



# Thyroid hormone receptor beta and NCOA4 regulate terminal erythrocyte differentiation

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**An effect of thyroid hormone (TH) on erythropoiesis has been known for more than a century but the molecular mechanism(s) by which TH affects red cell formation is still elusive. Here we demonstrate an essential role of TH during terminal human erythroid cell differentiation; specific depletion of TH from the culture medium completely blocked terminal erythroid differentiation and enucleation. Treatment with TR $\beta$  agonists stimulated premature erythroblast differentiation in vivo and alleviated anemic symptoms in a chronic anemia mouse model by regulating erythroid gene expression. To identify factors that cooperate with TR $\beta$  during human erythroid terminal differentiation, we conducted RNA-seq in human reticulocytes and identified nuclear receptor coactivator 4 (NCOA4) as a critical regulator of terminal differentiation. Furthermore, *Ncoa4*<sup>-/-</sup> mice are anemic in perinatal periods and fail to respond to TH by enhanced erythropoiesis. Genome-wide analysis suggests that TH promotes NCOA4 recruitment to chromatin regions that are in proximity to Pol II and are highly associated with transcripts abundant during terminal differentiation. Collectively, our results reveal the molecular mechanism by which TH functions during red blood cell formation, results that are potentially useful to treat certain anemias.**

thyroid hormone | erythropoiesis | NCOA4 | nuclear receptor

The production of red blood cells (RBCs) in mammals is tightly regulated by erythropoietin (Epo), a kidney-produced hormone that stimulates erythropoiesis by promoting the survival, proliferation, and terminal differentiation of colony-forming unit erythroid (CFU-E) progenitors (1, 2). The early phase of CFU-E erythroid differentiation is Epo dependent, whereas later stages are Epo independent (3). Binding of Epo to Epo receptors (EpoRs) on the surface of erythroid progenitors such as CFU-Es leads to the activation of multiple intracellular signal transduction pathways, including those mediated by signal transducer and activator of transcription 5 (Stat5) and phosphatidylinositol 3-kinase (PI-3 kinase), which are essential for erythroid progenitor survival and proliferation (4, 5).

A group of hormones that bind to and activate nuclear receptor (NR) transcription factors (TFs) also regulate erythropoiesis. Cortisol and its receptor glucocorticoid receptor (GR) are important during stress erythropoiesis and synthetic glucocorticoids have been used to treat severe anemias such as Diamond Blackfan anemia (DBA) (6–8). Corticosteroids promote the self-renewal of burst-forming unit erythroid (BFU-E) progenitors and prevent their differentiation to the more mature CFU-Es (9, 10). The GR synergizes with peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) to enhance BFU-E self-renewal, and PPAR- $\alpha$  agonists and corticosteroids increase red blood cell production both in cell culture and in vivo (11). Retinoid acid increases the numbers of human erythroid progenitors, including BFU-Es in vitro (11). Retinoid acid receptor alpha (*Rxra*) knockout mice are anemic during the embryonic period due to a deficiency in erythroid differentiation, although the molecular mechanism(s) is still unclear (12). In addition, the orphan nuclear receptors, testicular receptors

2 and 4 (TR2/TR4), are involved in globin switching in adult erythroid cells (13).

A function of TH in erythropoiesis has been known for more than a century (14, 15). Aberrant production of RBCs and anemia are often observed in patients with thyroid diseases (16). TH signals through TH nuclear receptors  $\alpha$  (TR $\alpha$ ) and  $\beta$  (TR $\beta$ ), both possessing different splicing isoforms (17). Although the molecular mechanism(s) underlying TH function on erythropoiesis is unknown, human genome-wide association studies (GWASs) have identified genetic variances in the TR $\beta$  locus associated with abnormal hematological traits (18). Knocking out TR $\alpha$  but not TR $\beta$  in mice results in reduction in the numbers of early erythroid progenitors in fetal livers (19).

Here we demonstrate that TH is required for terminal erythroid differentiation. Targeting thyroid hormone signaling by TH receptor agonists enhances the differentiation of erythroid progenitors. More importantly, treatment with TR $\beta$  agonist GC-1 enhanced erythroblast differentiation in vivo and alleviated anemic symptoms in a chronic anemia mouse model. Furthermore, we identified NCOA4 as a critical component of TH signaling during erythroid terminal differentiation. Genome-wide analysis suggested that TH promotes NCOA4 recruitment to chromatin regions that are in proximity to Pol II and are highly associated with genes encoding transcripts that are abundant during terminal differentiation. Our results reveal the

## Significance

**We have long known that thyroid hormone (TH) stimulates formation of red blood cells and patients with thyroid diseases are often anemic, but the underlying molecular mechanisms are unclear. This study uses pharmacologic and genetic approaches in primary cells and animal models to demonstrate essential roles of nuclear receptor coactivator 4 (NCOA4) and TH in late erythropoiesis. We show that TH is essential for the last steps in formation of red cells in culture, and that treatment of cells with drugs that activate a particular nuclear TH receptor, TR $\beta$ , stimulates erythroid differentiation and alleviates anemic symptoms in a chronic anemia mouse model, indicating potential clinical applications. Further, we show that TR $\beta$  functions together with NCOA4 to regulate red cell formation.**

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. GSE102586).

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molecular mechanism by which TH stimulates terminal red blood cell formation, results that are potentially useful to treat certain anemias.

## Results

**TH Is Required for Terminal Erythroid Differentiation.** To test whether TH is essential for human red blood cell formation, we used a four-stage culture system using human CD34<sup>+</sup> peripheral blood hematopoietic stem/progenitor cells purified from G-CSF-treated normal individuals (11). Terminal differentiation requires serum and begins at day (D) 14 when Epo is the only cytokine added to the culture medium. Replacement of normal FBS at day 14 with charcoal-stripped FBS completely blocked terminal differentiation, as measured by the percentage of enucleated cells (Fig. 1A). Consistent with a block in erythroid development, the size of erythroid cells cultured with charcoal-stripped FBS is significantly larger than that of the control group. Importantly, addition of thyroxine (T4) at day 14 completely rescued the failure of terminal differentiation of erythroid cells by charcoal-stripped FBS (Fig. 1A).

T4 also accelerates and enhances terminal erythroblast differentiation when added at day 14 to normal cultures (Fig. S1A), leading to over ~95% enucleation at day 16, while control cells in these human CD34<sup>+</sup> erythroid cultures have not begun to enucleate (Fig. 1B and Fig. S1B). Consistent with premature induction of terminal differentiation, treatment of mouse BFU-Es with T4 rapidly induced expression of erythroid genes such as *Hemoglobin Beta Chain (Hbb)*, *Solute Carrier Family 4, Anion Exchanger, Member 1 (Slc4a1)*, *Ferritin Light Chain (Ftl)*, and *Alpha Hemoglobin Stabilizing Protein (Ahsp)*, and reduced the expression of *Kit*, which is essential for erythroid progenitor self-renewal and is normally down-regulated during terminal differentiation (Fig. S1C). These data reinforce the essential function of TH in promoting terminal erythroid differentiation.

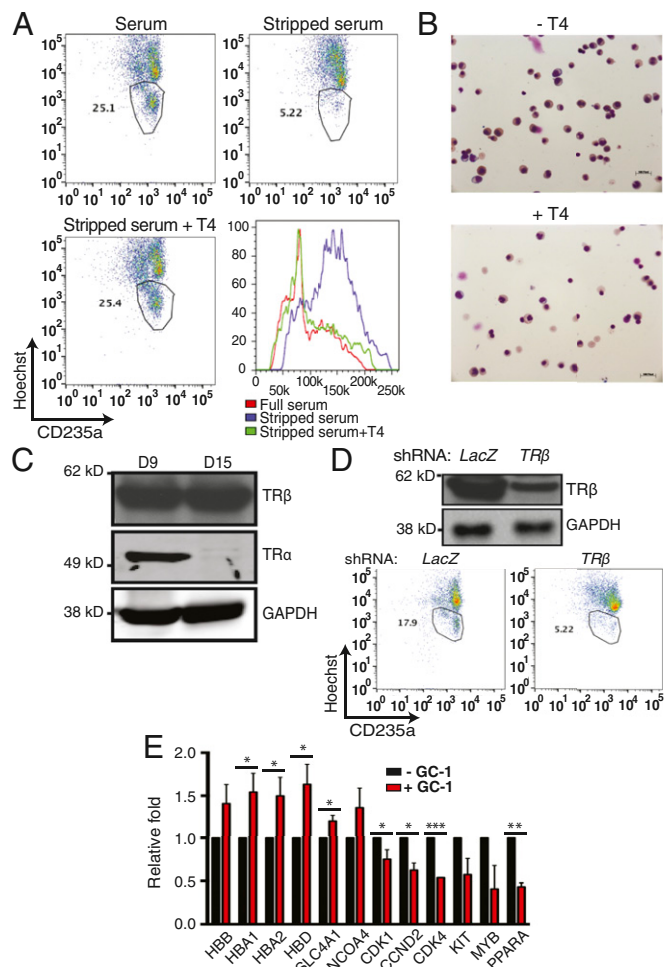
While both TR $\alpha$  and TR $\beta$  proteins are expressed in human CD34<sup>+</sup> progenitors, only TR $\beta$  protein is detectable in late erythroblasts (Fig. 1C). Knocking down TR $\beta$  in CD34<sup>+</sup> cells impaired the enucleation of human erythroblasts, indicating that TR $\beta$  is required for human erythroid terminal differentiation (Fig. 1D). Consistent with the notion that TR $\beta$  but not TR $\alpha$  is required for terminal differentiation, both T4 and the TR $\beta$  selective agonist GC-1 restore normal erythroblast differentiation, measured by enucleation, that is deficient in cultures in charcoal-stripped serum (Fig. S1D). These effects of TH receptor (THR) agonists are significantly abrogated upon TR $\beta$  knockdown (Fig. S1D). Taken together, these data suggest that TH is required for terminal differentiation of human erythroid progenitors and that this function is mediated by TR $\beta$  and not TR $\alpha$ .

Global run-on sequencing (GRO-seq) analysis showed up-regulation of many erythroid genes including *HBB*, *Hemoglobin Alpha Chain (HBA)*, and *SLC4A1* in human erythroblasts treated with GC-1 (Fig. 1E). In contrast, expression of genes involved in the cell cycle and essential for stem/progenitor self-renewal and normally down-regulated during terminal erythropoiesis, including *KIT*, *MYB*, and *PPARA* (11), were significantly down-regulated by GC-1 treatment. Together, these studies showed that activation of TR $\beta$  accelerates terminal human erythroid differentiation.

**TR $\beta$  Agonist GC-1 Alleviates Anemia in a Chronic Anemia Mouse Model.** We tested whether TR $\beta$  selective agonists are able to increase red cell production in a mouse model of chronic anemia, neonatal anemia (*Nan*) mice (20). GC-1 injection significantly increased hemoglobin levels, hematocrits, and RBC numbers, and reduced the numbers of reticulocytes in these anemic mice (Fig. 2A). Red cells in *Nan/+* mice are anisocytotic with many microcytes, spherocytes, and hypochromic cells (20). These abnormalities were alleviated by GC-1 treatment (Fig. 2B). Quantitatively, the red blood cell distribution width (RDW) was significantly increased above normal in *Nan/+* mice, and the RDW was significantly decreased, although not to normal, following GC-1 treatment

(Fig. 2C). These data suggest TH alleviates anemic symptoms in these mice through enhancing erythroid differentiation.

In mice the spleen is the primary site of stress erythropoiesis (21). Both spleen size and weight were dramatically increased in *Nan/+* mice (20) (Fig. S2A and B). GC-1 injection reduced both splenomegaly and spleen weight in *Nan/+* mice compared with control DMSO treatment (Fig. S2B). Colony-forming assays showed that GC-1-injected mice had fewer BFU-E progenitors in the spleen, suggesting that indeed GC-1 is able to alleviate



**Fig. 1.** TH promotes terminal erythroid differentiation through TR $\beta$ . (A) Human CD34<sup>+</sup> cells from G-CSF mobilized peripheral blood were cultured by the four-stage (21 d total) erythroid differentiation method as described in *Materials and Methods*. At day 14, cells were switched to terminal differentiation (Dif III) culture medium containing serum, stripped serum, or stripped serum supplemented with 1  $\mu$ M T4. Flow cytometry analysis was conducted at day 21. (Bottom Right) Forward scatterplots are shown for erythroid cells at day 21. (B) Benzidine-Giemsa staining demonstrating cell morphology of human erythroblasts at day 16 of the CD34<sup>+</sup> erythroid culture. Brown color of benzidine staining indicates hemoglobin accumulation. Cells were untreated or treated with 1  $\mu$ M T4 at day 14 for 72 h. (C) Western blotting demonstrating protein levels of TR $\alpha$  and TR $\beta$  in human erythroid cells at days 9 and 15 of culture. (D) Human CD34<sup>+</sup> cells were transfected by lentivirus vectors encoding shRNAs targeting either LacZ (control) or *TR $\beta$*  at day 1. (Top) Protein expression of TR $\beta$  determined by Western blotting at day 14. (Bottom) The percentage of enucleated cells was determined by flow cytometry analysis at day 21. (E) Gene expression in human erythroid cells untreated or treated at day 14 with 1  $\mu$ M GC-1 for 6 h is analyzed by GRO-seq. Data were normalized to cells treated with DMSO (error bars represent mean  $\pm$  SD from three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001).



stress erythropoiesis in vivo (Fig. S2C). In *Nan*<sup>+/+</sup> mice virtually all of the spleen cells are positive both for CD71 and Ter119, indicating that they are in the erythroid lineage. Treatment with GC-1 reduced this population significantly such that about half the cells are negative both for CD71 and Ter119 and thus presumably not undergoing erythroid differentiation (Fig. S2D). Thus, GC-1 not only alleviates the anemia characteristic of the *Nan*<sup>+/+</sup> mice, it also reduces extramedullary erythropoiesis.

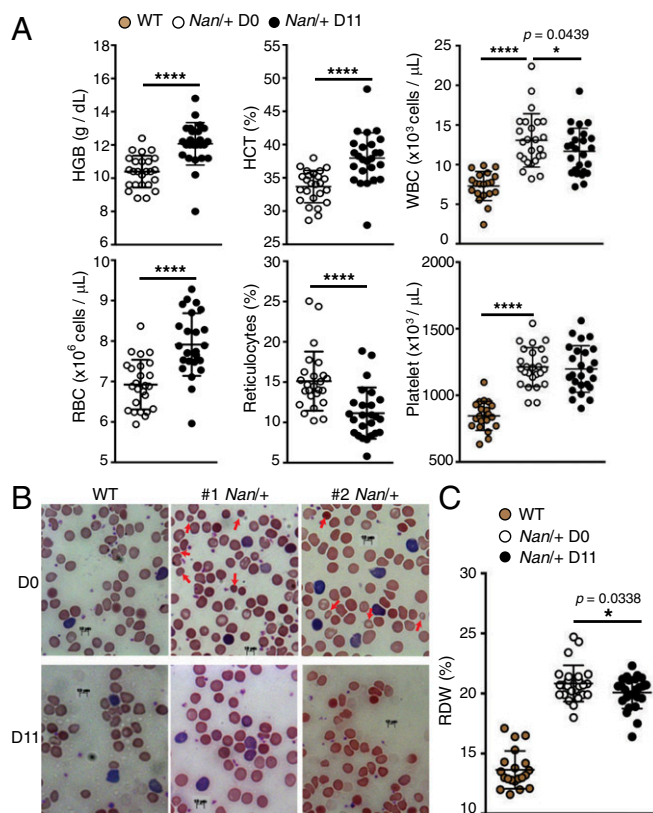
We next set out to understand how TH receptor agonist GC-1 increases RBC production in *Nan*<sup>+/+</sup> mice. *Nan*<sup>+/+</sup> BFU-E cells were isolated from embryonic day 14.5 (E14.5) fetal livers and cultured for 6 h in the presence or absence of GC-1. Consistent with previous studies, *Eklf* mRNA levels as well as levels of mRNAs encoding *Dmt1*, *Slc4a1*, and *Hbb*, were significantly down-regulated in cells from *Nan*<sup>+/+</sup> mice compared with those from WT littermates (20). Treatment with GC-1 significantly increased the expression of *Dmt1*, *Slc4a1*, and *Hbb* (20) (Fig. S2E). In contrast, GC-1 did not increase the expression of *Eklf* or *Gata1*. Therefore, our data suggested TH alleviates anemia in the *Nan*<sup>+/+</sup> mice by up-regulating expression of several key erythroid-important genes.

**NCOA4 Is Essential for Human Erythroblast Terminal Differentiation.** To further determine which transcriptional regulators are important for terminal erythropoiesis, we isolated reticulocytes produced in our culture of human adult CD34<sup>+</sup> stem/progenitor cells (11). RNA-seq analysis on these cells (Fig. S3A) revealed an abundance of *NCOA4* transcripts. Recently *NCOA4* has been suggested to regulate iron metabolism during mouse erythropoiesis (22–26). However, the function of *NCOA4* has not been tested in primary human erythroblasts, and our experiments, detailed below, suggest important nuclear functions of *NCOA4*.

Initial studies showed that the protein level of *NCOA4* in both nucleus and cytoplasm is increased during terminal differentiation (Fig. 3A), and the *NCOA4* transcript level is immediately up-regulated by GC-1 in human erythroid cells (Fig. 1E).

Knocking down *NCOA4* in human erythroblasts impaired human erythroid terminal differentiation in normal media (Fig. S3B). Similarly, knockdown of *NCOA4* blocked the enhancement of erythroid differentiation induced by GC-1 treatment of erythroid cultures placed in charcoal-stripped serum to remove thyroxine (Fig. 3B). RNA-seq analysis revealed that *NCOA4* knockdown reduced induction of erythroid genes whose activation was dependent on activation of *STAT5* downstream of activated *Epo* receptors (Fig. S3C). Knockdown of *NCOA4* also prevented the normal down-regulation of hypoxia-induced genes during terminal differentiation, genes that are important for erythroid progenitor self-renewal and that are normally down-regulated during terminal differentiation (Fig. S3C). We also validated our RNA-seq data by real-time PCR analysis (Fig. S3D), showing impaired induction of key erythroid-important genes following *Nco4* knockdown. More generally, genes up-regulated by at least 1.5-fold in human erythroblasts upon GC-1 treatment (Fig. 1E) were mostly down-regulated following *NCOA4* knockdown (Fig. 3C). In contrast, genes normally down-regulated by more than 50% during terminal differentiation were mostly up-regulated by *NCOA4* knockdown, suggesting an essential role of *NCOA4* during erythroid terminal differentiation regulated by TH (Fig. 3C). Taken together, these data suggest that *NCOA4* is important for TH signaling during human erythroid terminal differentiation by regulating erythroid gene expression; these functions are additional to any relating to iron metabolism (22–26).

**NCOA4 Is Recruited by THR Agonist GC-1 to Genomic Loci of Many Erythroid Genes.** We thus hypothesized that *NCOA4* is a key transcriptional regulator during terminal erythropoiesis. ChIP-seq analysis established that 12.8% of the *NCOA4* chromatin binding sites are associated with transcriptional start sites (TSSs) and 8.4% are in 5'-UTR regions (Fig. S4A). Treatment with GC-1 significantly increased the signal intensities of the top 300 *NCOA4* chromatin binding sites, which overlapped with binding sites for

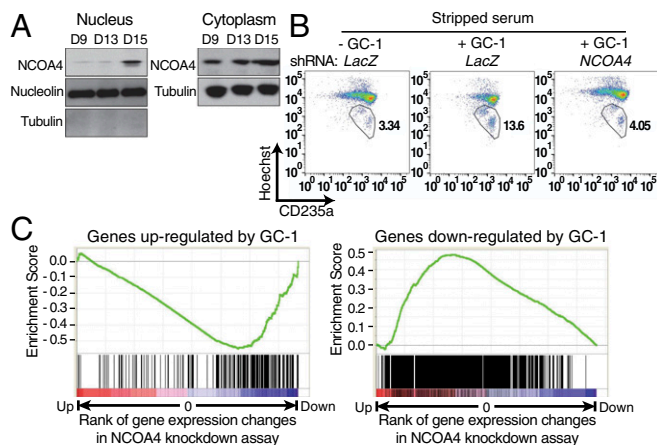


**Fig. 2.** TR $\beta$  agonist alleviates anemic symptoms in a chronic anemia mouse model. (A) *Nan*<sup>+/+</sup> mutant mice, 6–8 wk old, were injected with TR $\beta$  agonist GC-1 (500  $\mu$ g/kg) daily for 11 d. Hemoglobin (HGB), red blood cell numbers (RBCs), hematocrit (HCT), reticulocytes, white blood cells (WBCs), and platelets were measured on day 0 (open circles) and day 11 (black circles). WBCs and platelets of WT mice (brown circles) at the same ages were included. Each dot represents one mouse. (B) Peripheral blood smears from WT and *Nan*<sup>+/+</sup> mice treated with GC-1 at day 0 or day 11. Blood samples from the two *Nan*<sup>+/+</sup> mice (nos. 1 and 2) were collected individually for analysis at D0 or D11 after the GC-1 injection for comparison. Blood samples from mice injected with DMSO for 11 d were included as control. (C) Red blood cell distribution width (RDW) of WT, or GC-1-treated *Nan*<sup>+/+</sup> mice on day 0 and day 11. \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ .

Pol II as well as with histone marks associated with active transcription, such as H3K27Ac and H3K4Me3, but not with marks such as H3K4Me1 commonly associated with enhancers (Fig. 4A).

As many of these chromatin binding “peaks” are close to TSS segments, slightly downstream of TSSs, we hypothesized that *NCOA4* is recruited in response to TH treatment to regulate gene transcription in erythroblasts. Supporting this notion, these chromatin binding sites are associated with abundant transcripts (<3 kb from TSSs) in human reticulocytes (Fig. S4B). Association of *NCOA4* in the proximity of TSSs increased significantly in response to TH (Fig. 4B). As examples, enrichment of *NCOA4* signals at chromatin sites in several erythroid-important gene loci such as *HBB*, *SLC4A1*, and *HBA* was more pronounced upon GC-1 treatment, and this enrichment was highly associated with the occupancy of Pol II in these regions (Fig. 4C and Fig. S4C). Interestingly, DNA sequences underlying *NCOA4* peaks are enriched in DNA binding motifs for several transcription factors including TR $\beta$  (Fig. S4D). Therefore, our data suggest *NCOA4* is highly associated with genomic regions of many erythroid genes and might regulate expression of these genes together with Pol II and other transcription factors, including TR $\beta$ .

***Nco4* Knockout (*Nco4*<sup>-/-</sup>) Mice Are Anemic and Fail to Respond to TH Treatment.** Among all seven members of the *Ncoa* gene family, only the *Nco4* gene locus is associated with strong active



**Fig. 3.** NCOA4 is essential for human erythroblast terminal differentiation. (A) Western blot analysis of NCOA4 expression in both nucleus and cytoplasm at different days during human erythropoiesis. Human CD34<sup>+</sup> cells were cultured and differentiated into erythroblasts as described in *Materials and Methods*. (B) Knocking down NCOA4 disrupted GC-1-mediated terminal human erythroblast differentiation. Human CD34<sup>+</sup> cells were transfected by lentiviruses encoding shRNAs targeting either *LacZ* (control) or *NCOA4* at day 1 of culture. At day 14, cells were switched to terminal differentiation medium containing stripped serum and supplemented or not with 1  $\mu$ M GC-1. Flow cytometry analysis was conducted at day 17. (C) Gene enrichment analysis. (Left) Genes in human erythroblast cells up-regulated at least 1.5-fold by GC-1 treatment, as judged by GRO-seq analysis (Fig. 1E), were compared with the set of genes that were ranked by expression changes in the NCOA4 knockdown assay (Left to Right on x axis: genes that are “the most up-regulated” to “the most down-regulated” in NCOA4 knockdown compared with *LacZ* control cells). There is a significant correlation between the degree of GC-1-mediated gene activation and the degree of NCOA4 knockdown-mediated gene repression. (Right) Genes down-regulated greater than 1.5-fold by GC-1 treatment, as judged by GRO-seq analysis, were compared with the set of genes that were ranked by expression changes in the NCOA4 knockdown assay (Left to Right on x axis: genes that are the most up-regulated to the most down-regulated in NCOA4 knockdown cells compared with *LacZ* control cells). There is a significant correlation between the degree of GC-1-mediated gene repression and the degree of NCOA4 knockdown-mediated gene activation.

transcription markers in mouse Ter119<sup>+</sup> erythroblasts (27) (Fig. S5A). Although previous studies suggested that *Ncoa4* is important for erythropoiesis, the function of *Ncoa4* has not been validated in primary erythroblasts (22–26). Knocking down *Ncoa4* but not any other *Ncoa* tested impaired terminal erythropoiesis in our mouse erythroid culture (Fig. S5B and C). We next generated *Ncoa4*<sup>-/-</sup> mice using the CRISPR-Cas method (Fig. 5A). Consistent with previous reports (24–26), *Ncoa4*<sup>-/-</sup> mice were anemic during the immediate postnatal period (Fig. 5B). Erythroid progenitors were also significantly reduced in bone marrow (Fig. 5C). Moreover, GC-1 injection did not alleviate the anemia of *Ncoa4*<sup>-/-</sup> mice (Fig. 5D), supporting the notion that *Ncoa4* is required for TH signaling. GC-1 failed to induce erythroid gene expression in BFU-Es purified from *Ncoa4*<sup>-/-</sup> fetal livers (Fig. 5E). Taken together, these data suggest that *Ncoa4* is essential for TH function in erythropoiesis.

## Discussion

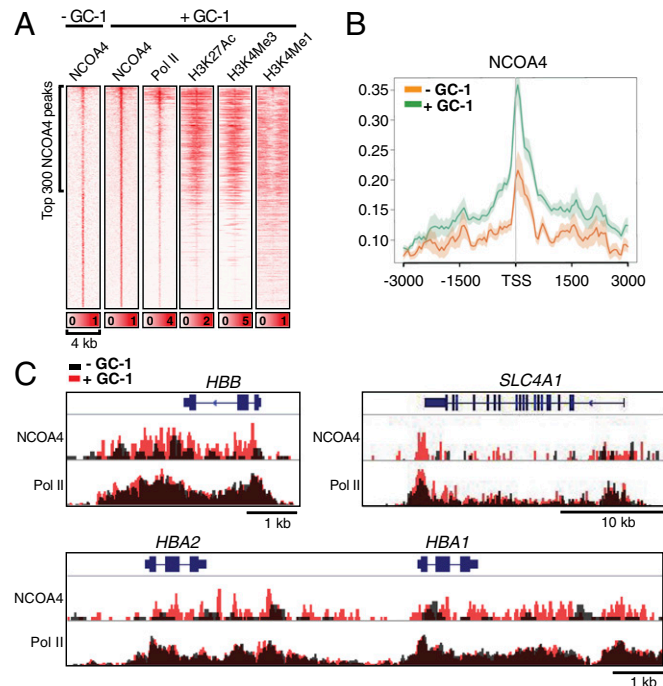
Anemia has long been observed in hypothyroidism and in patients resistant to thyroid hormone (RTH) (14, 28, 29), and a stimulatory effect of TH on erythropoiesis has been well documented (16, 30). Several studies showed that TH significantly enhances the number of erythroid progenitor BFU-Es and CFU-Es in cultured cells from both human and canine marrow. However, due in part to the cellular complexity of bone marrow (31), the stage(s) of erythropoiesis and the molecular mechanism(s) by which TH stimulates red cell production has remained obscure. In addition, GWASs identified human genetic variants in the

TR $\beta$  locus that are associated with abnormal hematocrits (18) and mutations in the *THRB* gene are common in RTH patients with anemia (32).

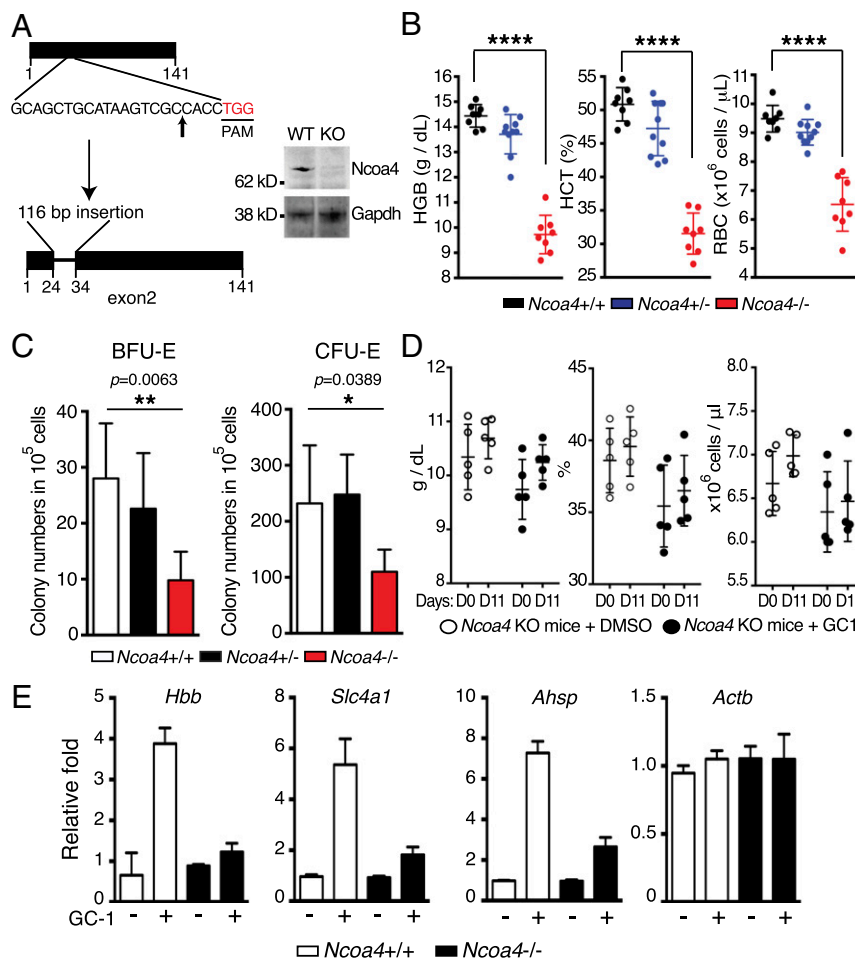
Here we established, first, that TH is essential for terminal human erythropoiesis, and functions through the TR $\beta$  receptor. Consistent with a key role for TR $\beta$ , both TH and the specific TR $\beta$  agonist GC-1 stimulate red cell formation in a mouse model of chronic anemia by stimulating erythroid gene expression. GC-1 also stimulated terminal erythropoiesis in our human erythroid cell culture system.

*Thrb* knockout mice exhibit normal erythropoiesis (19), whereas knocking out TR $\alpha$  resulted in a reduction in the numbers of early erythroid progenitors in fetal livers (19). Consistent with a role of TR $\alpha$  in early stages of erythroid development, our results showed that TR $\alpha$  protein levels are much higher in early human progenitors compared with late stage erythroblasts. Similarly, overexpression of TR $\alpha$  in murine erythroid progenitors supported sustained growth of these cells and prevented erythroid differentiation (33). It will be interesting to examine red cell production in *Thrb* knockout mice during erythroid stress, as earlier BFU-E progenitors play a key role in stress erythropoiesis (21).

Abnormal levels of TH are present in several types of anemia patients, and some anemia patients have been treated with



**Fig. 4.** TH recruits NCOA4 to genomic loci of many erythroid genes. (A) ChIP-seq analysis of NCOA4 occupancy compared with occupancy by Pol II and indicated histone modifications in human erythroid cells treated with or without GC-1 for 4 h. At day 14, cells were switched to regular terminal differentiation medium containing FBS and supplemented with or without 1  $\mu$ M GC-1. Density maps represent NCOA4 sites with GC-1 (+) and without GC-1 (-). Genome-wide NCOA4 binding sites in GC-1-treated cells are ranked based on ChIP-seq signal intensity (Top to Bottom, strong to weak binding signal). The center of the NCOA4-binding peaks is set as the reference point “0” to anchor the corresponding ChIP-seq signals of Pol II and histone modification marks from cells treated with GC-1, in the 2 kb upstream and downstream regions. Color scale units are arbitrary. (B) Transcription start site (TSS) plot. Displayed are the average ChIP enrichment signals of NCOA4 surrounding the TSSs in cells treated or not with GC-1. (C) Occupancy of NCOA4 and RNA Pol II of the *HBB*, *SLC4A1*, and *HBA2* gene loci in human erythroid cells treated with or without GC-1 for 4 h. At day 14, cultured human CD34<sup>+</sup> cells were switched to terminal differentiation medium containing regular FBS and with or without 1  $\mu$ M GC-1. After 6 h, cells were harvested for ChIP-seq analysis.



**Fig. 5.** *Ncoa4*<sup>-/-</sup> mice are anemic during both embryonic and perinatal periods and fail to respond to thyroxine treatment to promote erythropoiesis. (A) Schematic outline of Cas9/sgRNA-targeting in the *Ncoa4* gene. The sgRNA-targeting sequence is shown. The protospacer-adjacent motif (PAM) sequence is highlighted in red. Nine nucleotides (underlined) are eliminated and 116 nucleotides are inserted into the site. Western blotting showing mouse NCOA4 protein expression in bone marrow of WT and *Ncoa4* KO mice. (B) Hemoglobin measurement of mice with indicated genotypes. <sup>+/+</sup>, wild-type; *Ncoa4*<sup>+/-</sup>, heterozygous; *Ncoa4*<sup>-/-</sup>, homozygous. Each dot represents measurement from one mouse. \*\*\*\**P* < 0.0001. (C) Colony-forming assays were conducted to determine BFU-E and CFU-E colony numbers from 10<sup>5</sup> bone marrow cells of wild-type, *Ncoa4*<sup>+/-</sup>, or *Ncoa4*<sup>-/-</sup> mice. Error bars represent mean ± SD from three biological replicates; \**P* < 0.05, \*\**P* < 0.01. (D) Hematological analysis of *Ncoa4* KO mice treated with DMSO (control, white circles) or GC-1 (black circles). Blood samples were collected from individual mice at D0 and D11 after the treatment. Each dot represents a measurement from one mouse. (E) Real-time PCR analysis of erythroid gene expression in WT and *Ncoa4*<sup>-/-</sup> mouse BFU-Es treated or untreated with GC-1. Mouse BFU-E cells were isolated from WT and *Ncoa4*<sup>-/-</sup> mouse embryos. BFU-Es were treated with or without GC-1 for 6 h. Actin beta gene (*Actb*) is shown as a control.

synthetic TH (8, 34). Our results demonstrate that injection of the TRβ selective agonist GC-1 alleviated anemia in a chronic anemia mouse model. GC-1 not only raised hemoglobin levels, hematocrits, and red blood cell numbers, but also reduced stress erythropoiesis and anisocytosis of the *Nan* mice in vivo. The increase of red cells by GC-1 is likely due to a direct function on bone marrow erythroid cells (35). Selective TRβ agonists like GC-1 not only can effectively treat metabolic diseases such as dyslipidemia, atherosclerosis, and obesity, but also have a promising safety profile (36, 37). Thus, these data suggest that GC-1 or other selective TRβ agonists might be used to treat certain anemias due to deficiencies in erythroid differentiation (38, 39).

Nuclear receptor signaling often requires cooperation with coactivators and corepressors. Our loss-of-function studies in human erythroblasts, together with our analysis of *Ncoa4*<sup>-/-</sup> knockout mice, established that coactivator NCOA4 is essential for red blood cell formation. NCOA4 was first discovered as a coactivator of the androgen receptor (40). NCOA4 transcripts are highly abundant in hematopoietic cells, particularly the erythroid lineage (23). *Ncoa4*<sup>-/-</sup> knockout mice are anemic in embryos and in the early postnatal period, but eventually acquire normal levels of red

cells, similar to what we observed in *Stat5a*<sup>-/-</sup>*5b*<sup>-/-</sup> knockout mice, where the mice greatly increased the numbers of erythroid progenitors (41). Both NCOA4 loss-of-function assays in human erythroblasts and in mice demonstrated impaired TH signaling during terminal erythroid differentiation. Importantly, our ChIP-seq data revealed that NCOA4 is bound to chromatin in close proximity to Pol II and is recruited to many erythroid gene loci to regulate their expression in response to TH signaling during terminal erythroid differentiation. We detected TRβ binding motifs within NCOA4-associated genomic regions, together with binding motifs for other TFs. This indicates TRβ might form a complex with NCOA4 and possibly other factors to regulate gene expression. However, due to the inefficiency of TRβ antibodies in coimmunoprecipitation and ChIP-seq experiments, we could not yet determine the physical interaction or the colocalization on chromatin between NCOA4 and TRβ.

Interestingly, recent studies also suggest that NCOA4 might play other roles in regulating red cell formation. NCOA4 is present in autophagosomes and interacts with ferritin heavy and light chains and facilitates the delivery of ferritin into lysosomes under stress conditions (24, 25). Dowdle et al. (25) observed profound



accumulation of iron in splenic macrophages in *Ncoa4* KO mice (24, 25). Mancias et al. (26) reported that knocking out *Ncoa4* impairs hemoglobin formation in zebrafish and K562 cells.

In summary, our results have elucidated the molecular mechanisms by which TH functions to support terminal red blood cell formation and suggested that targeting TR $\beta$  by its selective agonists can potentially be used to treat certain types of anemias.

## Materials and Methods

**Reagents.** Details of the reagents are described in *SI Materials and Methods*.

**Ex Vivo Human CD34<sup>+</sup> Erythroid Culture.** The human CD34<sup>+</sup> cell erythroid differentiation method comprises four phases over 21 d: Expansion (days 0–4), differentiation (Dif) I (days 5–9), II (days 10–13), and III (days 14–21) as described before (11) and in *SI Materials and Methods*.

**Global Run-On Sequencing.** GRO-seq experiments were performed as previously reported (42) and detailed in *SI Materials and Methods*.

**Genotyping of *Nan1*+ Mice Embryos.** The *Nan1*+ mice were a gift from Luanne Peters, The Jackson Laboratory, Bar Harbor, ME. *Nan1*+ mice were mated to wild-type littermates. Individual embryos at E14.5 d were genotyped as described previously and in *SI Materials and Methods*. All mouse procedures were approved by the Animal Care and Use Committees of the Massachusetts Institute of Technology.

- Lodish H, Flygare J, Chou S (2010) From stem cell to erythroblast: Regulation of red cell production at multiple levels by multiple hormones. *IUBMB Life* 62:492–496.
- Hattangadi SM, Wong P, Zhang L, Flygare J, Lodish HF (2011) From stem cell to red cell: Regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. *Blood* 118:6258–6268.
- Wu H, Liu X, Jaenisch R, Lodish HF (1995) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 83:59–67.
- Socolovsky M, et al. (2001) Ineffective erythropoiesis in *Stat5a*(<sup>-/-</sup>)*5b*(<sup>-/-</sup>) mice due to decreased survival of early erythroblasts. *Blood* 98:3261–3273.
- Bouscary D, et al. (2003) Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. *Blood* 101:3436–3443.
- Bauer A, Ulrich E, Andersson M, Beug H, von Lindern M (1997) Mechanism of transformation by v-ErbA: Substitution for steroid hormone receptor function in self-renewal induction. *Oncogene* 15:701–715.
- Ganguli G, Back J, Sengupta S, Waslyk B (2002) The p53 tumour suppressor inhibits glucocorticoid-induced proliferation of erythroid progenitors. *EMBO Rep* 3:569–574.
- Vlachos A, Muir E (2010) How I treat Diamond-Blackfan anemia. *Blood* 116:3715–3723.
- Golde DW, Bersch N, Cline MJ (1976) Potentiation of erythropoiesis in vitro by dexamethasone. *J Clin Invest* 57:57–62.
- Flygare J, Rayon Estrada V, Shin C, Gupta S, Lodish HF (2011) HIF1 $\alpha$  synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. *Blood* 117:3435–3444.
- Lee HY, et al. (2015) PPAR- $\alpha$  and glucocorticoid receptor synergize to promote erythroid progenitor self-renewal. *Nature* 522:474–477.
- Makita T, et al. (2001) A developmental transition in definitive erythropoiesis: Erythropoietin expression is sequentially regulated by retinoic acid receptors and HNF4. *Genes Dev* 15:889–901.
- Cui S, et al. (2011) Nuclear receptors TR2 and TR4 recruit multiple epigenetic transcriptional corepressors that associate specifically with the embryonic  $\beta$ -type globin promoters in differentiated adult erythroid cells. *Mol Cell Biol* 31:3298–3311.
- Tudhope GR, Wilson GM (1960) Anaemia in hypothyroidism. Incidence, pathogenesis, and response to treatment. *Q J Med* 29:513–537.
- Peschle C, Zanjani ED, Gidari AS, McLaurin WD, Gordon AS (1971) Mechanism of thyroxine action on erythropoiesis. *Endocrinology* 89:609–612.
- Dainiak N, Hoffman R, Maffei LA, Forget BG (1978) Potentiation of human erythropoiesis in vitro by thyroid hormone. *Nature* 272:260–262.
- Brent GA (2012) Mechanisms of thyroid hormone action. *J Clin Invest* 122:3035–3043.
- van der Harst P, et al. (2012) Seventy-five genetic loci influencing the human red blood cell. *Nature* 492:369–375.
- Kendrick TS, et al. (2008) Erythroid defects in *TR $\alpha$* <sup>-/-</sup> mice. *Blood* 111:3245–3248.
- Siatecka M, et al. (2010) Severe anemia in the *Nan* mutant mouse caused by sequence-selective disruption of erythroid Kruppel-like factor. *Proc Natl Acad Sci USA* 107:15151–15156.
- Paulson RF, Shi L, Wu DC (2011) Stress erythropoiesis: New signals and new stress progenitor cells. *Curr Opin Hematol* 18:139–145.
- Nilsson R, et al. (2009) Discovery of genes essential for heme biosynthesis through large-scale gene expression analysis. *Cell Metab* 10:119–130.
- Weber GJ, et al. (2005) Mutant-specific gene programs in the zebrafish. *Blood* 106:521–530.
- Mancias JD, Wang X, Gygi SP, Harper JW, Kimmelman AC (2014) Quantitative proteomics identifies NCOA4 as the cargo receptor mediating ferritinophagy. *Nature* 509:105–109.
- Dowdle WE, et al. (2014) Selective VPS34 inhibitor blocks autophagy and uncovers a role for NCOA4 in ferritin degradation and iron homeostasis in vivo. *Nat Cell Biol* 16:1069–1079.

**ChIP-Seq and de Novo Motif Discovery.** ChIP-seq experiments in human erythroblast cells were conducted as described before (11) and in *SI Materials and Methods*.

**RNA-Seq and Quantitative Real-Time RT-PCR.** The procedures were conducted as described in *SI Materials and Methods*.

**Generation of *Ncoa4* Knockout Mice.** The procedures of in vitro transcription of Cas9 mRNA and sgRNA, one-cell embryo injection, and knockout mice genotyping are described in *SI Materials and Methods*.

**Immunoblotting.** The procedures were conducted as described in *SI Materials and Methods*.

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- Mancias JD, et al. (2015) Ferritinophagy via NCOA4 is required for erythropoiesis and is regulated by iron dependent HERC2-mediated proteolysis. *Elife* 4.
- Wong P, et al. (2011) Gene induction and repression during terminal erythropoiesis are mediated by distinct epigenetic changes. *Blood* 118:e128–e138.
- Moran C, et al. (2014) Resistance to thyroid hormone caused by a mutation in thyroid hormone receptor (TR) $\alpha$ 1 and TR $\alpha$ 2: Clinical, biochemical, and genetic analyses of three related patients. *Lancet Diabetes Endocrinol* 2:619–626.
- Cineme H, Bilir C, Gokosmanoglu F, Bahcebası T (2009) Hematologic effects of levothyroxine in iron-deficient subclinical hypothyroid patients: A randomized, double-blind, controlled study. *J Clin Endocrinol Metab* 94:151–156.
- Popovic WJ, Brown JE, Adamson JW (1977) The influence of thyroid hormones on in vitro erythropoiesis. Mediation by a receptor with beta adrenergic properties. *J Clin Invest* 60:907–913.
- Morrison SJ, Scadden DT (2014) The bone marrow niche for haematopoietic stem cells. *Nature* 505:327–334.
- Wu SY, Sadow PM, Refetoff S, Weiss RE (2005) Tissue responses to thyroid hormone in a kindred with resistance to thyroid hormone harboring a commonly occurring mutation in the thyroid hormone receptor beta gene (P453T). *J Lab Clin Med* 146:85–94.
- Bartunek P, Zenke M (1998) Retinoid X receptor and c-cerbA/thyroid hormone receptor regulate erythroid cell growth and differentiation. *Mol Endocrinol* 12:1269–1279.
- Akoum R, et al. (2007) Myelodysplastic syndrome and pancytopenia responding to treatment of hyperthyroidism: Peripheral blood and bone marrow analysis before and after antihormonal treatment. *J Cancer Res Ther* 3:43–46.
- Malgor LA, et al. (1975) Direct effects of thyroid hormones on bone marrow erythroid cells of rats. *Blood* 45:671–679.
- Lin JZ, et al. (2012) Thyroid hormone receptor agonists reduce serum cholesterol independent of the LDL receptor. *Endocrinology* 153:6136–6144.
- Baxter JD, Webb P (2009) Thyroid hormone mimetics: Potential applications in atherosclerosis, obesity and type 2 diabetes. *Nat Rev Drug Discov* 8:308–320.
- Lipton JM, Kudisch M, Gross R, Nathan DG (1986) Defective erythroid progenitor differentiation system in congenital hypoplastic (Diamond-Blackfan) anemia. *Blood* 67:962–968.
- Suragani RN, et al. (2014) Transforming growth factor- $\beta$  superfamily ligand trap ACE-536 corrects anemia by promoting late-stage erythropoiesis. *Nat Med* 20:408–414.
- Yeh S, Chang C (1996) Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. *Proc Natl Acad Sci USA* 93:5517–5521.
- Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in *Stat5a*<sup>-/-</sup>*5b*<sup>-/-</sup> mice: A direct role for Stat5 in Bcl-X(L) induction. *Cell* 98:181–191.
- Li W, et al. (2013) Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. *Nature* 498:516–520.
- Ingolia NT, Ghaemmaghami S, Newman JR, Weissman JS (2009) Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* 324:218–223.
- Zhang Y, et al. (2008) Model-based analysis of ChIP-seq (MACS). *Genome Biol* 9:R137.
- Heinz S, et al. (2010) Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* 38:576–589.
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108.
- Truett GE, et al. (2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* 29:52–54.
- Hsu PD, et al. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 31:827–832.