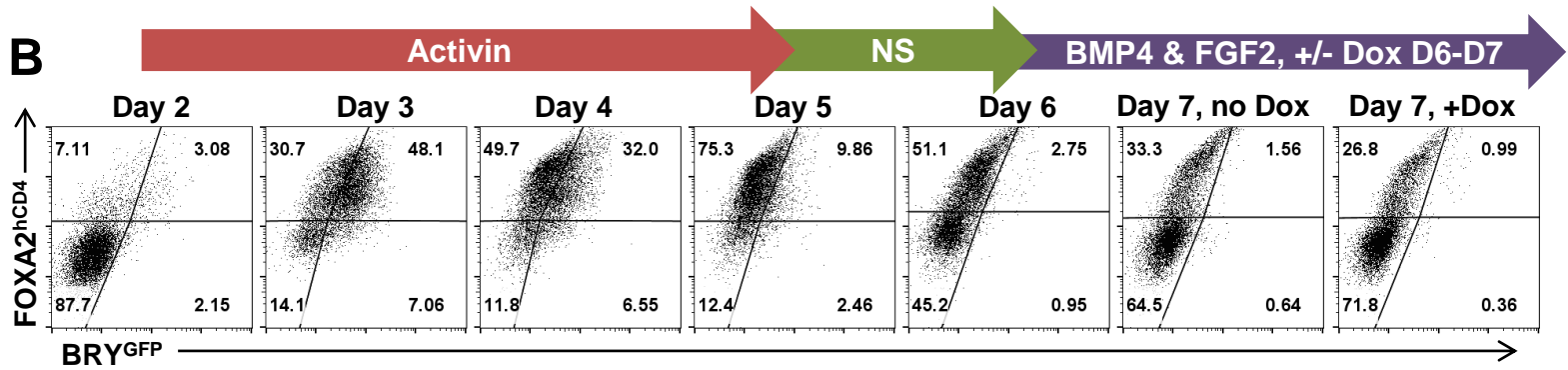
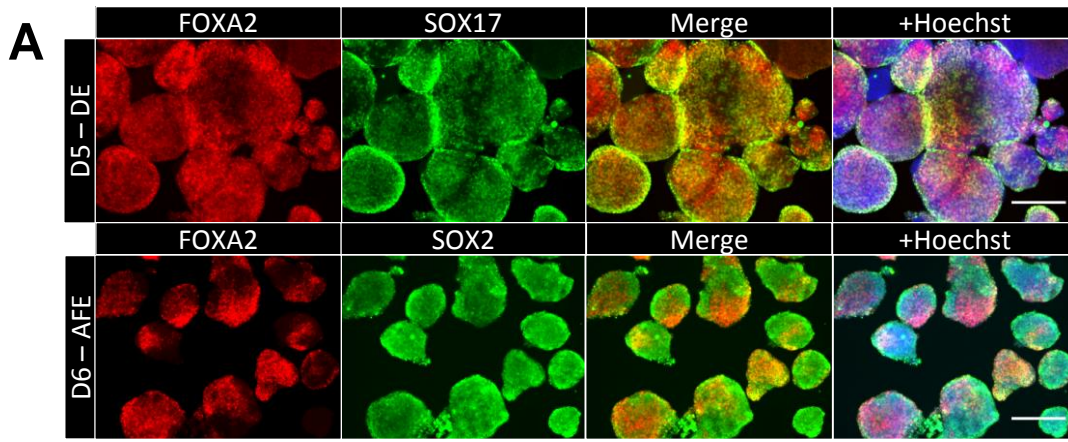
H



**Figure S2. (Related to Figure 2) Nkx2-1 overexpression at AFE stage results in efficient thyroid differentiation**

- (A) Gene expression by RT-qPCR of lung markers in the Dox-treated (D6-D7) and untreated conditions (n=5 independent experiments). Controls are FACS purified Nkx2-1<sup>GFP+/-</sup> lung populations and whole lung from an adult Nkx2-1<sup>GFP</sup> reporter mouse, as well as E13.5 Nkx2-1<sup>GFP+</sup> thyroid and adult thyroid. Fold changes are calculated relative to D0 undifferentiated cells, error bars represent SEM (n=5 independent experiments).
- (B) Cells transduced with the SPC-dsRed reporter lentivirus show minimal induction of the lung alveolar marker SPC by D27. Scale bars represents 100  $\mu$ m.
- (C) Gene expression by RT-qPCR of neuronal markers in the Dox-treated (D6-D7) and untreated conditions (n=5 independent experiments). Controls are FACS purified E9.5, E13.5 Nkx2-1<sup>GFP+</sup> forebrain cells. Fold changes are calculated relative to D0 undifferentiated cells, error bars represent SEM (n=5 independent experiments).
- (D) Immunostaining of D14 Dox-treated (D6-D7) conditions shows non-overlap of NKX2-1 with SOX2 (endodermal or lung lineage marker), ALB (liver marker) and TUJ1 (neural marker). Scale bars represent 100  $\mu$ m.
- (E) Percent positive populations of NKX2-1, PAX8, TG quantified by flow cytometry. Error bars represent SD (n=4 independent experiments).
- (F) Phase contrast micrographs showing the cell morphology at D22 of cells grown on two-dimensional gelatin coated plates (left panel) or embedded in three-dimensional Matrigel from D14-D22 (right panel).
- (G) Flow cytometry quantification of intracellular staining for NKX2-1<sup>+</sup> and PAX8<sup>+</sup> cells grown in 2D vs. 3D culture conditions (n= 3 wells from the same differentiation).
- (H) Gene expression data of cells cultured to D50 for T4 ELISA analysis as in Figure 2D,E (n=3 wells from the same differentiation).





**Key:**

- No Dox
- Dox D6-D7

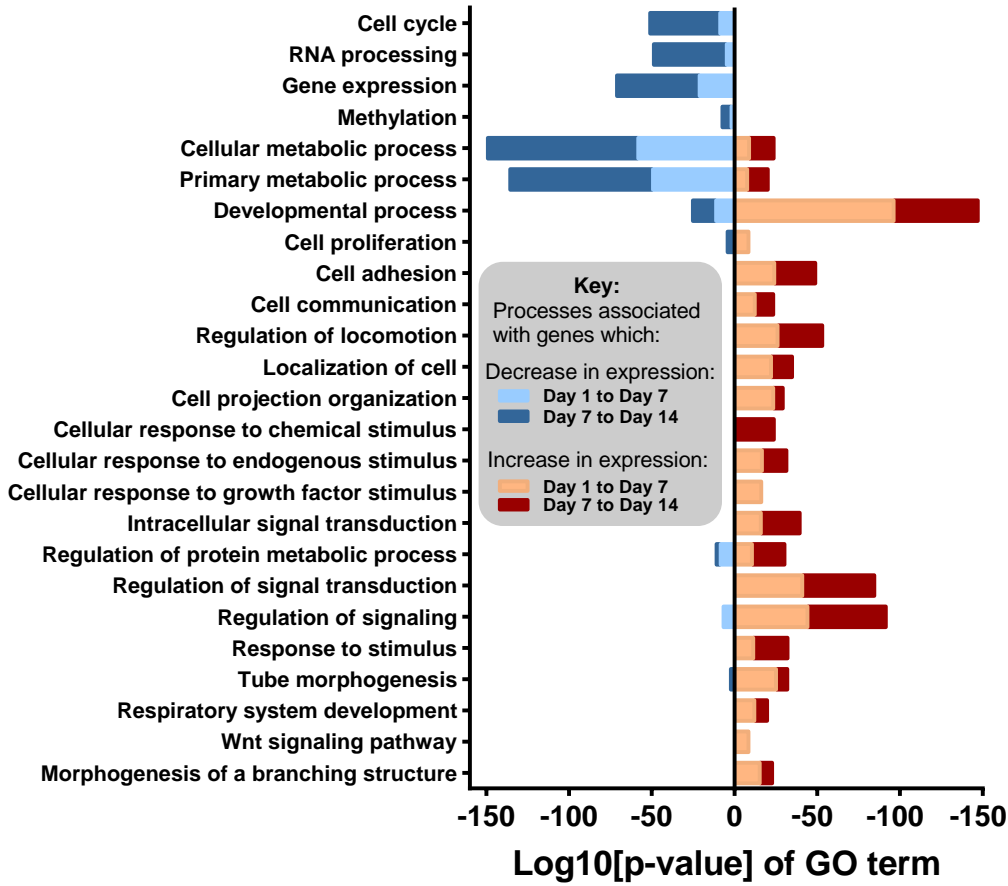
**Figure S3. (Related to Figure 3) Efficient specification is dependent on precise modulation of BMP/FGF signaling and derivation of the thyroid-competent Foxa2<sup>Neg</sup> AFE population**

- (A) Endoderm staining for markers of DE and AFE show efficient derivation. Scale bars represent 200  $\mu\text{m}$ .
- (B) Flow cytometry data showing the induction kinetic of Foxa2<sup>+</sup> definitive endoderm, stained using the hCD4 knock-in reporter. Using the Foxa2<sup>hCD4</sup> and Bry<sup>GFP</sup> reporters we were able to track the DE derivation through an anterior primitive streak (double positive) intermediate. When cells are hereafter exposed to NS treatment, expression of FOXA2 by percentage rapidly decreases during this 24 hr period, resulting in two populations (FOXA2<sup>High</sup> and FOXA2<sup>Neg</sup>).
- (C) D5-D6 FOXA<sup>hCD4</sup> kinetic (NS media vs. Activin). When cells remain in Activin in place of the NS treatment, loss of FOXA2 does not occur to the same extent, indicating this drop is in response to the NS treatment.
- (D) Experimental schematic of AFE vs. mesodermal population derivation and media used for specification and outgrowth. Early mesoderm at D4 (“Mesoderm” branch) was cultured +/- Dox for 24 hrs, either in mesoderm-promoting media (DMEM+FBS) or in the thyroid specification media (BMP4/FGF2) for comparison to the thyroid protocol (“Endoderm” branch), also +/- Dox D6-D7.
- (E) Flow cytometry comparison of FOXA2, BRY expression in AFE vs. mesodermal stage populations.
- (F) RT-qPCR for endogenous Nkx2-1 expression for the conditions described in (E), duplicate samples. Fold changes were calculated relative to D0 undifferentiated cells.
- (G) Flow cytometry quantification at D14 of NKX2-1<sup>+</sup> and PAX8<sup>+</sup> cells from cultures grown (as in Fig 3G) with both specification factors compared to FGF2 alone. Error bars represent SD of biological triplicates within the same differentiation
- (H) Cell counts (cells/well of a 6-well plate) harvested at D14 (same conditions as in G).

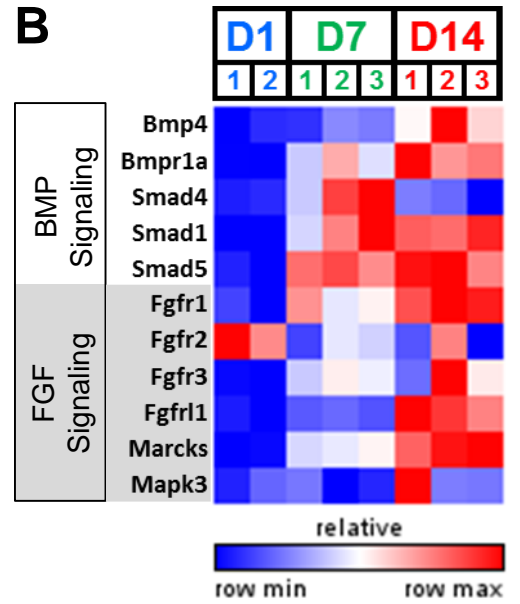
**A**

RNA-Seq Data:  
Gene Ontology in Sample Sets

Figure S4



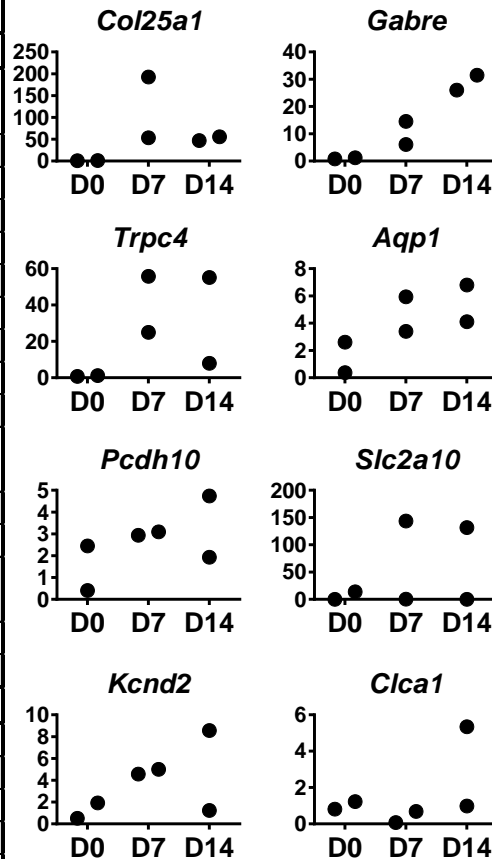
**B**



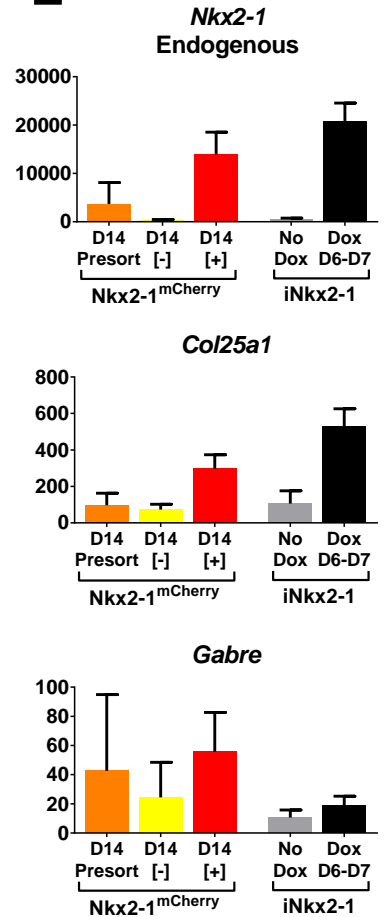
**C**

"Late" Cluster GSEA Analysis	
Enriched GO Molecular Functions	FDR q-val
SEQUENCE-SPECIFIC DNA BINDING	
TRANSCRIPTION FACTOR ACTIVITY	1.80E-02
GROWTH FACTOR ACTIVITY	1.81E-02
COLLAGEN BINDING	2.46E-02
SEQUENCE-SPECIFIC DNA BINDING	4.07E-02
ION CHANNEL ACTIVITY	4.14E-02
CALCIUM ION BINDING	5.64E-02
HEPARIN BINDING	6.22E-02
PROTEIN HETERODIMERIZATION ACTIVITY	6.58E-02
METALLOENDOPEPTIDASE ACTIVITY	6.95E-02
PROTEIN HOMODIMERIZATION ACTIVITY	7.71E-02
RNA POLYMERASE II CORE PROMOTER INVOLVED IN POSITIVE REGULATION OF TRANSCRIPTION	7.77E-02
G-PROTEIN COUPLED RECEPTOR ACTIVITY	1.31E-01
IDENTICAL PROTEIN BINDING	1.44E-01
TRANSMEMBRANE SIGNALING RECEPTOR ACTIVITY	1.59E-01
METALLOPEPTIDASE ACTIVITY	1.76E-01
DNA BINDING	1.83E-01
Enriched KEGG Pathways	FDR q-val
PROTEIN DIGESTION AND ABSORPTION	0.00E+00
NEUROACTIVE LIGAND	0.00E+00
ECM-RECEPTOR INTERACTION	2.89E-03
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	3.19E-02
PI3K-AKT SIGNALING PATHWAY	7.35E-02
PURINE METABOLISM	1.05E-01

**D**



**E**





**Figure S4. (Related to Figure 4) RNA-Sequencing data reveals thyroid developmental processes**

- (A) Functional Analysis: Gene Ontology processes associated with genes with significant differential expression ( $P < 0.05$ ) between time points. Terms shown represent relevant biological processes of interest. Bars represent differentially expressed processes between conditions, full length of each bar is the combined value (stacked) of both sets.
- (B) Differential gene expression from RNA-Seq of selected genes involved in BMP and FGF signaling.
- (C) GSEA pathway analysis of the “Late” cluster gene set.
- (D) RT-qPCR validation data of cell surface markers identified in the “Late” cluster (n=2 independent experiments).
- (E) Further gene expression analysis of select targets from (G) with purified Nkx2-1<sup>mCherry+</sup> from the thyroid protocol for validation (n=3 independent experiments).

## Supplemental Tables

**Table S1. Related to Figure 4. Transcription factors from the “Early” and “Late” clusters identified from RNA-Sequencing** (GEO accession number GSE92572). Relative gene expression across samples, low (blue) to high (red), is indicated.

<sup>a</sup>Genes referenced in Longmire et al.

<sup>b</sup>Genes referenced in Millien et al.

<sup>c</sup>Genes referenced in Fagman et al.

**Table S2. Related to Figure 4. Putative cell surface markers factors from the “Early” and “Late” clusters identified from RNA-Sequencing** (GEO accession number GSE92572). Relative gene expression across samples, low (blue) to high (red), is indicated.

<sup>a</sup>Genes referenced in Longmire et al.

<sup>c</sup>Genes referenced in Fagman et al.

## Supplemental Experimental Procedures:

### mESC Maintenance

Undifferentiated mESCs were maintained on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in serum-containing media consisting of DMEM (Life Technologies, 11995-073) with 15% FBS (Thermo Fisher Scientific, NC0712155), 200 mM L-glutamine (Invitrogen, 25030-164) and 100 µg/ml Primocin (Thermo Fisher Scientific, NC9392943), supplemented with LIF-containing conditioned media (Millipore, ESG1106) at 1 U/ml or an equivalent concentration of conditioned LIF produced in our lab. mESCs were passaged as needed when cultures reached appropriate confluency using 0.05% trypsin (Invitrogen, 25300-120) for continued expansion on MEFs or to start differentiation experiments. ESCs or differentiated cells were counted using the Countess II Automated Cell Counter (Thermo Fisher Scientific, AMQAX1000) according to the manufacturer's instructions.

### Karyotype Analysis

Cytogenetic analysis (Cell Line Genetics) was performed on twenty G-banded metaphase cells from the chosen mESC line. The majority (18/20) cells demonstrated an apparently normal male karyotype, while two cells demonstrated loss of the Y chromosome (Figure S1B), a common feature of cultured mouse ESCs. Cells were expanded for a minimal ( $\leq 4$ ) number of passages before being used in experiments to avoid genetic instability.

### Mouse ESC Directed Differentiation

To initiate directed differentiation, ESC colonies cultured on mouse embryonic fibroblasts (MEFs) were trypsinized to form a single cell suspension and MEFs were depleted for 30 min at 37°C in mESC media on a tissue culture dish. The supernatant containing the ESCs was rinsed and cells were transferred at a density of 500,000 cells per 10 cm petri dish into complete serum-free differentiation media (cSFDM) containing 75% IMDM (Life Technologies, 12440061) and 25% Ham's Modified F12 medium (Thermo Fisher Scientific, MT-10-080-CV) supplemented with N2 (Life Technologies, 17502-048), B27 with RA (Life Technologies, 17504-044), 0.05% BSA (Life Technologies, C15260-037), 2 mM L-glutamine (Invitrogen 25030-164), 0.05 mg/ml ascorbic acid (Sigma A4544-25G),  $4.5 \times 10^{-4}$  M monothioglycerol (MTG) (Sigma M6145) and 100 µg/ml Primocin (Fisher NC9392943). Cells were grown in this media for 48 hours, where they formed embryoid bodies (EBs). At day 2, EBs were monodispersed by trypsinization and cells were re-suspended in cSFDM containing 50 ng/ml Activin A (R&D systems 338-AC) to re-form EBs and induce definitive endoderm. After 72 hrs of Activin A exposure, endoderm induction was confirmed by expression of Foxa2-hCD4 by flow cytometry (Gadue et al., 2006; Gouon-Evans et al., 2006). EBs were rinsed with base media and transferred to anteriorization media containing cSFDM supplemented with 100 ng/ml Noggin (Fisher Scientific, 1967-NG-025) and 10 µM SB431542 (Fisher, 16-141-0) for BMP and TGFβ inhibition. After 24 hr exposure to these factors, EBs were trypsinized for monodispersion and plated at a density of 200,000 cells per 9.5 cm<sup>2</sup> onto gelatin-coated cell culture dishes (Corning Costar, 3516 or 3513). Plating EBs or monodispersed cells at this time point did not significantly affect Nkx2-1 induction or thyroid specification (data not shown). Specification media from day 6 to day 14 consisted of cSFDM supplemented with 10 ng/ml hBMP4, 250 ng/ml rhFGF2 (R&D Systems, 233-FB-01M) and 100 ng/ml Heparin Sodium Salt (Sigma Aldrich H3149-100KU) (Kurmann et al., 2015). Upon initial plating at day 6, ROCK inhibitor (Y27632, TOCRIS 1254) was added to the media until day 7 to aid in survival of monodispersed cells. Dox (Doxycycline Hyclate, Sigma, D9891-5G) was added to treated conditions as indicated at a concentration of 1 µg/ml and removed after 24 hr by rinsing cultures twice with basal media and replacing with Dox-free specification media. Media was replaced as needed until day 14, when cells were harvested for analysis or passaged to day 22 or later for further expansion and maturation in cSFDM supplemented with 250 ng/ml rhFGF2, 100 ng/ml rhFGF10 (R&D 345-FG), and 100 ng/ml Heparin Sodium Salt (FGF2+10 media). As indicated in the text, these day 14 cells were either passaged onto gelatin-coated tissue culture plates at a density of about 21,000 cells/cm<sup>2</sup> or mixed into undiluted growth factor reduced Matrigel drops (Corning, 356231) at a density of 80,000 cells per 100 µl. At day 22 or 30, cells were harvested for analysis as described in the following analysis subsections.

For extended maturation to day 50, cells were grown and plated into Matrigel droplets at day 14 as described above and subsequently cultured as previously described (Kurmann et al., 2015). Media from D14-22 consisted of cSFDM supplemented with 250 ng/ml rhFGF2, 100 ng/ml rhFGF10, 100 ng/ml Heparin Sodium Salt, 1 mU/ml bTSH (Los Angeles Biomedical Research Institute National Hormone & Peptide Program, AFP-8755B), 25 ng/ml rhEGF (R&D Systems, 236-EG), 50 ng/ml IGF1 (R&D Systems, 791-MG), and 10 µg/ml Insulin (Sigma, 10516-5ml). Media from D22-D30 consisted of cSFDM supplemented with 250 ng/ml rhFGF2, 100 ng/ml rhFGF10, 100 ng/ml Heparin Sodium Salt, 1 mU/ml bTSH, 25 ng/ml rhEGF, 50 ng/ml rmlGF1, and 5 µg/ml ITS (Thermo Fisher Scientific, 41400045). Media from D30-D50 consisted of Ham's Modified F12 medium supplemented with 10 mM HEPES (Sigma H0887), 0.25% BSA, 0.8 mM CaCl<sub>2</sub> (Sigma, C1016), 50 nM Dexamethasone (Sigma, D4902), 100 ng/ml

Heparin Sodium Salt, 1 mU/ml bTSH, 25 ng/ml rhEGF, 50 ng/ml rmIGF1, and 5 µg/ml ITS. NaI (Sigma-Aldrich, 383112) was added at a concentration of 10 µM from D40-D50.

Directed differentiation to mesoderm consisted of a two day LIF depletion in cSFDM to allow formation of EBs, followed by two days mesoderm induction with 2 ng/ml Activin A, 3 ng/ml hBMP4, and 0.3 ng/ml rmWnt3a (R&D Systems, 1324-WN). For the purpose of experiments described, cultures at day 6 (endoderm) or day 4 (mesoderm) were plated either into thyroid specification media (FGF2+BMP4 as described above) or mesodermal outgrowth media (cSFDM supplemented with 20% FBS). Cultures were treated with Dox for 24 hrs in parallel to untreated cultures, and analyzed after 8 days of outgrowth.

### Immunostaining

**2-Dimensional culture:** Samples were grown from day 6 to day 14 or 22 (passaged on day 14) on gelatin-coated tissue culture dishes and fixed in 4% paraformaldehyde (PFA) (Ted Pella, 18505) for 15 minutes, then rinsed twice with PBS and stored at 4°C if staining was not performed the same day. Cells were permeabilized with 0.2% Triton X-100 detergent (Thermo Fisher Scientific, 28314) in PBS for five minutes, rinsed twice, and then blocked with 1% BSA in PBS for 30 minutes. The samples were then incubated with the primary antibody in 1% BSA at dilutions listed below overnight at 4°C. Pax8 and Tg antibodies were blocked in 1% BSA with 0.1% Triton X. Fluorophore-conjugated secondary antibodies were added at the concentrations listed below and incubated for one hour at 37°C and rinsed twice with PBS. To counterstain nuclei, cultures were incubated for 5 min with 1 µg/ml DAPI (Life Technologies, D1306) or 500 nM PI (Life Technologies P3566). Cultures were rinsed twice with PBS and stored in PBS at 4°C until imaged.

**3-Dimensional culture:** Cells were grown on gelatin-coated tissue culture dishes from day 6 to day 14, and then passaged into undiluted Matrigel where they were grown until day 22 or 30. Matrigel was dissolved using 2 mg/ml Dispase (Invitrogen 17105-041) in DMEM incubated at 37°C for 30 min, or Cell Recovery Solution (Corning CB-40253) added for 1 hr at 4°C. The resulting free-floating cell structures were fixed in 4% PFA for 15 minutes, and rinsed twice with PBS. Cells were then embedded into agarose (Sigma, A9414) melted at 67°C, and cooled at 4°C or on ice for at least one hour. The agar/cell pellets were then processed into paraffin similar to a tissue sample using standard paraffin embedding procedures. For staining, slides were deparaffinized and steamed for 30 min in Target Retrieval Solution (Dako, S1699). Individual sections on slides were segregated using a PAP pen (Abcam, ab2601) and were blocked with 1% BSA in PBS for 30 minutes. The samples were then incubated with the primary antibody in 1% BSA at dilutions listed below overnight at 4°C. The next day, slides were rinsed twice in PBS and incubated with fluorophore-conjugated secondary antibodies for one hour at 37°C followed by two rinses with PBS. Cells sectioned onto slides were mounted with either SlowFade Diamond Antifade with DAPI (Life Technologies, S36964) or were counterstained with DAPI or Hoechst (Life Technologies, H3570) at 20 µg/ml for 10 min and subsequently mounted with Prolong Diamond Antifade Mountant (Life Technologies, P36961), sealed the following day, and stored at 4°C until imaging.

Primary Antibodies						
Protein Target	Company	Catalog Number	Host Species	Clonality	Dilution: Flow Cyt.	Dilution: ICC/IHC
NKX2-1	Dako	M3575	Mouse	monoclonal		1:200
NKX2-1	Abcam	ab76013 (Isotype ab172730)	Rabbit	monoclonal	1:50	1:200
PAX8	Abcam	ab122944 (Isotype: ab172730)	Rabbit	monoclonal		1:200
PAX8	Biocare	ACI438C (Isotype: NC490)	Mouse	monoclonal	1:50	1:200
TG	Abcam	ab80783	Mouse	monoclonal	1:50	1:200
ECAD	Abcam	ab76319	Rabbit	monoclonal		1:100
ALB	Dako	A0001	Rabbit	polyclonal		1:1000
TUJ1	Sigma	T2200	Rabbit	polyclonal		1:1000
SSEA1	Millipore	90230	Mouse	monoclonal		1:50
NANOG	Abcam	Ab80892	Rabbit	polyclonal		1:50
T4	Thermo Scientific	MA5-14716	Mouse	monoclonal		1:100

NIS	Imanis Life Sciences	REA008	Rabbit	polyclonal		1:2000
FOXA2	Abcam	Ab108422	Rabbit	monoclonal		1:300
SOX17	R&D Systems	AF1924	Goat	polyclonal		1:50
SOX2	BD Pharmingen	561469	Mouse	monoclonal		1:100
hCD4	Life Technologies	MHCD0404 (Isotype: MG2A04)	Mouse (RPE)	monoclonal	1:10	
SIRPA	BD Biosciences	560107 (Isotype: 553925)	Rat (PE)	monoclonal	1:50	
EPHB2	BD Biosciences	564699 (Isotype: 550854)	Mouse (APC)	monoclonal	1:50	
<b>Secondary Antibodies</b>						
Alexa Fluor 488	Invitrogen	A10667	goat anti mouse		1:100	1:300
DyLight 550	Abcam	ab96884	goat anti-rabbit			1:300
Alexa Fluor 647	Invitrogen	A21245	goat anti-rabbit		1:100	

### Image Acquisition

Fluorescent micrographs were acquired on the Nikon Eclipse *Ti* inverted microscope (Boston University Cellular Imaging Core) or a Nikon Eclipse *Ni* upright microscope using the NIS-Elements software. Acquired ND2 files were processed with Fiji (Schindelin et al., 2012; Schneider et al., 2012) to optimize clarity and create merged micrographs. Large-scale composite micrographs (Fig. S3) were acquired using the Nexcelom Celigo Cytellect Imaging Cytometer microwell plate imager.

Phase contrast micrographs and phase contrast / fluorescent micrographs were acquired using a Nikon Eclipse TS100 (Diagnostic Instruments Inc., Model #18.2 Color Mosaic) microscope with a Lumen200 Fluorescent Illumination (Prior Scientific) and the SPOT Insight Firewire 2 camera (Diagnostic Instruments Inc., Model 18.2) and the SPOT Software Version 4.6.1.38.

### Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

To harvest cells, cultures on tissue culture dishes were rinsed and trypsinized with 0.05% trypsin until dissociated. FBS (Fisher, SH3007003E) was added to quench enzymatic activity, and cells were rinsed with basal media (DMEM) and centrifuged to pellet. Cell pellets were stored at -80°C until ready for processing.

RNA was extracted using QIAshredder columns (QIAGEN, 79656) and the RNeasy Plus Mini Kit (QIAGEN, 74136) according to the QIAGEN manual instructions. Purified RNA was eluted in 30 µl nuclease-free water, quantified on the Nanodrop (ND1000), and diluted as needed. cDNA was prepared using Taqman Reverse Transcription Reagents (Applied Biosystems, N808-0234). cDNA samples were prepared with TaqMan Fast Universal Master Mix (Life Technologies, 4367846) and diluted to 6.25 ng per 25 µl reaction for real-time analysis on the StepOnePlus Real Time PCR System (Applied Biosystems). Relative expression analysis calculations were performed according to the  $\Delta\Delta CT$  method (Livak and Schmittgen, 2001) utilizing 18S rRNA as the internal reference gene and undifferentiated ESCs collected prior to each differentiation as the reference sample. If expression was undetected based on the set threshold, the value was set to the maximum number of cycles (40) to allow fold change calculations. Data was graphed for visualization as shown in the figures using the GraphPad Prism 6 software.

Custom probes specific for endogenous and exogenous Nkx2-1 were designed using the Custom Taqman Assay Design Tool. Sequences were entered that spanned the transgene to the 3' UTR to create the endogenous specific probe, and the transgene to the polyA tail of the transgene to create the exogenous specific probe.

Initial validation for the RNA-Sequencing was performed using Custom TaqMan Array Plates, 96 well format (Thermo Fisher Scientific, 4413255, "Early" cluster Design ID: PPFGSW4, "Late" cluster Design IDs: PP4FSDO, PP5OQJW).

RT-qPCR Taqman Probes		
Expression Target	Company	Catalog Number
18S rRNA	Thermo Fisher	4319413e
Endogenous Nkx2-1	Thermo Fisher	4441117 Assay ID: AJLJIJO
Exogenous Nkx2-1	Thermo Fisher	4331348 Assay ID: AIVI6DF
Pax8	Thermo Fisher	Mm00440623_m1

Tg	Thermo Fisher	Mm00447525_m1
Foxe1	Thermo Fisher	Mm00433948_m1
Hhex (Hex1)	Thermo Fisher	Mm00433954_m1
Slc5a5 (Nis)	Thermo Fisher	Mm00475074_m1
Tpo	Thermo Fisher	Mm00456355_m1
Tshr	Thermo Fisher	Mm00442027_m1
Iyd	Thermo Fisher	Mm00503564_m1
Dio1	Thermo Fisher	Mm00839358_m1
Sftpa (Spa)	Thermo Fisher	Mm00499170_m1
Sftpb (Spb)	Thermo Fisher	Mm00455681_m1
Sftpc (Spc)	Thermo Fisher	Mm00488144_m1
Scgb3a2	Thermo Fisher	Mm00504412_m1
Trp63 (P63)	Thermo Fisher	Mm00495788_m1
Scgb1a1 (Cc10)	Thermo Fisher	Mm00442046_m1
Abca3	Thermo Fisher	Mm00550501_m1
Col25a1	Thermo Fisher	Mm00472589_m1
Gabre	Thermo Fisher	Mm00489935_m1

**Target sequence of *Nkx2-1* 3' UTR used to design the *Nkx2-1* endogenous-only probe:**

GTTCTACCTTGCTTTATGGTCGGACCTGGTGAGACGTGAGATGCGCTTGA  
 GCCCCGCGACCTCAACGCTTCCCCTCTGCCTTCCGCAAAGACCACCAT  
 TCGCCCGCTGCTCCACGCGCTTCTACTTTTTTAAGAATCTGTTTATGTT  
 TAGACCAAGGAAAAGTACACAAAGACCAAAGTCTGGACGACTTCTTCTT  
 CTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT  
 TCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT  
 TCCTCTCCTCCTCTTCTCCTTCTTGTCCCCGCTCGTTCTTTCTTTCT  
 CCCCCTCCTCTTCTGTTTCCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT  
 TCTTTACTATCTAAAACCTGACACTTTTTGTTTTTAAACATAAAAAGAA  
 AATAGAAACAGCCAAGCAAATTC AACCTTTACGGATTCTTTAAACAGAG  
 AAGGACAGAGAACAATTTGGGGTGTCTTTCTGGTAGTTCAAATGGGTTC  
 CCAAGCTTAGGCATGGCACAGTTTTGGAGCCTGTTCTATGCTTCCATGGC  
 CCTGAACTCTAAAGACGGAAAACCTTTCTGTGGATGCACCCTGCCAGCAA  
 AGTGAGCTTGCTTGTAATACCAGGATTTTTCGTTTGTGTTGTATGTTTCA  
 GAAGGGAGGACAGACGCTGGAGATAGGAAAGTCTTCAGCATAAACCATTT  
 GTACCTGACACAAAGGAAGTGTCCCCTCCCAGGCGCCCTCTGGCCCTACA  
 GGTTCAGTCCAGGCTGGCCTTTCAGAAAATTGTTTTAGGTTTGATGTGAA  
 CTTGTAGCTGTAATAATGCTGTTAAAAGTTGGACTAAATGCCTAGTTTTTA  
 GTAACCTGTACATTATGTTGTA AAAAGAACCCAGTCCCAGTCCCTAGTC  
 CCTCACTTTTTCAAGGGGCATTGACAAACCTGTGTATATTATTTGGCAGT  
 TTGGTATTTGCAGCACCAATCCTTTTTTTTTTTCTGTTGTA ACTTATGTA  
 GATATTTGGCTTAAATATAGTTCCTAAGAAGCTTCTAATAAATTATACGA  
 ATTA AAAAAGATGGTTTTTTTTTCTTGATTA

**Target sequence spanning *Nkx2-1* transgene to polyA tail used to design the *Nkx2-1* exogenous-only probe:**

GCTTTATGGTTCGGACCTGGTGAGTCGACCCGGCGGCCGCTTCGAGCAGA  
 CATGATAAGATACATTGATGAGTTTGGACAAACCACA ACTAGAATGCAGT  
 GAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATT

**Intracellular Flow Cytometry**

Samples were monodispersed by trypsinization and fixed in 1.6% PFA in PBS for 30 min at 37°C. Samples were stored for up to two weeks in PBS with 0.5% BSA and 0.02% Sodium Azide (NaN<sub>3</sub>) at 4°C before staining. To stain, samples were rinsed twice in 1X Saponin buffer (Permeability Wash Buffer 10x, Biolegend 421002). Primary antibodies were added in 1X Saponin buffer at the dilutions stated below and incubated for one hour at room temperature. Samples were rinsed twice with 1X Saponin buffer and then incubated with the secondary antibodies in 1X Saponin for 30 min at room temp. They were then rinsed twice with 1X Saponin and transferred to PBS with 2% FBS into filter-top FACS tubes (Falcon, 352235). Flow cytometry data was acquired from the BD FACSCalibur using the BD CellQuest software. Exported sample data was gated and analyzed as shown in the figures and text using the



FlowJo v10 software. Flow cytometry controls included unstained cells, cells incubated with the secondary antibody only (no primary), isotype controls, and negative controls (undifferentiated cells or cell populations known not to express the protein of interest).

### **Live Cell Surface Marker Staining**

Cultures were monodispersed with trypsin and rinsed with FACS buffer containing HBSS with 2% FBS. Cells were incubated with conjugated primary antibody for 30 min at 4°C. Cells were rinsed twice with FACS buffer and then transferred to filter-top polystyrene FACS tubes to run on the BD FACSCalibur. Flow cytometry controls included unstained cells and cells incubated with an isotype.

### **Live Cell Sorting**

Cells were prepared as described in Surface Marker Staining. 10  $\mu$ M Calcein Blue, AM (Life Technologies, C1429) was added to stain cells for inclusive live gating. Sorting was performed in the Boston University Flow Cytometry Core Facility using the FACSARIA II SORP high speed cell sorter. Cells sorted at day 6 were replated onto gelatin-coated plates with 24 hours exposure to ROCK inhibitor.

### **Purification of Nkx2-1<sup>GFP+</sup> Thyroid and Forebrain mouse embryonic cells**

All mouse work was approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine. Thyroid epithelial cells and forebrain cells were isolated from mouse embryos using the Nkx2-1<sup>GFP</sup> reporter mouse, originally described in (Longmire et al., 2012). Briefly, breeding cages were set up with one male and three female mice and the morning of the day a vaginal plug was detected was defined as embryonic day E0.5. Thyroid epithelial cells were sorted from E13.5 embryos and forebrain cells from E9.0 and E13.5 embryos. Timed-pregnant females were euthanized by inhalation of isoflurane and subsequent cervical dislocation. Embryos were delivered by cesarean and the three Nkx2-1<sup>GFP</sup>-expressing domains (forebrain, thyroid, and lung) were dissected out with the help of an Olympus stereo fluorescence imaging microscope (model SZX16, Olympus America, Central Valley, PA) and kept in HBSS with 10% FBS. Tissues were digested using Collagenase A (0.1%, Roche 103578) and Dispase II (2.4 U/ml, Roche 295825) for 60 min at 37°C with periodic trituration. Monodispersed cells were resuspended in HBSS with 2% FBS, filtered through 30  $\mu$ m FACS strainers and stained with PI (for dead cell exclusion) directly before sorting. Sorting was performed in the Boston University Flow Cytometry Core Facility using the FACSARIA II SORP high speed cell sorter.

### **Measurement of T4 by ELISA**

T4 levels in mature organoids were measured as previously described (Kurmann et al., 2015). Organoids were removed from the Matrigel using Dispase and were lysed with 330  $\mu$ l per sample of cold Barbitol buffer (Sigma, B5934) using the QIAGEN TissueLyser system. Lysate from the organoids was sonicated and centrifuged for 20 min at 13,000 rpm. The supernatant was divided into three groups for genomic DNA (gDNA) extraction, and enzyme degraded and non-degraded samples.

For enzymatic degradation to break down T4 from TG-T4, 150  $\mu$ l of protease solution (Kurmann et al., 2015) and 15  $\mu$ l of Toluene (Sigma, 244511) were added to 100  $\mu$ l of lysate and incubated for 48 hours at 37°C with shaking followed by 5 min at 98°C for enzyme inactivation. T4 concentration was measured using AccuDiag T4 ELISA kit (Diagnostic automation/Cortez Diagnostics, Inc., 3149-18). 25  $\mu$ l of degraded or non-degraded organoid lysates were used for measurement and normalized to gDNA concentration.

Genomic DNA was extracted using Archive Pure DNA tissue kit (5Prime, 2300860). Briefly, 300  $\mu$ l of Cell Lysis Solution was added to 100  $\mu$ l of organoid lysate followed by treatment with RNase A Solution. After incubation for 5 min at 37°C, 100  $\mu$ l of Protein Precipitation Solution was added to the lysate. After centrifugation, the supernatant containing the gDNA was mixed with 300  $\mu$ l of isopropanol and centrifuged for one hour at 4°C. The gDNA pellet was washed with 70% ethanol and hydrated with 15  $\mu$ l of DNA Hydration Solution. The concentration of gDNA was measured using the Nanodrop spectrophotometer.

### **SPC-dsRed Lentivirus Reporter**

The SPC-dsRed lentivirus was generated as previously described (Longmire et al., 2012). In brief, a published 3.7 kb SPC promoter fragment was cloned upstream of the dsRed construct and packaged into the virus. Cells were transduced with the lentivirus on day 9 while in specification media. 8  $\mu$ l of the virus per 1 ml media in a 6 well plate, with 5  $\mu$ g/ml Polybrene (Sigma, H9268-50g) was incubated with the cells for 24 hrs. Dose was determined by parallel transduction with a CMV-dsRed surrogate reporter to estimate transduction efficiency. Cells were subsequently monitored for dsRed expression using fluorescence microscopy. At day 14, cells were passaged into FGF2+10 media

until day 22, then changed to DCI+K media to further stimulate maturation, as previously described (Longmire et al., 2012).

### **RNA Sequencing Preparation and Analysis**

RNA samples were prepared for RNA-Sequencing as described in the Supplemental Experimental Procedures for RT-qPCR. Samples were selected from biologically replicate runs (separate differentiations), in duplicate or triplicate sets. D1 RNA was limited to duplicate samples due to available space on the Flow-Cell. Sequencing libraries were prepared from total RNA samples using Illumina® TruSeq® RNA Sample Preparation Kit v2. Briefly, the mRNA was isolated using magnetic bead-based poly(A) selection, fragmented, and randomly primed for reverse transcription, followed by second-strand synthesis to create double-stranded cDNA fragments. These cDNA fragments were then end-repaired, added with a single 'A' base, and ligated to Illumina® Paired-End sequencing adapters. The products were purified and PCR-amplified to create the final cDNA library. The libraries from individual samples were pooled in groups of four for cluster generation on the Illumina® cBot using Illumina® TruSeq® Paired-End Cluster Kit. Each sample was sequenced four per lane on the Illumina® HiSeq 2500 to generate more than 25 million Paired-End 100-bp reads. The RNA-Seq reads were aligned to mouse genome mm9 by Tophat v2.0.4 (Kim et al., 2013). Then the reads were summed to the gene level by HTSeq package (Anders et al., 2015). This data has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE92572>.

A generalized linear model implemented by edgeR package was used to extract the differentially expressed genes between each two-condition comparison (D14 vs. D0, D7 vs. D0, D14 vs. D7) with a false discovery rate (FDR) cutoff at 0.05 (Robinson et al., 2010). Finally, a hierarchical clustering was performed on the combined differentially expressed gene list across all samples. Data were log-transformed with gene expression counts, mean centered, and row scaled. To generate the heat map, hierarchical agglomerative clustering was performed via Pearson correlation similarity metric with average linkage used to determine pairwise distance. Java Treeview (Saldanha, 2004) was used to generate the heat map visualization and for extraction of the gene lists comprising the main clusters.

### **Identification of Putative Cell Surface Markers and Transcriptional Regulators**

Cell surface markers (cut-off of  $p < 0.001$ ) were identified using DAVID (Huang et al., 2009) through categorization in GO term "Integral to Plasma Membrane" (GO:0005887) and manually curated to exclude genes encoding for proteins not transmembrane or plasma membrane bound. Transcription factors (cut-off of  $p < 0.001$ ) were selected using correlation (4/5 or 5/5) with the five reference lists: Animal Transcription Factor DataBase (AnimalTFDB v2.0 2014-06-30) (Zhang et al., 2012), Riken Transcription Factor Database, TFdb (Kanamori et al., 2004), TFcheckpoint (Transcription Factor Checkpoint, (Chawla et al., 2013)), and classification by DAVID within GO terms "Transcription Factor Activity" (GO:0003700) and "DNA Binding" (GO:0003677). Small-scale heat maps in the figures were generated using GENE-E (<http://www.broadinstitute.org/cancer/software/GENE-E/>).

### **Gene Ontology Analysis**

Lists of significantly ( $FDR < 0.05$ ) differentially expressed genes were entered into the Gene Ontology Consortium online resource (Ashburner et al., 2000; Consortium, 2015). GO terms with a p-value of  $< 0.05$  were entered into the REVIGO website for consolidation (Supek et al., 2011), using PANTHER Overrepresentation Test (release 2015-04-30) and GO Ontology database (release 2015-08-06). The  $\log_{10}$  p-values of the GO term output were used for the graphical representation of associated biological processes (Figure S4A).

### **GSEA analysis**

Genes from the "Early" and "Late" clusters were ranked on logFC values. Gene sets were selected from 'gene set data for pathway analysis in mouse' R package (Bares V and Ge X (2015). gskb: Gene Set data for pathway analysis in mouse. R package version 1.2.0). Specifically, we used all the provided 259 KEGG gene sets from the 'Metabolic pathways' set and all 12525 GO gene sets from the 'Gene Ontology'. The command-line version of GSEA (Mootha et al., 2003; Subramanian et al., 2005) was used in the 'Preranked' mode (specifically: gsea2-2.1.0.jar -Xmx512m xtools.gsea.GseaPreranked) and 'classic' scoring scheme, as advised for RNA-Seq data in the FAQ section of GSEA online documentation. We ran GSEA with the default parameters. Gene sets included in the figure were selected based on a cutoff of  $FDR < 0.2$ .

### **Principal Component Analysis**

Before PCA, expression values were normalized across all samples to a mean of zero and a standard deviation of one. PCA of the normalized data was performed using the `sklearn.decomposition.PCA` command in the Python package `scikit-learn`.

## Gene Expression Projections

Here we give a brief overview of the projection method plots shown in Figure 4F-H, please see (Lang et al., 2014; Pusuluri et al., 2015) for complete mathematical details. The projection method allows one to compare experimentally acquired gene expression data versus a reference basis. A projection score of 1.0 indicates a perfect match of gene expression, while 0.0 indicates no match. The projection method has several key advantages relative to other techniques such as PCA. First, it provides a biologically interpretable numeric value of the similarity between the data and the reference basis. Second, it removes the inherent correlations of the reference basis by measuring orthogonal projections onto the reference basis. This means that each reference cell type has a projection of 1 with only itself and has a projection of 0 with all other reference cell types.

To create the reference basis cell types, we first started with the basis used in (Lang et al., 2014; Pusuluri et al., 2015). We removed the following cell types: Common Lymphoid Progenitor (CLP), Common Myeloid Progenitor (CMP), Granulocyte-Monocyte Progenitor (GMP), and Megakaryocyte-Erythroid Progenitor (MEP). Second, we included E8.25 ectoderm, E8.25 foregut, and E14.5 Nkx2-1<sup>+</sup> thyroid RNA-Seq samples from our lab. The basis thus consists of 62 cell types.

The dataset consists of a mix of Affymetrix GeneChip Mouse Gene 1.0 ST and RNA-Seq data and was normalized as follows. First, raw microarrays and RNA-Seq were separately processed following standard techniques for each data type. Next, only genes common to all datasets were kept, leading to N=12039 genes. In order to make robust comparisons across platforms the raw expression output was converted to a rank order. Next, we wanted to convert this rank order to the z-score of a log-normal distribution. We converted the rank to a percentile (for N genes, by dividing by N+1), and then this percentile into a normal z-score. For mathematical convenience, we used a biased estimator (i.e. we normalized by N and not N-1) since then the Euclidean norm of each microarray gene expression was N. Therefore, for the data analysis each sample is described by a Gaussian distribution with a Euclidean norm of N = 12039.

As mentioned above, a projection score of 1.0 indicates a perfect match of gene expression, 0 indicates no match, and -1.0 indicates perfectly anti-correlated gene expression. There are no direct equivalents of p-values to measure the significance of a projection. However, one can compare the expression of random sets of gene expression versus the reference basis. A random set of 10,000 different gene expression profiles (each with N=12039 genes) had a mean projection of 0 (within machine precision) with any of the reference cell types, while the standard deviation of the projections was 0.0184. Therefore, a projection greater in magnitude of 0.092 (5 stds) is highly unlikely to be due to random expression patterns. This is also supported by the fact that the variation in the projections of each experimental replicate was minimal.

## Simple mathematical model for bistable switch

The experimental data in the main text is consistent with the idea that Nkx2-1 positively regulates its own expression, giving rise to a bistable switch. To demonstrate this, we constructed a simple model of a genetic switch consisting of a gene which produces a protein that can bind its own promoter and increase gene expression (Figure 4F). When the feedback loop is cooperative, this molecular architecture can give rise to bistability, with two stable steady-states possible: (i) a low-expression steady-state where the positive feedback loop is off and proteins are expressed at low levels and (ii) a high-expression steady-state where the positive feedback loop is turned-on and proteins are expressed at high levels (Ferrell, 2002; Maamar et al., 2007; Mehta et al., 2008; Tyson et al., 2003).

Mathematically, we represent the deterministic dynamics of such a bistable switch using a simple differential equation for the number of mRNA molecules,  $m$ , and the number of protein molecules,  $p$ :

$$\frac{dm}{dt} = \alpha_{pulse}(t) + \alpha_0 + \frac{a_m p^q}{p^q + K_d^q} - \tau_m^{-1} m \quad (1)$$

$$\frac{dp}{dt} = a_p m - \tau_p^{-1} p \quad (2)$$

with  $\alpha_{pulse}(t)$  the generally (time-dependent) transcription rate from the pulsing construct,  $\alpha_0$  the basal transcription rate,  $a_m$  the maximal transcription rate due to the positive feedback loop,  $K_d$  the half-maximal protein concentration for the positive feedback loop to turn on,  $q$  the Hill coefficient describing cooperative binding,  $\tau^m$  the mRNA lifetime,  $a_p$  the rate at which proteins are translated from an mRNA molecule, and  $\tau^p$  the protein lifetime. For all simulations, we take (where rates are in units of molecules per hour)  $\alpha_0 = 0.5$ ,  $a_m = 5$ ,  $a_p = 20$ . Additionally,  $\tau^p = 6\text{hr}$ ,  $\tau^m = 0.5\text{hr}$ ,  $q = 3$ , and  $K_d = 150$ . Furthermore, we assume there is a finite time window during which the cell can switch to high to a protein state. Very little is known about how this

occurs at the molecular level. Inspired by measurements of the transition to competence in the bacteria *Bacillus subtilis* (Maamar and Dubnau, 2005; Maamar et al., 2007), we model this process by assuming that the basal transcription rate is turned off after a critical time cutoff,  $t = 25\text{hr}$  so that  $\alpha_0 = 0.5\theta(t_{\text{cutoff}} - t)$ , where  $\theta(t)$  is the Heaviside function. We emphasize that this is just one of many ways that this regulation could occur and our results do not depend strongly on this choice. To model pulsing for a duration  $t_{\text{pulse}}$ , we use  $a_{\text{pulse}} = 4 * \theta(t - t_{\text{pulse}})$ .

One can easily verify that with this choice of parameters, the dynamics are bistable with critical concentration  $p_{\text{crit}} \approx 102$  separating the low-expression and high-expression fixed points. When  $p < p_{\text{crit}}$ , the cell is attracted to a low-expression fixed point. However, when  $p > p_{\text{crit}}$ , cell are attracted to a high expression state. Due to stochastic effects resulting from low number of molecules, cells can transition between these steady-states.

To understand the stochastic switching dynamics, we performed a stochastic simulation of gene expression dynamics for this system using Gillespie's algorithm (Gillespie, 1977; Mehta et al., 2008). We considered three distinct conditions: (i) no pulse where  $t_{\text{pulse}} = 0$ , (ii) short pulses where  $t_{\text{pulse}} = 2\text{hr}$ , and (iii) a long pulse where  $t_{\text{pulse}} = 24\text{hr}$ . For each of these conditions, we performed 1000 simulations and calculated the fraction of cells with high protein expression ( $p > p_{\text{crit}}$ ) at  $t = 100\text{hr}$  (Figure 4G). As expected, the fraction of high-expression cells increased with the pulse duration. We also examined the time traces for cells that switched or did not switch in both the pulsed and non-pulsed conditions, representative traces are shown (Figure 4H).

As shown in Figure 4F, although a certain percentage of cells is expected to reach the "On" state without an exogenous NKX2-1 pulse, this percentage could be increased by short or long NKX2-1 pulses. Figure 4H simulations exemplify this effect, as cells displayed heterogeneous behaviors with some cells reaching the "On" expression state (purple lines) and others reaching the "Off" expression state in all conditions. On the other hand, the "On" expression state is favored in the pulsed condition and it is achieved faster than in the non-pulsed condition.

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