Single-Cell Network Analysis Identifies DDIT3 as a Nodal Lineage Regulator in Hematopoiesis.

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Graphical Abstract

Highlights
- We present a method for inferring gene regulatory networks (GRNs) from single cells
- Lineage cross-antagonism is a key property of GRNs of early lineage commitment
- Ddit3 is a regulatory node in erythroid lineage programming
- A Ddit3-Gata2 regulatory axis antagonizes myeloid and enables erythroid programs

Authors
Cristina Pina, José Teles, Cristina Fugazza, ..., Mattias Ohlsson, Carsten Peterson, Tariq Enver

Correspondence
t.enver@ucl.ac.uk

In Brief
Pina et al. develop a gene regulatory network inference method using single-cell gene expression data and identify Ddit3 as a regulatory node in erythroid lineage programming. The authors explore this inference and show that Ddit3 can antagonize myeloid programming and enable erythroid signatures and forms a regulatory axis with Gata2.

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Single-Cell Network Analysis Identifies DDIT3 as a Nodal Lineage Regulator in Hematopoiesis

Cristina Pina,1,3,4 José Teles,1,2,5 Cristina Fugazza,1,3,6 Gillian May,1 Dapeng Wang,1 Yanping Guo,1 Shamit Soneji,1,7 John Brown,1 Patrik Edén,2 Mattias Ohlsson,2 Carsten Peterson,2 and Tariq Enver1,*

1Stem Cell Laboratory, UCL Cancer Institute, University College London, London W1CE 6BT, UK
2Computational Biology and Biological Physics, Department of Astronomy and Theoretical Physics, Lund University, 223 62 Lund, Sweden
3Co-first author
4Present address: National Health Service Blood and Transplant, Department of Haematology, University of Cambridge, Cambridge CB2 0PT, UK
5Present address: Sainsbury Laboratory, University of Cambridge, Cambridge CB2 1LR, UK
6Present address: Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, 20126 Milano, Italy
7Present address: Lund Stem Cell Center, Lund University Hospital, Biomedical Centre, 221 84 Lund, Sweden
*Correspondence: t.enver@ucl.ac.uk
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SUMMARY

We explore cell heterogeneity during spontaneous and transcription-factor-driven commitment for network inference in hematopoiesis. Since individual genes display discrete OFF states or a distribution of ON levels, we compute and combine pairwise gene associations from binary and continuous components of gene expression in single cells. Ddit3 emerges as a regulatory node with positive linkage to erythroid regulators and negative association with myeloid determinants. Ddit3 loss impairs erythroid colony output from multipotent cells, while forcing Ddit3 in granulo-monocytic progenitors (GMPs) enhances self-renewal and impedes differentiation. Network analysis of Ddit3-transduced GMPs reveals uncoupling of myeloid networks and strengthening of erythroid linkages. RNA sequencing suggests that Ddit3 acts through development or stabilization of a precursor upstream of GMPs with inherent Meg-E potential. The enrichment of Gata2 target genes in Ddit3-dependent transcriptional responses suggests that Ddit3 functions in an erythroid transcriptional network nucleated by Gata2.

INTRODUCTION

Development and differentiation are characterized by genetic circuitry or gene regulatory networks (GRNs) that have inherent forward momentum encoded by a number of regulatory motifs (Davidson, 2010). To self-renew and maintain differentiation potential, stem cells must structure their GRNs so as to arrest or buffer this forward trajectory. Networks at early multipotent stages may bear little relation to those of mature differentiated cells, making comparison between them difficult. Detailed time-series data are useful in this regard (Bruno et al., 2004; May et al., 2013), but there is likely substantial asynchrony between individual cells at any given time point. Cells also may undergo lineage commitment through different initial gene expression trajectories (Pina et al., 2012). Together, these factors can confound attempts to infer network architectures and gain molecular insights into commitment and subsequent lineage specification from averaged gene expression profiles.

Analysis of gene expression in single cells offers a different approach that, in principle, makes use of cellular heterogeneity as a source of variation for establishing gene-gene associations. Recent studies have used all expression data (Guo et al., 2013; Moignard et al., 2013, 2015) or inferred pairwise gene associations using only co-expressing cells (Ståhlberg et al., 2011). It has been suggested that levels of expression are better accounted for in cells that co-express both genes, and may be obscured by presence/absence effects when all cells are considered (Rusnakova et al., 2013). We have tried to address this constraint in our exploration of gene expression networks around the erythroid versus myelo-monocytic lineage choice of multipotent hematopoietic progenitor cells. Additionally, we have focused on capturing networks from closely related cells in the vicinity of the commitment boundary, to gain insight into the evolution of GRNs relevant to lineage specification.

The erythroid-myeloid bifurcation is an intensively studied paradigm, and both transcription factors (TFs) and regulatory motifs involved in physiological transcriptional programming of these alternative fates have been described (Wolff and Humeniuk, 2013). Key players include Gata factors, the Ets family protein Pu.1, and C/ebp family members, whose potency has been demonstrated in the experimental reprogramming of blood lineages (Graf and Enver, 2009). We used two distinct cell commitment scenarios to obtain high resolution around the early phase of commitment and lineage specification. First, we identified and prospectively isolated cells spontaneously committing to both lineages under culture conditions that maintain self-renewing (SR) cells and lack pro-differentiative cytokines. Second, we...
used inducible variants of the lineage-affiliated TFs Gata1 and Pu.1 to drive cells into lineage specification, again in the absence of pro-differentiative cytokines. This approach allows timed sampling after instigation of a discrete lineage trigger, and it may provide a more homogeneous molecular entry into commitment and lineage development.

We describe state-distinct networks in multipotent and early lineage-committed cells and, in particular, highlight the existence of lineage-conflicting programs at the emergence of lineage choice. We further identify an axis involved in lineage specification that includes Gata2 and Ddit3, a C/ebp family member previously implicated in stress response (Zinszner et al., 1998) and described as a potential target of erythropoietin signaling in erythro-leukemic cell lines (Coutts et al., 1999).

RESULTS

We have explored commitment in the non-transformed bone marrow (BM)-derived hematopoietic multipotent cell line FDCPmix. FDCPmix is karyotypically normal, IL-3 dependent, and capable of multilineage differentiation in response to the appropriate environmental cues. Under maintenance culture conditions, SR and lineage-committed cells (erythroid- or myeloid-committed progenitors [ECPs or MCPs]) co-exist in culture (Figure 1A) and can be isolated on the basis of their surface phenotype (Figure 1B). SR cells are Kit+Gr1− cells (Figure 1B) with proliferative capacity in bulk and clonal cultures (Figures S1A and S1B) and are uniquely able to faithfully reconstitute the cellular heterogeneity observed in maintenance cultures (Figure S1C). Lineage-committed cells devoid of SR potential are Gr1+ MCPs, with an early myeloid morphology (Figure S1D) and no erythroid differentiation capacity (Figure S1E), and kit−Gr1− ECPs, with accelerated erythroid differentiation (Figure S1E) but minimal or no contribution to neutrophil cultures (Figure S1F). The transcriptional signatures of SR, MCP, and ECP compartments are readily distinct (Figure S1G) and confirm their lineage affiliation (Table S1).

We next explored cellular heterogeneity within these cell compartments using single-cell multiplex qRT-PCR. The results showed substantial cell-to-cell heterogeneity (Figure 1C) and overlap (Figure 1F) within and among all three compartments; nevertheless, the compartments may be robustly identified using single and dual-gene classifiers (Table S1). The transcriptional heterogeneity observed within individual populations may highlight the capture of multiple contemporaneous molecular programs underlying lineage commitment under self-renewal conditions. Since TFs are potent instigators of lineage determination or reprogramming, we next examined cellular and transcriptional heterogeneity in FDCPmix cells driven to erythroid or myeloid lineages through expression of inducible Gata1 or

Figure 1. Single-Cell Transcriptional Profiling Captures Molecular Spaces of Lineage Commitment

(A) Depiction shows the two modes of commitment surveyed in this study: (top) hierarchically related SR cells, ECPs, and MCPs in equilibrium in a multipotent cell culture system; (bottom) unilineage commitment of SR cells driven by a single TF.

(B) Flow cytometry plot shows co-existing SR, ECP, and MCP cells in an FDCPmix culture under SR conditions.

(C) Heatmap of expression profiles of 26 genes in individual FDCPmix SR, ECP, and MCP cells. The gene panel represents the consensus analyzed in sufficient cell numbers in all compartments between replicate experiments. Data are Z score-normalized ΔCt values; undetectable expression, gray.

(D) Flow cytometry plots show FDCPmix SR cells transduced with control empty vector or with a GATA1-ERT fusion after 21-hr activation with tamoxifen (4-OHT, 1 μM).

(E) Cloning efficiency of control and Gata1-ERT GFP+ cells cultured under SR and neutrophil differentiation (N) conditions during a time course of 4-OHT activation. Sampling times correspond to those of single-cell qRT-PCR analysis. At each time point, 60 individual cells of each genotype were plated into SR or N conditions, and individually seeded wells were inspected at regular intervals for a 7-day period.

(F) PCA plot of the transcriptional profiles of individual FDCPmix cells undergoing distinct modes of lineage commitment. The first two PC explain 31% of the data variance; n = 82 (SR), 60 (ECP), 59 (MCP), 147 (Gata1-ERT), and 103 (Pu.1-ERT). A consensus set of 22 genes was analyzed in all five compartments.
Pu.1 estrogen receptor fusions, respectively (Figure S1H). We analyzed the transcriptional programs of single cells captured at various time points (Figure S1I) after induction. In parallel, we studied the temporal dynamics of lineage commitment in this setting and functionally tested their commitment status by evaluating the following: (1) their retention of self-renewal potential, i.e., their capacity to re-initiate maintenance cultures; and (2) their lineage potential in response to various cytokine cues. This experimental design affords a dynamic appreciation of cellular and molecular mechanisms employed in lineage specification.

Activation of Gata1 in SR cells led to phenotypic changes at as early as 4–6 hr (Figure 1D), accompanied by the loss of neutrophil potential (at 6 hr) and followed by the loss of SR capacity (at 45 hr) (Figure 1E). Enforced Pu.1 activity resulted in the loss of clonogenic SR potential and elicited a myeloid differentiation bias in a more extended time frame (Figures S1J and S1K). Single-cell transcriptional profiling during TF-driven commitment confirmed the lineage identity of the cells obtained, which broadly separated away from the SR state along erythroid (Gata1) and myeloid (Pu.1) axes (Figure 1F). The analysis also revealed significant heterogeneity of molecular programs throughout the process of commitment. Gata1-driven cells co-occupied the same transcriptional space as ECP cells and showed a similar extent of cell-to-cell variation. In contrast, Pu.1-driven cells, while similarly heterogeneous to MPPs, appeared to occupy a distinct territory (data not shown). This presumably reflects the neutrophilic status of MPPs and a monocytic bias of Pu.1-ERT-differentiated cells, consistent with prior reports of Pu.1-driven cell fate (Laslo et al., 2006).

We exploited the heterogeneity of cells at early stages of spontaneous and TF-driven lineage commitment to explore the transcriptional networks controlling lineage specification. Inspection of patterns of expression for individual genes revealed a fraction of cells in which the gene is off, and a fraction of cells expressing the gene to varying levels (on) (Figure 2A). The on/off status can be described as binary while the distribution of on values represents a continuous component of the data. Thus, for any given gene pair, both binary and continuous relationships are possible; this is exemplified for Gata1 and Epor in Figure 2B. We sought to capture both kinds of information to infer putative transcriptional networks. Methodologically, we used odds ratio (OR) to quantify on/off gene-to-gene associations (Figure 2C) and Spearman rank correlation to measure correlations between gene expression levels (Figure 2D). We combined gene associations obtained by both methodologies to infer putative regulatory networks characterizing SR states and the different modes of lineage commitment (Figure 2E).

At coarse grain, the networks revealed increased connectivity in the lineage-committed compared to the SR state. Also, commitment appeared associated with a higher frequency of negative associations between genes (Figures 2F and S2A), including known lineage-determining factors (Table S1). While this may be, to some extent, a function of the genes analyzed, it also may reflect mechanistically distinct processes governing acquisition of lineage identity versus exit from self-renewal. Negative associations are less prominent in the full-activation time courses of TF-driven commitment, as the networks capture not only the early processes of lineage specification, but also the later consolidation of the differentiation program. This likely increases the proportion of positive associations between lineage-affiliated genes. In contrast, detailed temporal analysis of Gata1-ERT-driven lineage specification revealed that cross-antagonistic associations between lineage determinants peak at 6 hr (Figure 2G), coincident with early loss of neutrophil differentiation potential (Figure 1E) en route to lineage commitment, suggesting that resolution of lineage conflicts is an early step in acquisition of lineage identity. In this respect, Ddit3 emerges as an interesting candidate in lineage cross-antagonism: it is positively associated with Gata2, both in SR and committed cells (Figure S2B), and negatively associated with the neutrophil determinant Cebpa, either directly or through Gata2, in MPPs and at early stages of erythroid lineage commitment (Figure S2B; Table S1). Since Ddit3 has not previously been tabled as a central regulator of erythro-myeloid lineage specification, we functionally tested its impact in loss- and gain-of-function experiments.

Knockdown of Ddit3 (Figures S3A and S3B) in FDCPmix cells resulted in the loss of erythroid and mixed-lineage colonies, with no change to myelo-monocytic potential (Figure 3A). The same loss of erythroid potential in colony-forming assays was observed in stem and progenitor cells (KLS) from mouse BM upon knockdown (Figure 3B) and constitutive knockout (Figure 3C) of Ddit3 expression. The data are compatible with a requirement for Ddit3 in the erythroid lineage, while it is dispensable for the development of the myeloid lineage. The negative association observed between Ddit3 and Cebpa in inferred transcriptional networks from early stages of lineage specification (Figures 2E and S2B; Table S1) suggests that Ddit3 contributes to the erasure of myeloid potential. We tested this in myeloid-committed granulo-monocytic progenitors (GMPs) by enforcing Ddit3 expression (Figure S3C), resulting in a transient re-acquisition of self-renewal potential (Figure 3D) and a dramatic change in the nature of the colonies obtained (Figure 3E), with the predominance of large GM colonies of immature appearance (Figure 3F). Cells in these colonies expressed immature surface markers and were predominantly lineage-negative Kit+/CD34+/CD16/32+, thus presenting an essentially GMP phenotype albeit with variable levels of Sca1 expression; in contrast, cells in control colonies exhibited a differentiated Gr1+Mac1+ phenotype (data not shown). Taken together, the data suggest that ectopic expression of Ddit3 in GMPs blocks lineage progression and transiently re-activates self-renewal capacity.

We used single-cell gene expression profiling of GMPs, either wild-type or transduced with a control vector or a Ddit3-expressing lentivirus, to interrogate the transcriptional program changes imposed by enforced expression of Ddit3 and to inspect its role in remodeling of the transcriptional networks underlying lineage progression and/or identity. Enforcement of Ddit3 changed the expression of two-thirds of genes (Figures S3D and S3E) predicted as its neighbors in our inferred transcriptional networks (Figure S2B), attesting to the robustness of our inference approach. Principal component analysis (PCA) of the populations of individual wild-type and transduced GMP cells separated Ddit3-expressing cells from controls (Figure 3G). This separation is mostly attributable to the increased expression of
Figure 2. Combined Single-Cell Transcriptional Network Inference Methods Implicate Ddit3 in Lineage Specification

(A) Representative gene expression distributions for Epor, Gata1, and Tal1 in ECPs are shown.

(B) Scatterplot of Gata1 and Epor single-cell expression highlights the dual aspect of the data with both binary (on/off) and continuous (expression-level) components.

(C) Contingency table summarizing on/off combination patterns of individual cells for Epor and Gata1. OR quantifies the diagonal versus off-diagonal of this matrix to infer significant positive and negative associations in the binary component of the data. Gata1 and Epor show significant positive association (OR = 3.18; lower95CI > 1).

(D) Scatterplot of Epor and Tal1 expression ranks in co-expressing cells. Epor and Tal1 show significant positive correlation in the continuous component of the data inferred by Spearman rank correlation (r = 0.56; p = 0.002).

(E) Single-cell transcriptional networks in SR, ECP, MCP, Gata1-ERT, and Pu.1-ERT compartments were inferred by combined use of OR and Spearman rank correlation. Solid red lines, positive associations; dashed black lines, negative associations. Node size is proportional to the relative connectivity in each network.

(F) Proportion of negative interactions in the networks in (E) is shown.

(G) Proportion of negative interactions in Gata1-ERT networks at each time point is shown.
early erythroid regulators Gata2, Tal1, Zfpm1/Fog1, and Gfi1b and the relative loss of M, GM, and G-CSF receptors (Csf1r, Csf2ra, and Csf3r, respectively) as well as of C/ebp family members (Figure 3H). The relative gain in the expression of erythroid-affiliated genes and loss of myeloid Csf receptors and C/ebp family TFS further developed with prolonged expression of Ddit3 in GMPs under differentiation conditions that support multilineage output (Figures S3F and S3G). These data confirm Ddit3 as a positive regulator of erythroid lineage specification at the expense of myeloid fate, providing an experimental validation of the predictive power of the networks we derived by analyzing the heterogeneity of single cells undergoing lineage specification.

We next asked if the relative gain in importance of erythroid-affiliated regulators in Ddit3-transduced GMPs was associated with a global remodeling of the transcriptional networks underlying GMP lineage identity. Indeed, we observed an overall loss in network connectivity specific to the activity of Ddit3 (Figure 4A). Moreover, there was a relative gain in connectivity of Gata2 at the expense of myeloid hubs, as quantified in Figure S4A. For a broader appreciation of the transcriptional changes induced by Ddit3, we performed RNA sequencing (RNA-seq). GMPs transduced with Ddit3 or control vector (CSlim) were cultured for up to 5 days under conditions supportive of multilineage output. Similarly to cells obtained from colony-forming assays, Ddit3-transduced cells retained a GMP-like phenotype, while control cells acquired differentiated myeloid surface markers (Figure S4B). The global transcriptional profiles of cells with enforced expression of Ddit3 (Figure S4C) were clearly distinct from control-transduced and wild-type GMPs. Gene set enrichment analysis (GSEA) showed that Ddit3 expression is associated with global loss of GMP programs and concomitant upregulation of Meg-E-affiliated signatures (Figure 4B). Interestingly, signatures representative of pre-GM cells, the developmental precursors of GMPs (Figure 4C), also were upregulated (Figure 4B). These data suggest that Ddit3 acts through the development or stabilization of a more primitive precursor with inherent Meg-E potential (Figure 4C). Analysis of the networks derived from wild-type GMPs (Figure S4D) as well as Ddit3-transduced cells exposed to conditions supportive of multilineage output for 2 days (Figure 4A) revealed increased importance of specific erythroid versus myeloid regulatory nodes.

Figure 3. Ddit3 Is Required in Early Erythroid Specification and Blocks Myeloid Lineage Progression

(A–C) Lineage potential of multipotent mouse BM cells upon the loss of Ddit3 expression. CFC assays of FDCPmix cells (n = 3) (A) and primary KLS cells (n = 3) (B) upon Ddit3 knockdown and of Ddit3 knockout KLS cells (n = 4) (C) are shown. Error bars, SD.

(D) Re-plating capacity of primary BM GMPs upon enforced expression of Ddit3 read in CFC assays (CSlim, empty vector; n = 4). Colonies were scored 7–10 days after plating of transduced cells (plate 1). The cellular content of the colonies obtained was re-seeded into successive CFC assays (plates 2–4) until the exhaustion of colony production.

(E) Distribution of colony types in CFC plate 1. Most GM colonies obtained upon Ddit3-enforced expression have a blast-like appearance. Error bars, SEM.

(F) Representative images of GM colonies in (E) are shown.

(G) PCA plot of the transcriptional profiles of individual GMPs, either untransduced (WT) or transduced with CSlim- or Ddit3-expressing lentiviral vectors, analyzed for the expression of 44 genes. The first two PC explain 24% of the data variance; n = 114 (CSlim), 84 (Ddit3), and 118 (WT).

(H) Gene loadings of PC1 and PC2 in (G). Genes with the most extreme positions along each axis contribute the most to cell separation along the respective PC.

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in those single cells that co-expressed the two genes, and derived networks from multipotent and lineage-committed compartments (Table S1). Within the ECP compartment, this analysis revealed the following: (1) an association between the two genes that was not seen when cells were used irrespective of Ddit3/Gata2 status, and (2) the involvement of both these genes in...
isolated GMP populations and that these are preferentially
selected for by Ddit3. This seems unlikely, given the kinetics of
the changes in cells and gene expression observed. Alterna-
tively, Ddit3 may regulate a subset of the erythroid program
that is simply overlaid on the existing GMP program, resulting
in a mixed-lineage program that is reminiscent of pre-GM cells.
In such a model, Ddit3 effects a pre-GM state from GMPs,
recapitulating its physiological role in lineage programming.
Recently, Nerlov and colleagues have suggested that high levels
of erythropoietin (EPO) may have instructive effects on lineage
specification in vivo (Grover et al., 2014), and interestingly
Ddit3 has been suggested as a target of EPO signaling in
erythro-leukemic cells (Coulls et al., 1999). The strong network
association observed between Ddit3 and Gata2 may indicate
that Ddit3 acts on an early erythroid signature primed by Gata2
in cells of mixed-lineage potential (May et al., 2013) and present
in the pre-GM state. Elements of this signature may be required
for erythroid lineage progression and, thus, explain the erythroid
defect observed upon Ddit3 loss of function.
It is interesting to speculate as to the existence of cross-antag-
onistic interactions between Gata2-centered networks and
C/ebp-driven myeloid programs putatively effected through
Ddit3. Ddit3 heterodimerizes with C/ebp family members to
form complexes that cannot bind DNA, thus blocking activation
of C/ebp-driven programs, a mechanism that has been described
to block differentiation in mesenchymal lineages (Han et al., 2013;
Shirakawa et al., 2006). A combination of Ddit3 structure-function
mutant studies and direct investigation of Gata2 and C/ebp DNA
binding in Ddit3-expressing GMPs will contribute to clarifying
dislodgement of C/ebp complexes from their target genes as a
putative mechanism of GMP lineage remodeling.

EXPERIMENTAL PROCEDURES

Mice
B6.129S-Ddit3tm1Dron/J (Ddit3 KO) mice (Jackson ImmunoResearch Labo-
ratories) and C57BL/6 mice were maintained in the John Radcliffe Hospital
and CR-UK London Research Institute animal facilities in accordance with
Home Office regulations.

Cell Culture and Lentiviral Transductions
FDCPmx culture conditions, lentiviral transductions with Gata1-ERT and Pu.1-
ERT constructs, and tamoxifen activation were performed as described
previously (May et al., 2013). Lentiviral transductions of FDCPmx cells with
Ddit3-small hairpin RNA (shRNA) were performed under maintenance culture
conditions; transductions of primary BM cells were performed in serum-free
expansion medium (SFEM) (STEMCELL Technologies) supplemented with
mouse stem cell factor (SCF) and Flt3L (50 ng/ml). GFP* cells were sorted after
2 days for downstream assays. In some experiments, GFP* GMPs were cultured
for up to 5 days in Iscove’s modified Dulbecco’s medium (IMDM) + 10% fetal
calf serum (FCS) supplemented with mouse SCF (50 ng/ml), mouse IL-3 and
IL-6 (10 ng/ml), and human recombinant erythropoietin (EPOX, 10 U/ml).
Colonies-forming cell (CFC) assays used M3234 supplemented with rat SCF
(100 ng/ml); mouse IL-3 (0.01 ng/ml) and EPOX (10 U/ml) (FDCPmix); and
M3434 or M3234 supplemented with mouse SCF (100 ng/ml), mouse IL-3, IL-
11, GM-CSF, and Tpo (10 ng/ml), and EPOX (10 U/ml). All mouse cytokines
were from PeproTech and CFC media were from STEMCELL Technologies.

Single-Cell qRT-PCR
Transcriptional profiling of up to 48 genes in individual cells was performed
on a Fluidigm platform and the data retrieved and quality-controlled as
described previously (Teles et al., 2014). The ΔCt values were calculated
to the mean of the three control genes utilized. Heatmap representation of
Z score-normalized ΔCt values was performed in Genesis; PCA plots used the Statistical Toolbox in MATLAB (MathWorks). The Taqman probes were listed in the Supplemental Experimental Procedures.

**Classification and Network Inference**

Single-cell gene expression data were linearly transformed as described previously (Teles et al., 2013). Logistic regression linear classifiers were used to infer the best predictor genes in the separation between two cell populations (Teles et al., 2013). Single-cell transcriptional networks were inferred by calculating significant pairwise associations using both continuous (Spearman rank correlations) and binary (OR) components of linearly transformed expression data. Spearman rank correlations were calculated between all pairs of genes co-expressed by a minimum of ten cells in a given population. Correlation coefficients >0.4 with p < 0.01 were considered to be significant. OR and respective 95% confidence intervals (CIs) were calculated based on presence/absence patterns of expression for all pairs of genes in a given population. Significant positive and negative associations were called when Lower95CI > 1 and Upper95CI < 1, respectively. Network representations of significant pairwise associations in both methods were produced using Cytoscape (Smoot et al., 2011). Additional methods are described in the Supplemental Experimental Procedures.

**ACCESSION NUMBERS**

The accession numbers for the microarray and RNA-seq data reported in this paper are GEO: GSE68754 and SRA: SRP045672, respectively.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.016.

**AUTHOR CONTRIBUTIONS**

C. Pina and C.F. designed and performed experiments and analyzed data. J.T. developed and implemented computational methods and analyzed data. G.M., Y.G., and J.B. performed experiments. D.W. and S.S. analyzed transcriptome data. P.E., M.O., and C. Peterson developed computational methods. T.E. supervised the project. C. Pina and T.E. wrote the paper with input from J.T., C.F., G.M., and C. Peterson.

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