A systems biology pipeline identifies regulatory networks for stem cell engineering

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A major challenge for stem cell engineering is achieving a holistic understanding of the molecular networks and biological processes governing cell differentiation. To address this challenge, we describe a computational approach that combines gene expression analysis, previous knowledge from proteomic pathway informatics and cell signaling models to delineate key transitional states of differentiating cells at high resolution. Our network models connect sparse gene signatures with corresponding yet disparate, biological processes to uncover molecular mechanisms governing cell fate transitions. This approach builds on our earlier CellNet and recent trajectory-defining algorithms, as illustrated by our analysis of hematopoietic specification along the erythroid lineage, which reveals a role for the EGF receptor family member, ErbB4, as an important mediator of blood development. We experimentally validate this prediction and perturb the pathway to improve erythroid maturation from human pluripotent stem cells. These results exploit an integrative systems perspective to identify new regulatory processes and nodes useful in cell engineering.

stem cell biology, cell engineering and regenerative medicine often invoke developmental principles to differentiate cells toward target identities. However, much remains to be learned about how signaling pathways integrate to determine cell fate. The past decade of cell engineering has shown that expression of individual genes, or sets of genes, is often insufficient to functionally reprogram cell identity, underscoring the need for new approaches to quantitatively describe and manipulate cell state. We previously established CellNet4–6 to assess the fidelity of engineered cells by interrogating key gene regulatory networks (GRNs) that define native populations. CellNet extracts cell-type specific GRNs from transcriptional profiling data, compares the GRNs to those of bona fide primary cells and tissues to assign a similarity score, and identifies dysregulated transcriptional regulators that account for the differences between engineered cells and their native counterparts. The network-level CellNet algorithm confers robustness to biological and technical variability and encodes topological information about regulator-target relationships. A limitation of CellNet is that training data from a small number of terminal cell and tissue types obscure the phenotypic heterogeneity that arises during dynamic biological processes such as cell differentiation. More recent efforts have aimed to describe intermediate developmental states using trajectory-based methods, which employ cell–cell similarity metrics to infer dynamics4–6. However, these algorithms rely on single-cell transcriptomics to provide sufficiently powered datasets and largely forgo network analytics.

Here, we extend CellNet to quantitatively define network dynamics along a differentiation pathway. We show that publicly accessible gene expression datasets capture population-level differentiation states with high dynamic resolution and broad biological scope, including responses across a spectrum of experimental variables such as chemical and genetic perturbations. Our pipeline goes beyond the establishment of GRNs to enable quantification of differentiation dynamics and identification of key signaling pathways governing cell fate changes (Fig. 1a). We apply this general approach to characterize erythropoiesis, a dynamic process that generates red blood cells (RBCs) throughout the lifetime of the organism. We focused on this system because its temporal stages of differentiation, defined by distinct immunophenotypes, have been comprehensively characterized. Our analyses confirm key processes involved in distinct stages of erythropoiesis and elucidate novel dynamic patterns of gene expression. To improve erythroid maturation in vitro, we constructed an interaction network connecting the dynamic molecular signatures that distinguish late erythroblasts from reticulocytes. Our network analytics identifies a role for ErbB signaling during erythropoiesis, which we validate in human, murine and zebrafish models and apply to the maturation of RBCs derived from human induced pluripotent stem cells (iPSCs).

Results

CellNet delineates stem cells and progeny. To analyze the dynamics of stem cell differentiation, we began by establishing GRNs for hematopoietic stem cells and differentiated progeny using CellNet as previously described. We augmented the original CellNet compendium of microarray datasets from 16 human cell and tissue types to include 164 publicly available erythroid microarray datasets.
ets (Supplementary Tables 1 and 2). The erythroid data represented five manually classified phenotypes: early (CFU-E), intermediate (IntE) and late (LateE) erythroid progenitors, as well as reticulocytes and the K526 erythroleukemia cell line (Supplementary Table 3). Our rationale for augmenting the original compendium arose from the paucity and lack of biological variability in publicly available erythroid-specific sequencing datasets at the time of data compilation.

Application of the original CellNet classifier identified erythroid cells as hematopoietic stem and progenitor cells (HSPCs) with high probability (Supplementary Fig. 1a). However, after re-training the classifier with the augmented compendium and establishing an erythroid-specific GRN, CellNet robustly distinguished HSPCs and erythroid cell types (Supplementary Fig. 1b,c), with little overlap between the two GRNs other than cofactors mediating the canonical ‘GATA switch’ that governs erythroid specification from HSPCs (Supplementary Fig. 1d,e,g). The erythroid GRN comprised 235 genes that were highly enriched for biological processes such as hemoglobin synthesis, oxygen transport, cell cycle and hematopoietic development (Supplementary Fig. 1f), with subnetworks...
To derive dynamic network models, we exploited topological regulatory information encoded in the erythroid GRN as a second-layer approach to the lineage-correlated loadings (Fig. 1e). Early erythropoiesis is dominated by a few, highly connected regulators, with more distributed regulation during the proerythroblast (C4) and late erythroblast (C5) stages (Fig. 1e). Canonical regulators, such as GATA1 and E2F2, are also highly connected in the mature (C6) network, suggesting that early regulators impart persistent influence (Fig. 1f). GATA1 has been implicated in erythroid maturation, with a distinct network from that of early erythropoiesis GATA1 and E2F2 also recur as a central regulator in both the intermediate erythroblast (C4) and reticulocyte (C6) stages; however, there is a clear rewiring of its targets from a diffuse cluster of coregulated genes to a more compact network during maturation (Fig. 1f). Thus, the integration of network biology and GRN-based feature selection with dimensionality reduction uncovers dynamic changes in network activity and architecture accompanying cell fate changes.

Identification of pathways mediating cell fate transitions. We further explored the capacity for network analytics to identify biological processes that mediate stem cell differentiation. We focused on the late erythroblast (C5) to reticulocyte (C6) transition, as relatively little is known about the integrated mechanisms controlling terminal erythroid maturation. Moreover, microarray datasets derived from the in vivo reticulocyte transcriptome18 (Supplementary Fig. 5) provided comparisons that are not readily accessible as these are transient, mobile populations.

To construct signatures of this transition, we employed the least absolute shrinkage and selection operator (LASSO) as a feature selection method that minimizes covariate correlation. The resulting 27-gene signature (Fig. 2a and Supplementary Table 10) accurately predicted the late erythroblast and reticulocyte cell states without overfitting, based on a partial least squares discriminant (PLSDA) model (Supplementary Fig. 6). This method produced a sparse gene set that lacked unifying annotations. We therefore adopted a ‘bottom-up’ approach using local network information to connect our signature genes (Fig. 2b). This propagation of LASSO targets is similar to network biology approaches to predict drug targets and disease-associated genes and is based on the hypothesis that genes in close proximity topologically are functionally related.

To identify common regulators, we investigated the local topology of the first-order subnetwork in the global CellNet GRN, from which cell-type specific GRNs were originally identified (Fig. 2c). Contrary to our hypothesis, there was largely a one-to-one connection between all connected regulators and LASSO targets (Fig. 2d). The few statistically enriched genes belonged to networks of coregulated transcription factors, such as the pluripotency factors (that is, NANOG, SOX2, LIN28)22,23 that are associated with a single LASSO target, SALL2. This topology suggests that LASSO targets are associated with discrete biological processes, rather than being downstream of common regulators. Accordingly, this same analysis identified common regulators between ontologically related genes (Supplementary Fig. 7). However, further dissection of gene modules with modest coregulation of LASSO targets revealed that late erythroblast targets (Fig. 2e, Module 1) were associated with regulators of hematopoietic differentiation and P53-apoptotic pathways, whereas reticulocyte LASSO genes (Fig. 2e, Module 2) were downstream of metabolic and lipid pathways important for RBC maintenance.

Based on this largely one-to-one topology of the transcriptional regulator-target network, we hypothesized that common signaling networks may lie upstream of the transcriptional layer. Therefore, we generated an interaction network using the STRING database (Fig. 2f, Supplementary Fig. 8 and Supplementary Table 11). We employed the Prize Collecting Steiner Forest (PCSF) algorithm24, which is particularly suited for modeling multiple, independent pathways acting

**GRNs capture cell state dynamics.** The augmented CellNet algorithm classified all stages of erythropoiesis with high probability (Supplementary Fig. 1h–j). We hypothesized that subnetworks, or smaller gene modules, within the erythroid GRN correspond to distinct stages of differentiation, analogous to the cell-type-specific GRNs identified CellNet. To dissect the erythroid GRN, we projected the data into a principal component space (Fig. 1b, Supplementary Fig. 2b). Unsupervised Gaussian Mixture Model (GMM)–based clustering identified six discrete phenotypes, which were significantly enriched for manual, literature-based designations of erythroid stage—C1–C2: CFU-E/early proerythroblast; C3–C4: intermediate proerythroblast; C5: late erythroblast; C6: reticulocyte (Fig. 1c and Supplementary Table 3). K562, an erythroblastic leukemia cell line, clustered in C1 and studies of hemoglobin-perurbed cells clustered in C3, a PC1-shifted intermediate cluster. We therefore focused on clusters C2, C4, C5 and C6 for the purpose of studying physiological and developmental erythropoiesis.

In contrast to whole genome-based dimensionality reduction techniques commonly used in trajectory algorithms, the erythroid GRN served as a feature selection upstream of PCA, which identifies genes correlated with developmental stages. By GMM clustering, erythroid network genes clustered into three distinct groups (Supplementary Table 4), with early (C1) and intermediate (C2) differentiation clusters associated with cell cycle and hemoglobin synthesis, respectively (Fig. 1d and Supplementary Table 5). The reticulocyte cluster (C6) comprised genes that were not significantly enriched for any biological processes. Likewise, ranking gene importance to each phenotypic cluster (Supplementary Fig. 2d–g and Supplementary Table 6) failed to yield annotations for the reticulocyte cluster (C6). We also implemented K-means clustering to identify sets of genes with similar dynamic expression across biological clusters (C2, C4, C5 and C6) and identified coordinated regulation of genes related to processes such as stress responses, autophagy and apoptosis during differentiation (Supplementary Fig. 2h).

We confirmed that dimensionality reduction similarly captured biologically meaningful clusters in developmentally staged, purified populations analyzed by bulk RNA-seq (GSE53983). Sample localization in the PCA space was driven by similar genes, as shown by significant correlations with biological analogs from microarray data—proerythroblast: C2/S1/S2; intermediate erythroblast: C4/S3; late erythroblast: C5/S4; positive Pearson’s r, with P<0.05 (Supplementary Fig. 3c). Of note, expert knowledge was required for the interpretation and comparison of biologically analogous samples across microarray and RNA-seq datasets (for example, C4 and C5 both correlate with S3; C2 correlates with both S1 and S2), likely because the granularity and variance are strongly tied to the data source and experimental design. This further highlights the need for large data compendia, including purified populations, primary cells, in vitro differentiated cells, genetically perturbed cells and rare or unique populations (that is, reticulocytes), to fully sample the biological space within a given cell type. We also demonstrated that, in addition to compatibility with different data types, our pipeline is generally applicable to other biological systems (Supplementary Fig. 4 and Supplementary Tables 7–9).

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Fig. 2 | ErbB signaling is implicated in erythroid differentiation. a, Gene signature distinguishing clusters C5 (late-E) and C6 (reticulocyte; red) from Fig. 2a, as determined by LASSO. b, Schematic of network propagation approaches across different regulatory layers, including (1) nuclear regulators, (2) signaling regulators and (3) associated targets, corresponding to networks from c, f and i, respectively. c, First-order connection network derived from connecting the LASSO signature genes within the CellNet global GRN. Significantly enriched regulators (P < 0.05 by Fisher’s test for connections between the LASSO signature genes within the CellNet global GRN) are shown in black, with community-derived (that is, locally high density) modules depicted above in varying colors and the LASSO genes in red and blue, corresponding to the representation in e. d, The degree of all regulators and LASSO gene module in the network compared to the global GRN) are shown in black, with community-derived (that is, locally high density) modules depicted above in varying colors and the LASSO genes in red and blue, corresponding to the representation in e. e, Module within the network are highlighted and annotated with significantly enriched biological processes. P values ranking node enrichment (Fisher’s test for connections in the LASSO network relative to the full STRING network) and corresponding gene ontology annotations. h, Enriched signaling pathways from the Reactome database. i, Coexpression network comprising genes highly correlated r > 0.90] with the LASSO signature. j, Enrichment analyses for Reactome processes, kinase perturbation (downregulated genes on kinase knockdown from LINCS L1000) and ligand regulation (upregulated on ligand stimulation from LINCS L1000).
in synergy toward a unified biological response. The resulting network was enriched for biological processes such as apoptotic signaling, stress responses and cell cycle, consistent with previous analyses (Fig. 2g). Uniting these processes, P53 is a highly interconnected central node (P < 0.001, see Supplementary Fig. 8f). The network was also significantly enriched for Reactome signaling pathways relevant for erythropoiesis, such as Notch1, RhoA, TGFβ and BCR, as well as novel candidate pathways, including epidermal growth factor receptor (EGFR)/ErbB4, TLR and RIG-I/MDA5 (Fig. 2h).

Finally, we used a 'guilt by association' approach to define networks that were highly correlated with the LASSO signature (Fig. 2i, Supplementary Fig. 9 and Supplementary Table 12). Highly enriched transcription factor binding (ENCODE and ChEA) and kinase regulation (LINCS L1000) further implicated proliferative and apoptotic processes (that is, E2F, P53 and FOXM1/WE1E1). Moreover, several enriched kinases included members of the MAPK/ERK pathway (that is, SRC, ErbB3/ErbB4), and the ligand activation signatures (epidermal growth factor (EGF), TGFA, BTC) further supported a role for ErbB signaling in regulation of the coexpression network (Fig. 2j). This analysis demonstrates the utility of combining sparse gene signatures with network propagation approaches to identify novel biological processes that potentially mediate dynamic fate changes, thereby establishing hypotheses to be experimentally confirmed.

**ErbB4 is necessary for efficient erythropoiesis.** Although our network models identified several enriched signaling nodes and candidate pathways in erythropoietic maturation, the preponderance of evidence pointed to ErbB signaling. Although significantly enriched, EGFR/ErbB4 was not among the top candidate pathways (Fig. 2h, see comprehensive list in Supplementary Table 8); however, when combined with expert knowledge that ErbB signaling is frequently associated with P53 (ref. 29), and the apoptotic- and proliferative- processes that were repeatedly identified in our network models, ErbB signaling emerged as a lead candidate. To determine whether ErbB signaling was necessary for erythropoiesis, we perturbed erythroblasts differentiated from bone marrow HSPCs (CD34+) with ErbB inhibitors (Fig. 3a). Maturation (GlyA/CD71−) was only affected by pan-ErbB inhibitors (Afatinib, Dacomitinib, Neratinib), implicating ErbB4 rather than EGFR/ErbB2.

ErbB4 signaling has not previously been implicated in blood development or homeostasis. We characterized ErbB4 in human, mouse and zebrafish erythropoiesis. Using an erythroid in vitro differentiation protocol for human HSPCs (CD34+), we observed increasing ERBB4 mRNA expression as erythroid cells matured (Supplementary Fig. 10a). Native human bone marrow erythroid fractions also exhibited increased ERBB4 expression in the most mature population (GlyA/CD71−) (Supplementary Fig. 10b).

Reciprocally, pharmacological inhibition of ErbB signaling with Neratinib for 1 week shifted the bone marrow differentiation profile in mice, with an increase in immature and a decrease in mature erythroid populations (Fig. 3b), as well as changes to the peripheral hematopoietic fractions (Supplementary Fig. 11).

We next determined whether ErbB4 signaling also functioned during erythroid ontogeny, a process that initiates in multiple waves from restricted progenitors during embryogenesis35. Morpholino inhibition of ErbB4 in zebrafish embryos significantly decreased the frequency of Gata1+ erythroid cells (Supplementary Fig. 12a) and of more differentiated globin-expressing cells (Fig. 3c,d) at 48–56 h post fertilization (hpf), without affecting neutrophils (Supplementary Fig. 12b). These data indicate that ErbB4 signaling is necessary for robust erythropoiesis during embryonic and adult hematopoiesis.

**ErbB4 deficiency induces stress erythropoiesis.** To morestringently characterize ErbB4 in adult erythropoiesis, we employed a genetic mouse model derived via αMHC-driven expression of human HER4 to circumvent embryonic lethality from heart defects in the whole body ErbB4 knockout (ErbB4−/−/HER4heart)34. Consistent with the effects of Neratinib treatment, we observed an increase in early proerythroblast populations, with fewer mature orthochromatic and normoblastic cells in the ErbB4−/− bone marrow (Supplementary Fig. 13a,b). Nucleated RBCs and a high percentage of reticulocytes were present in peripheral blood, indicating moderate stress erythropoiesis in homozygotes and blood counts revealed significant changes in hemoglobin distribution (Fig. 4a and Supplementary Fig. 13c). ErbB4−− mice had enlarged spleens (Supplementary Fig. 13d), a >two-fold expansion of early erythroblasts (GlyA/CD71+; gate II) (Fig. 4b) and overcrowded red pulp (Fig. 4c), suggesting extramedullary erythropoiesis. Morphological analysis demonstrated early developmental blocks across multiple lineages in ErbB4−− bone marrow (Fig. 4d). CD41+ megakaryocytes in ErbB4-deficient spleen (P = 0.007 compared to wild type; Fig. 4e) decreased significantly, accompanied by a myeloid-skewed leukocyte profile and increased platelets in the periphery (Fig. 4f). These results demonstrate dysregulated multi-lineage hematopoietic phenotypes in ErbB4−/−/HER4heart mice.

Mitotic and proliferative processes downstream of ErbB matures iRBCs. To interrogate the molecular mechanisms downstream of ErbB, we performed global gene expression analysis of in vitro differentiated RBCs perturbed with pan versus selective inhibitors. Transcriptomic analysis confirmed that the erythroid GRN was modulated by pan-ErbB inhibitors, but not by Lapatinib, a dual EGFR/ErbB2 inhibitor (Supplementary Fig. 14a,b). Although cells were treated between the intermediate and late erythroblast stages, early network cluster genes (G1, see Supplementary Fig. 14c) were significantly decreased, suggesting that ErbB signaling plays a role during multiple stages of differentiation. Analysis of pathways dysregulated by pan-ErbB inhibition revealed upregulation of P53 signaling (Fig. 5a), with concomitant downregulation of mitotic and proliferative pathways (Fig. 5b). Consistent with our previous computational analysis, these data connect ErbB signaling with P53 and proliferative pathways in human erythropoiesis.

As mechanistic analyses identified the Wnt pathway as a putative downstream target of ErbB4 (Fig. 5b), we exploited the pharmacologic accessibility of this pathway to enhance erythropoiesis in vitro. A critical barrier to blood generation as a cell-based biotechnology stems from a block in erythroid maturation from iPSC cells (iRBCs), often requiring the use of feeder cells. We promoted maturation of iRBCs in a feeder-free system using bioprocess-compatible hematopoietic progenitors, which undergo continuous expansion under doxycycline-induced overexpression of five transcription factors (Fig. 5c)36. Activation of Wnt signaling via the agonist CHIR99021 increased the maturation of iRBCs, resulting in a 1.8-fold (P = 4.8×10−3) increase in GlyA/CD71− orthochromatic erythroblasts (Fig. 5d). Concomitantly, cells decreased in size with an increased nuclear-to-cytoplasmic ratio (Fig. 5e). Collectively, these data demonstrate that systems-level identification of druggable signaling pathways in developmental processes, such as erythropoiesis, is directly applicable to stem cell biomanufacturing and regenerative cell therapies.

**Discussion**

Here, we establish the utility of systems-level analytics to elucidate biological processes that mediate dynamic stem cell and developmental transitions. Our computational pipeline provides a road-map for the derivation of network models that connect sparse gene signatures with corresponding, yet disparate, biological processes, to captures the multi-factorial nature of cell state transitions. With cell engineering in hematopoiesis as an example, we highlight how to connect critical elements (for example, LASSO gene signatures)
to pathways/processes (for example, networks derived via PCSF and correlation). Our network models suggested and we experimentally confirmed a previously unanticipated role for ErbB4 in hematopoiesis.

Our advanced pipeline integrates network topological architecture with pseudotemporal information to provide multiple layers of information about cell differentiation, which is complementary to purely trajectory-based algorithms and highlights the changing roles of transcriptional regulators across dynamic stages of development. Moreover, the LASSO feature reduction as a foundation for network modeling ensures that the resulting models are informed by genes most vital to distinguishing divergent cell states. In contrast to traditional differential gene expression approaches, LASSO produces a sparse, sharply focused gene set and, when combined with PCSF, produces a signaling network comprising branches associated with distinct biological processes. Together, these approaches provide a more global depiction of the systems-level processes associated with cell fate transitions.

By applying our pipeline to study hematopoietic specification, we established a role for ErbB4 signaling in erythropoiesis in multiple in vitro and in vivo models. Many of our computational approaches did not directly identify ErbB4; however, network propagation from our maturation signature repeatedly identified ErbB ligands and ErbB-associated signaling, including MAPK/ERK, mitotic processes, P53 and apoptosis. This highlights the need for future development of unsupervised metrics to prioritize candidates from aggregate data, which currently requires expert knowledge as an integral part of the process. Although there were no annotated processes enriched within the reticulocyte gene cluster, it included the N-methyl-D-aspartate (NMDA) receptor, GRIN3B, which is commonly implicated, along with ErbB4, in neurological development and pathophysiology. Anemia is a common side effect of antipsychotic drugs and studies of glutamate-mediated ion channels supports their functional role in erythropoiesis. This opens the possibility of new avenues of crosstalk between neurological and hematopoietic systems, akin to the regulation of hematopoietic stem cell production by the central nervous system. Our dynamic analyses also revealed that oxidative stress pathways peak at the late erythroblast stage; ErbB4 is a known stress responsive pathway in the heart and abrogates oxidative damage in the brain. Although a recent meta-analysis of genome-wide association study data identified neuregulin-4 (NRG4), an ErbB4-specific ligand, as a putative locus in aberrant human RBC phenotypes, the pathway has not been previously characterized in erythropoiesis.

Cell engineering has broadly focused on inducing transcription factors as the emissaries of phenotype. To this end, CellNet successfully predicts candidate and aberrant transcription factors. However, even the most-studied form of reprogramming, induced pluripotency, remains exquisitely sensitive to culture conditions and hematopoietic systems, akin to the regulation of hematopoietic development of unsupervised metrics to prioritize candidates from aggregate data, which currently requires expert knowledge as an integral part of the process. Although there were no annotated processes enriched within the reticulocyte gene cluster, it included the N-methyl-D-aspartate (NMDA) receptor, GRIN3B, which is commonly implicated, along with ErbB4, in neurological development and pathophysiology. Anemia is a common side effect of antipsychotic drugs and studies of glutamate-mediated ion channels supports their functional role in erythropoiesis. This opens the possibility of new avenues of crosstalk between neurological and hematopoietic systems, akin to the regulation of hematopoietic stem cell production by the central nervous system. Our dynamic analyses also revealed that oxidative stress pathways peak at the late erythroblast stage; ErbB4 is a known stress responsive pathway in the heart and abrogates oxidative damage in the brain. Although a recent meta-analysis of genome-wide association study data identified neuregulin-4 (NRG4), an ErbB4-specific ligand, as a putative locus in aberrant human RBC phenotypes, the pathway has not been previously characterized in erythropoiesis.

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Fig. 4 | ErbB4 genetic deficiency leads to blood defects in the ErbB4<sup>−/−</sup> HER4<sup>lox/lox</sup> mouse model. a. Morphology of the peripheral blood from smears stained with May–Grunwald–Giemsa indicating the increased reticulocyte fraction (purple), quantified as Ter119<sup>+</sup> Thiazole Orange (TO)<sup>+</sup> cells (ErbB4<sup>+/−</sup> n = 3; ErbB4<sup>−/−</sup> n = 5; ErbB4<sup>+/−</sup> n = 4; *P < 0.05 compared to wild type by one-way ANOVA). b. Erythroid fractions within the spleen of wild type, heterozygous and homozygous mice, demonstrating increased extramedullary erythropoiesis (gate II, CD71<sup>+</sup>Ter119<sup>+</sup>). c. Morphology of the spleen stained with hematoyxin and eisin showing dense red pulp. d. Bone marrow composition from cytospins stained with May–Grunwald–Giemsa demonstrating the presence of immature fraction hematopoietic fractions across multiple lineages. e. Proportion of CD41<sup>+</sup> megakaryocytes in the spleen. f. Quantification of lymphocytes, neutrophils and platelets in the peripheral blood via Hemavet (ErbB4<sup>+/−</sup> n = 3; ErbB4<sup>−/−</sup> n = 5; ErbB4<sup>+/−</sup> n = 4; *P < 0.05, **P < 0.01 compared to wild type by one-way ANOVA). Scale bars correspond to 10 µm (a), 100 µm (c) and 50 µm (d).

Fig. 5 | Modulation of pathways downstream of ErbB signaling augments iPS-derived RBC generation. RNA-seq of bone marrow CD34<sup>+</sup> cells treated with Lapatinib (increased affinities for EGFR and HER2), as well as Neratinib, Dacomitinib and Afatinib (pan-ErbB inhibitors) for 24 h. a,b. Expression of genes from the Hallmark pathways (GSEA) significantly (*FDR < 0.25) upregulated (a) and downregulated (b) (compared to DMSO vehicle) and common to pan-ErbB inhibitor treated cells. c. Cell numbers (log fold change) of expanded iPS-derived CD34-5F cells<sup>+</sup> over 14 d. d. Erythroid profile with and without CHIR99021 treatment during the final week of differentiation, with significantly increased proportion of mature (GlyA<sup>+</sup>, CD71<sup>+</sup>) erythrocytes. *P < 0.001 by unpaired, two-sided t-test. e, morphology of May–Grunwald–Giemsa stained, iPS-derived orthochromatic erythroblasts. c, n = 7 and d, n = 6 independent replicates from two experiments. Scale bar in e, 20 µm.
maintaining elusive populations, such as HSCs. Although engraftable HSCs can be generated with transcription factors alone\(^1\), reprogramming is enhanced by perturbation of developmental pathways, such as TGFβ and BMP4 (ref. \(^4\)). Similarly, AKT-activated endothelial cells support self-renewal and maintenance of HSCs through angiocrine factors\(^6\). The prevalence of growth factor supplementation and cocultures across hematopoietic differentiation protocols further highlights the need to identify and recapitulate cell-extrinsinc signals. Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41587-019-0159-2.

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**Author contributions**

M.A.K., D.A.L. and G.Q.D. conceived the project. M.A.K., L.T.V., J.M.F., J.B., A.J.C. and S.L. performed experimental work and data interpretation. K.-K.W., J.J.C., P.C., T.E.N., D.A.L. and G.Q.D. supervised research and participated in project planning. M.A.K., T.E.N., D.A.L. and G.Q.D. prepared the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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An Analysis

Nature Biotechnology

Methods

GRN reconstruction and CellNet analytics. In total, 164 erythroid Affymetrix microarrays (Supplementary Tables 1 and 2) from the HGU133plus2 platform were acquired from the Gene Expression Omnibus (GEO) and compiled with the original human CellNet compendium. Microarrays were preprocessed, the global GRN was calculated via the context likelihood of relatedness (CLR) inference algorithm, and subnetworks were detected via InfoMap community detection, as previously described. Unless specified, all high dimensionality data analyses were accomplished using the R computational environment (v.3.2.2), with specified packages from GRN and CellNet bioconductor. All graph-related representations, and network analytics were visualized and calculated with the igraph package. Cell- and tissue-specific GRNs were established via enrichment using the chi-squared statistical test. As implemented in CellNet, a random forest classifier was trained based on the GRN for each cell type and trained with a randomly selected subset comprising approximately 50% of the microarrays. The classification performance was then evaluated on the remaining independent set of microarrays and/or parallel biological replicates (for example, animals or cell cultures). The exact replicate numbers and statistical tests are specified in the figure legends.

Human CD34+ RBC differentiation. Human CD34+ progenitors derived from mobilized peripheral blood (AllCells) were expanded for 4d in StemSpan SFEM (StemCell Technologies) with the addition of interleukin (IL)-3 (10 ng/ml), IL-6 (50 ng/ml), thrombopoietin (TPO) (50 ng/ml), stem cell factor (SCF) (50 ng/ml) and Fli3 (50 ng/ml). Unless specified, all cytokines were from PeproTech. Erythroid differentiation was accomplished using a previously published, three-stage protocol. Briefly, all stages of differentiation consisted of a basal erythroid differentiation medium (EDM) consisting of IMDM with 15% FBS, 1% BSA, 2 mM l-glutamine, 500 μg/ml holo-transferrin and 10 μg/ml insulin. Stage 1 consists of EDM plus the addition of dexamethasone (1 μM), β-estradiol (1 μM), IL-3 (5 ng/ml), SCF (100 ng/ml) and erythropoietin (EPO) (6 U) for 5 days (days 0–5). Stage 2 consists of EDM plus the addition of SCF (50 ng/ml) and EPO (6 U) for 4d (days 5–9). Stage 3 consists of EDM plus the addition of EPO alone (2 U) for 8 d (days 9–17). At all stages, cells were cultured in 24-well plates in 1 ml of media. After 24 h, cells were washed by automated compensation with anti-mouse Igk and negative beads and resuspended in basal EDM. The EDM was then evaluated on the remaining independent set of microarrays. The classification performance was then evaluated on the remaining independent set of microarrays.

Statistical analyses. All statistical analyses were calculated in R, using two-sided, unpaired t-test, ANOVA or Fisher’s exact test. Data are presented as standard error boxplots representing the median and ranging from the 25th to 75th percentiles, with the whiskers extending to 1.5x interquartile range. The sample sizes represent a minimum of three independent replicates, corresponding to distinct experiments and/or parallel biological replicates (for example, animals or cell cultures).

iPS-SF generation and RBC differentiation. Human iPS-SF cells were generated as previously described12 from MSC-iPS obtained from the Boston Children’s Hospital Human Embryonic Stem Cell Core and verified by immunohistochemistry for pluripotency markers, teratoma formation and karyotyping. Briefly, iPS cells were differentiated as embryoid bodies using a hematopoietic induction protocol and CD34+ cells were sorted from bulk embryoid body culture by magnetic activated cell sorting using human CD34 microbeads (Miltenyi Biotec.), as per the manufacturer’s instructions. The embryoid body progenitors were seeded on retroenexin-coated (10 μg/cm2) 96-well plates (2 × 105–5 × 105 cells per well) in SFEM (StemCell Technologies) containing 50 ng/ml SCF, 50 ng/ml FL3, 50 ng/ml TPO (all R&D Systems) 30 ng/ml IL-3 and 10 ng/ml IL-1 (both from PeproTech) and infected with 5 PFU lentiviral particles. Lentiviral particles for the 5F plasmids (HOXA9, ERG, RORA, SOX4 and MYB cloned into pluducer-23 doxycycline-inducible vector) were produced by transfecting 293T-17 cells (ATCC) with generation packaging plasmids. The multiplicity of infection (MOI) for each factor was: ERG MOI = 5, HOXA9 MOI = 5, RORA MOI = 3, SOX4 MOI = 3, MYB MOI = 3. Following 24 h of incubation, 5F cells were cultured in SFEM with 50 ng/ml SCF, 50 ng/ml FL3, 50 ng/ml TPO, 50 ng/ml IL-3, 50 ng/ml IL-6 and 10 ng/ml IL-3 (5Fpep) and 2 μg/ml doxycycline (Dox, Sigma). Cultures were maintained at a density of <1 x 105 cells/ml and the medium was changed every 3–4 d.

RBC differentiation from iPS-SF followed a slightly modified protocol that was previously optimized for translational approaches aimed at translation in vitro-derived RBCs13. In this protocol, the EDM was instead consisting of IPDM with 15% inactivated plasma (solvent detergent pooled plasma AB from the Rhode Island Blood Center), 2 mM l-glutamine, 330 μg/ml holo-transferrin and 10 μg/ml insulin, 2 IU/ml heparin (Sigma) and 31U/ml rEPO. Stage 1 (days 0–8) was performed at 1–3 x 105 cells/ml supplemented with 10 ng/ml SCF and 5 ng/ml IL-3. Stage II (days 8–11) was plated at 1–3 x 105 cells/ml and supplemented with 100 ng/ml SCF. Stage III (days 11–18) was plated at 1 x 105 cells/ml in the basal EDM. All analyses were conducted at day 18 of differentiation and CHIR99021 (3 μM) was added throughout stage III (days 9 and 13).

Flow cytometry and cell sorting. Human erythropoiesis, including differentiation from bone marrow CD34+ cells and native bone marrow samples, was analyzed with the following antibody panel: CD71 PE (M-A712, BD) and CD235a/ Glycoporphin A PE-Cy7 (114E8-7-6, Coulter) or CD235a/Glycoporphin A FITC (114E8-7-6, Coulter). Mouse erythropoiesis from Nteratinb treated and H460 tumors (follwing with the following antibodies: CD71 PE, CD45 PE-Cy7, CD34 PE, mTer119 PE-Cy5 (Ter119; eBioscience). All staining was performed with <1 x 105 cells per 100 μl staining buffer (PBS + 2% FBS) with 1:100 dilution of each antibody for 30 min at room temperature in the dark. Compensation was performed by automated compensation with anti-mouse Igk and negative beads.
Inhibitors. All inhibitors were added to cell cultures at 1 μM on days 9 and
13 of differentiation, corresponding to the beginning and middle of stage 3
(supplemented with EPO only, as described above). DMSO was used for a vehicle
control in all cell culture studies. Details on ordering information and affinities are
provided in Supplementary Table 13.

RNA-sequencing. RNA was extracted after 24 h of incubation with ErbB inhibitors
(day 10 of erythroid differentiation) using Trizol reagent (Invitrogen) and the
RNeasy Plus kit (Qiagen). Quality of RNA was monitored via QC for high RNA
integrity number values and low levels of DNA contamination. RNA-seq libraries
were prepared using the SMARTSeq v4 kit as per the manufacturer's protocol with
10 ng input RNA. Libraries were sequenced using the 200-cycle paired-end kit on the
Illumina HiSeq2500 system. RNA-seq reads were analyzed with the Tuxedo Tools
following a standard protocol on the Harvard Medical School Orchestra Cluster.
Reads were mapped with TopHat v.2.1.0 and Bowtie2 v.2.2.4 with default parameters
against build hg19 of the human genome, and build hg19 of the RefSeq human
genome annotation. Samples were quantified with the Cufflinks package v.2.2.1.
Differential expression was performed using Cuffdiff with default parameters.

PCR. RNA was extracted as described above and complementary DNA was
synthesized using the SuperScript VILO cDNA Synthesis Kit (Thermo), per the
manufacturer’s instructions. Real-time PCR was run using SYBR green technology with
QuantiTect primers for the ErbB receptor family (Qiagen) on the QuantStudio
Flex Real-Time PCR System.

Zebrafish studies. Zebrafish were maintained according to institutional animal
care and use committee-approved protocols. The Tg(globin:eGFP) line was
provided by L.I. Zon, Children’s Hospital, Harvard Medical School, Boston. MOs
(GeneTools) were microinjected at the one-cell stage as described previously69.
ErbB4 MOs were generated from previously published sequences51. Embryos were
harvested at 48–56 hpf and were processed with matched sibling controls for
o-dianisidine staining and evaluation of globin:eGFP intensity. Staining intensity was
quantified using the Cufflinks package v.2.2.1.

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Data availability
All RNA-seq data have been deposited to the GEO database under GSE108128.
Corresponding author(s): George Q. Daley

Reporting Summary

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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Software and code

Policy information about availability of computer code

Data collection

164 erythroid Affymetrix microarrays (Supplementary Tables 1&2) from the HGU133plus2 platform were acquired from the Gene Expression Omnibus (GEO) and compiled with the original human CellNet compendium. Microarrays were preprocessed, the global gene regulatory network (GRN) was calculated via the Context Likelihood of Relatedness (CLR) inference algorithm, and subnetworks were detected via InfoMap community detection, as previously described. Unless specified, all high dimensionality data analytics were accomplished using the R computational environment (version 3.2.2), with specified packages (see Online Methods) from CRAN and Bioconductor.
Data analysis

All statistical analyses were calculated in R, using two-sided, unpaired t-test or ANOVA, where appropriate, with \( p < 0.05 \) considered significant. Data are presented as standard boxplots representing the median and ranging from the 25th to 75th percentiles, with the whiskers extending to 1.5*IQR. The sample sizes represent a minimum of three independent replicates, as specified in the figure legends. Gene set enrichment analysis (GSEA) was run according to default parameters in their native implementations. Statistical enrichment of gene lists in gene ontology (GO) enrichment was performed using Fisher’s exact test followed by correction for multiple hypothesis testing.

RNA-seq reads were analyzed with the Tuxedo Tools following a standard protocol on the Harvard Medical School Orchestra Cluster. Reads were mapped with TopHat version 2.1.0 and Bowtie2 version 2.2.4 with default parameters against build hg19 of the human genome, and build hg19 of the RefSeq human genome annotation. Samples were quantified with the Cufflinks package version 2.2.1. Differential expression was performed using Cuffdiff with default parameters.

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RNA-seq data has been deposited to the Gene Expression Omnibus (GEO) database under GSE108128. Raw data is available in the Supplementary Tables.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | At least \( n=5 \) mice were used per cohort for neratinib treatments. For zebrafish studies, at least \( n>6 \) embryos were used and replicated across multiple clutches. HER4heart mouse studies were analyzed with at least \( n=4 \). |
| Data exclusions | No data were excluded |
| Replication | Number of replicates per experiment and statistical analyses are described in each figure legend. |
| Randomization | Mice and zebrafish were assigned randomly to groups and not blinded. |
| Blinding | Mice and zebrafish were assigned randomly to groups and not blinded. |

Reporting for specific materials, systems and methods

| Materials & experimental systems |
| n/a | Involved in the study |
| ☑️ | Unique biological materials |
| ☑️ | Antibodies |
| ☑️ | Eukaryotic cell lines |
| ☑️ | Palaeontology |
| ☑️ | Animals and other organisms |
| ☑️ | Human research participants |

| Methods |
| n/a | Involved in the study |
| ☑️ | ChiP-seq |
| ☑️ | Flow cytometry |
| ☑️ | MRI-based neuroimaging |
Antibodies

| Antibodies used | Human erythropoiesis, including differentiation from BM CD34+ cells and native bone marrow samples, was analyzed with the following antibody panel: CD71 PE (M-A712; BD), and CD235a/Glycophorin A PE- Cy7 (11E4B-7-6; Coulter) or CD235a/Glycophorin A FITC (11E4B-7-6; Coulter). Mouse erythropoiesis from Neratinib treated and HER4heart mice was analyzed with the following antibody panel: mCD71 FITC (C2; BD), mTer119 PE-Cy5 (Ter-119; eBioscience). |
| Validation | Antibodies were all previously validated using cord blood and peripheral blood mononuclear cells as positive controls. |

Eukaryotic cell lines

| Policy information about cell lines | Cell line source(s) |
| --- | --- |
|  | iPS-5F cells were generated as previously described (Doulatov et al., Cell Stem Cell, 2014; Vo et al., Nature, 2017) from MSC-iPS (Park et al., Nature, 2008) obtained from the Boston Children's Hospital Human Embryonic Stem Cell Core (hESC) and verified by immunohistochemistry for pluripotency markers, teratoma formation and karyotyping. Primary cells (CD34+ cord blood) were obtained from AllCells. |

| Validation | Antibodies were all previously validated using cord blood and peripheral blood mononuclear cells as positive controls. |
| Authentication | MSC-iPS were verified by immunohistochemistry for pluripotency markers, teratoma formation and karyotyping |
| Mycoplasma contamination | All lines routinely tested negative for mycoplasma contamination. |
| Commonly misidentified lines | N/A |

Animals and other organisms

| Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research | Laboratory animals |
| --- | --- |
|  | All mice were housed in pathogen-free animal facilities, and all experiments were performed with the approval of the Animal Care and Use Committee at Harvard Medical School and Dana-Farber Cancer Institute and/or the BCH animal care committee. At least n=4 animals were used per cohort. Mice were assigned randomly to groups and not blinded. Neratinib was delivered to B6 albino mice via oral gavage at 60 mg/kg daily for 1 week. Hydroxypropyl methylcellulose (HPMC) was used as a vehicle control for Neratinib in mouse treatments. ErbB4-/-HER4heart mutant mice were generated in 2003 by Martin Gassmann (University of Basel) and colleagues and obtained from Dr. Gabriel Corfas at the University of Michigan. |

| Wild animals | N/A |
| Field-collected samples | N/A |

Flow Cytometry

| Plots | Confirm that: |
| --- | --- |
| | ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC). |
| | ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers). |
| | ☒ All plots are contour plots with outliers or pseudocolor plots. |
| | ☒ A numerical value for number of cells or percentage (with statistics) is provided. |

Methodology

| Sample preparation | CD34+ progenitors derived from mobilized peripheral blood (AllCells) were expanded and differentiated via an established erythroid differentiation protocol. All staining was performed with < 1x106 cells per 100 μL staining buffer (PBS + 2% FBS) with 1:100 dilution of each antibody for 30 min at RT in dark. Compensation was performed by automated compensation with anti-mouse Igk and negative beads (BD). |
| Instrument | Acquisition was performed on a BD Fortessa cytometer and all sorting was performed on a BD FACS Aria II cell sorter using a 70-mm nozzle. |
| Software | All flow cytometry data was analyzed using FlowJo 8.7. |
| Cell population abundance | No sorting was performed. |
Gating strategy

All cells were first gated on FSC/SSC according to cell size and granularity, using stained human cord blood mononuclear cells (MNCs) as a positive control and reference for cell size, granularity and staining intensity. Unstained samples were used to set up negative gates. Dead cell populations were excluded using DAPI staining.

 Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.