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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 TRβ 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 TRβ 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*$^{-/-}$ 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 phosphatidylinositide 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-α) to enhance BFU-E self-renewal, and PPAR-α 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 α (TRα) and β (TRβ), 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 TRβ locus associated with abnormal hematological traits (18). Knocking out TRα but not TRβ 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 TRβ 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, TRβ, stimulates erythroid differentiation and alleviates anemic symptoms in a chronic anemia mouse model, indicating potential clinical applications. Further, we show that TRβ functions together with NCOA4 to regulate red cell formation.

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The authors declare no conflict of interest.

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$^+$ 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. 1*A*). 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. 1*A*).

T4 also accelerates and enhances terminal erythroblast differentiation when added at day 14 to normal cultures (Fig. S1*A*), leading to over ∼95% enucleation at day 16, while control cells in these human CD34$^+$ erythroid cultures have not begun to enucleate (Fig. 1*B* and Fig. S1*B*). 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. S1*C*). These data reinforce the essential function of TH in promoting terminal erythroid differentiation.

While both TRα and TRβ proteins are expressed in human CD34$^+$ progenitors, only TRβ protein is detectable in late erythroblasts (Fig. 1*C*). Knocking down TRβ in CD34$^+$ cells impaired the enucleation of human erythroblasts, indicating that TRβ is required for human erythroid terminal differentiation (Fig. 1*D*). Consistent with the notion that TRβ but not TRα is required for terminal differentiation, both T4 and the TRβ selective agonist GC-1 restore normal erythroblast differentiation, measured by enucleation, that is deficient in cultures in charcoal-stripped serum (Fig. S1*D*). These effects of TH receptor (THR) agonists are significantly abrogated upon TRβ knockdown (Fig. S1*D*). Taken together, these data suggest that TH is required for terminal differentiation of human erythroid progenitors and that this function is mediated by TRβ and not TRα.

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. 1*E*). 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 TRβ accelerates terminal human erythroid differentiation.

TRβ Agonist GC-1 Alleviates Anemia in a Chronic Anemia Mouse Model. We tested whether TRβ 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. 2*A*). Red cells in *Nan*/+ mice are anisocytotic with many microcytes, spherocytes, and hypochromic cells (20). These abnormalities were alleviated by GC-1 treatment (Fig. 2*B*). 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. 2*C*). 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. S2 *A* and *B*). GC-1 injection reduced both splenomegaly and spleen weight in *Nan*/+ mice compared with control DMSO treatment (Fig. S2*B*). 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

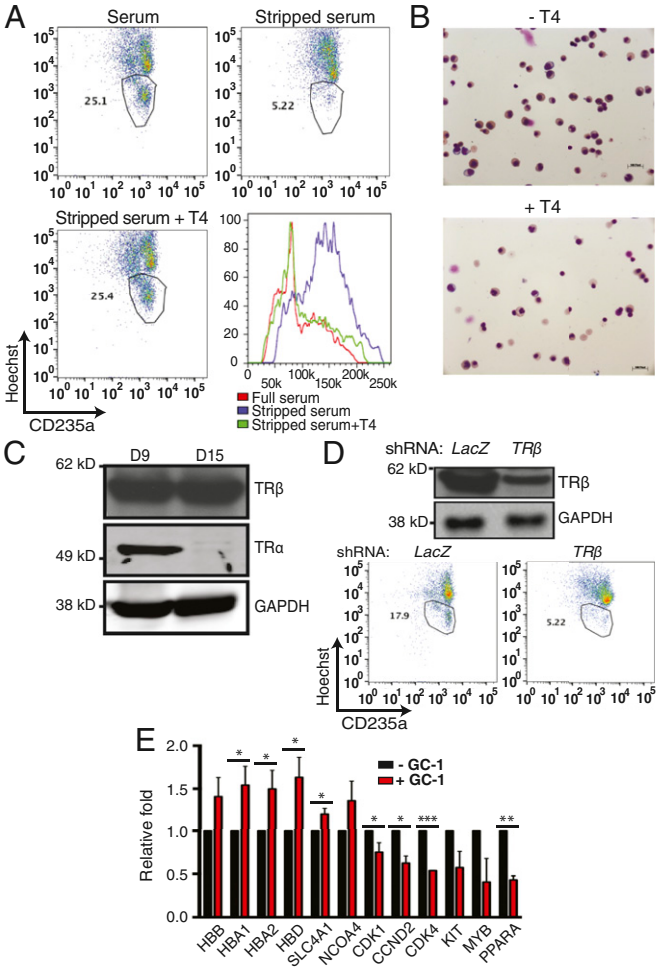

Fig. 1. TH promotes terminal erythroid differentiation through TRβ. (*A*) Human CD34$^+$ cells from G-CSF mobilized peripheral blood were cultured by the four-stage (21 d total) erythroid differentiation method as described in *Materials and Methods*. At day 14, cells were switched to terminal differentiation (Dif III) culture medium containing serum, stripped serum, or stripped serum supplemented with 1 μM 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$^+$ erythroid culture. Brown color of benzidine staining indicates hemoglobin accumulation. Cells were untreated or treated with 1 μM T4 at day 14 for 72 h. (*C*) Western blotting demonstrating protein levels of TRα and TRβ in human erythroid cells at days 9 and 15 of culture. (*D*) Human CD34$^+$ cells were transduced by lentivirus vectors encoding shRNAs targeting either LacZ (control) or *THRB* at day 1. (*Top*) Protein expression of TRβ 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 μM GC-1 for 6 h is analyzed by GRO-seq. Data were normalized to cells treated with DMSO (error bars represent mean ± SD from three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

stress erythropoiesis in vivo (Fig. S2*C*). In *Nan*/+ 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. S2*D*). Thus, GC-1 not only alleviates the anemia characteristic of the *Nan*/+ mice, it also reduces extramedullary erythropoiesis.

We next set out to understand how TH receptor agonist GC-1 increases RBC production in *Nan*/+ mice. *Nan*/+ 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 *Dmtn*, *Slc4a1*, and *Hbb*, were significantly down-regulated in cells from *Nan*/+ mice compared with those from WT littermates (20). Treatment with GC-1 significantly increased the expression of *Dmtn*, *Slc4a1*, and *Hbb* (20) (Fig. S2*E*). In contrast, GC-1 did not increase the expression of *Eklf* or *Gata1*. Therefore, our data suggested TH alleviates anemia in the *Nan*/+ 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$^+$ stem/progenitor cells (11). RNA-seq analysis on these cells (Fig. S3*A*) 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. 3*A*), and the NCOA4 transcript level is immediately up-regulated by GC-1 in human erythroid cells (Fig. 1*E*).

Knocking down NCOA4 in human erythroblasts impaired human erythroid terminal differentiation in normal media (Fig. S3*B*). 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. 3*B*). 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. S3*C*). 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. S3*C*). We also validated our RNA-seq data by real-time PCR analysis (Fig. S3*D*), showing impaired induction of key erythroid-important genes following Ncoa4 knockdown. More generally, genes up-regulated by at least 1.5-fold in human erythroblasts upon GC-1 treatment (Fig. 1*E*) were mostly down-regulated following NCOA4 knockdown (Fig. 3*C*). 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. 3*C*). 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. S4*A*). Treatment with GC-1 significantly increased the signal intensities of the top 300 NCOA4 chromatin binding sites, which overlapped with binding sites for

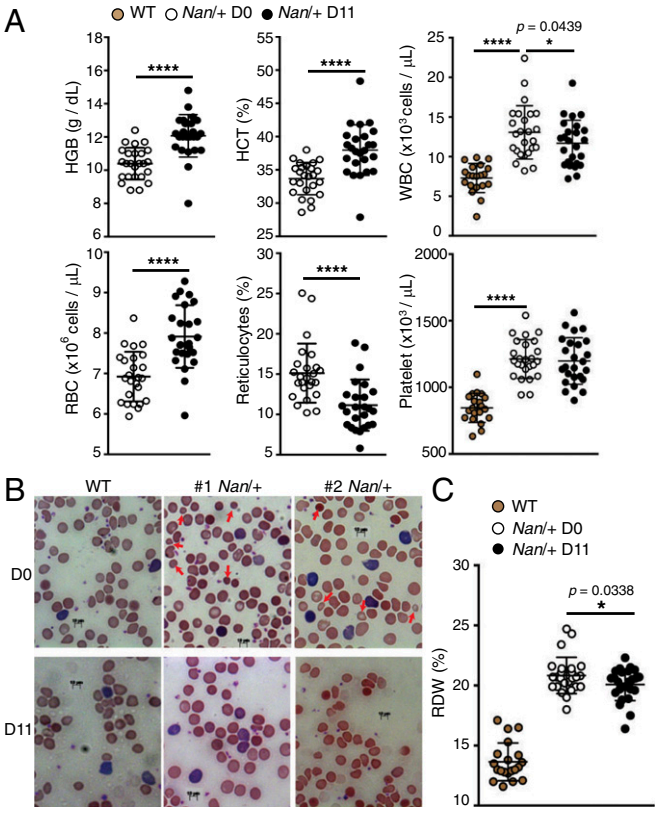

Fig. 2. TRβ agonist alleviates anemic symptoms in a chronic anemia mouse model. (*A*) *Nan*/+ mutant mice, 6~8 wk old, were injected with TRβ agonist GC-1 (500 μg/kg) daily for 11 d. Hemoglobin (HGB), red blood cell numbers (RBCs), hematocrit (HCT), reticulocytes, white blood cells (WBCs), and platelets 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*/+ mice treated with GC-1 at day 0 or day 11. Blood samples from the two *Nan*/+ 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*/+ 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. 4*A*).

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. S4*B*). Association of NCOA4 in the proximity of TSSs increased significantly in response to TH (Fig. 4*B*). 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. 4*C* and Fig. S4*C*). Interestingly, DNA sequences underlying NCOA4 peaks are enriched in DNA binding motifs for several transcription factors including TRβ (Fig. S4*D*). 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 TRβ.

***Ncoa4* Knockout (*Ncoa4*$^{-/-}$) Mice Are Anemic and Fail to Respond to TH Treatment.** Among all seven members of the *Ncoa* gene family, only the *Ncoa4* gene locus is associated with strong active

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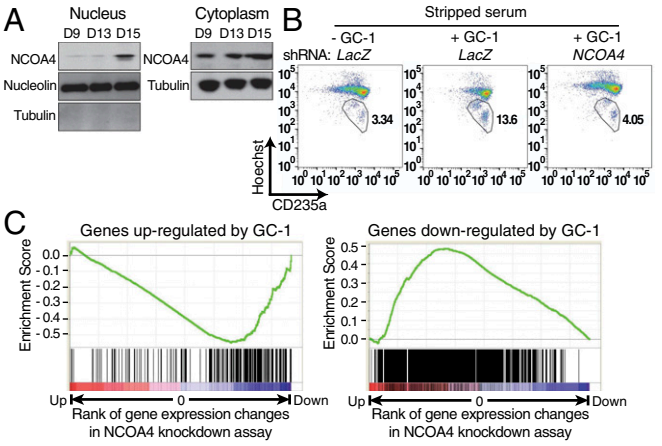

Fig. 3. NCOA4 is essential for human erythroblast terminal differentiation. (*A*) Western blot analysis of NCOA4 expression in both nucleus and cytoplasm at different days during human erythropoiesis. Human CD34$^+$ cells were cultured and differentiated into erythroblasts as described in *Materials and Methods*. (*B*) Knocking down NCOA4 disrupted GC-1–mediated terminal human erythroblast differentiation. Human CD34$^+$ cells were transduced 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 μM 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. 1*E*), 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+ erythroblasts (27) (Fig. S5*A*). 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. S5 *B* and *C*). We next generated *Ncoa4*$^{-/-}$ mice using the CRISPR-Cas method (Fig. 5*A*). Consistent with previous reports (24–26), *Ncoa4*$^{-/-}$ mice were anemic during the immediate postnatal period (Fig. 5*B*). Erythroid progenitors were also significantly reduced in bone marrow (Fig. 5*C*). Moreover, GC-1 injection did not alleviate the anemia of *Ncoa4*$^{-/-}$ mice (Fig. 5*D*), supporting the notion that Ncoa4 is required for TH signaling. GC-1 failed to induce erythroid gene expression in BFU-Es purified from *Ncoa4*$^{-/-}$ fetal livers (Fig. 5*E*). 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 TRβ 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 TRβ receptor. Consistent with a key role for TRβ, both TH and the specific TRβ 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 TRα resulted in a reduction in the numbers of early erythroid progenitors in fetal livers (19). Consistent with a role of TRα in early stages of erythroid development, our results showed that TRα protein levels are much higher in early human progenitors compared with late stage erythroblasts. Similarly, overexpression of TRα 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

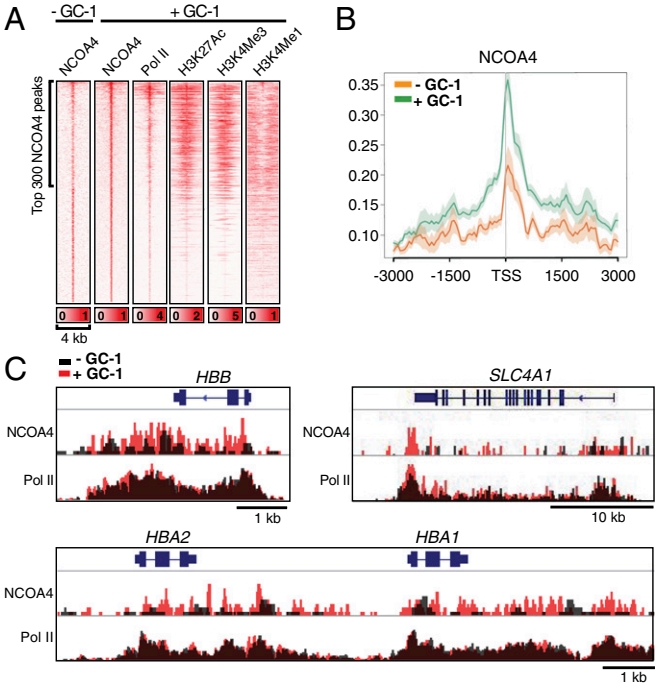

Fig. 4. TH recruits NCOA4 to genomic loci of many erythroid genes. (*A*) ChIP-seq analysis of NCOA4 occupancy compared with occupancy by Pol II and indicated histone modifications in human erythroid cells treated with or without GC-1 for 4 h. At day 14, cells were switched to regular terminal differentiation medium containing FBS and supplemented with or without 1 μM 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 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 HBA* gene loci in human erythroid cells treated with or without GC-1 for 4 h. At day 14, cultured human CD34$^+$ cells were switched to terminal differentiation medium containing regular FBS and with or without 1 μM GC-1. After 6 h, cells were harvested for ChIP-seq analysis.

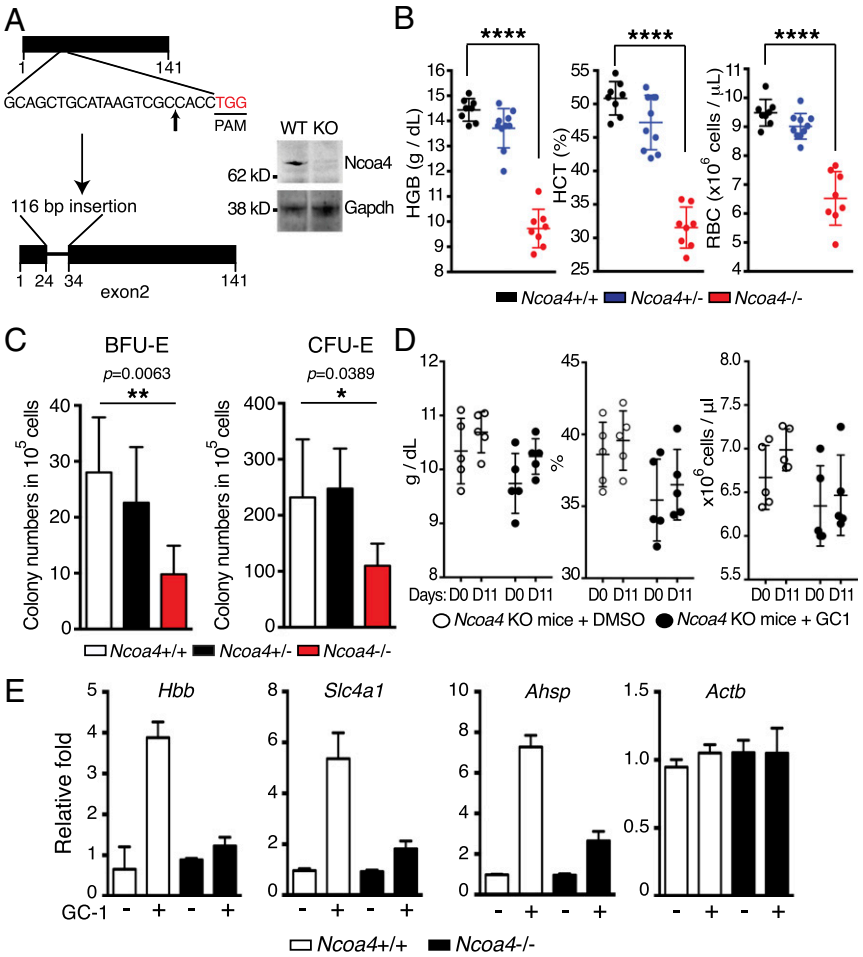

Fig. 5. *Ncoa4*$^{-/-}$ mice are anemic during both embryonic and perinatal periods and fail to respond to thyroxin treatment to promote erythropoiesis. (*A*) Schematic outline of Cas9/sgRNA-targeting in the *Ncoa4* gene. The sgRNA-targeting sequence is shown. The protospacer-adjacent motif (PAM) sequence 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. $^{+/+}$, wild-type; Ncoa4$^{+/-}$, heterozygous; Ncoa4$^{-/-}$, 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 10^5 bone marrow cells of wild-type, Ncoa4$^{+/-}$, or Ncoa4$^{-/-}$ 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*$^{-/-}$ mouse BFU-Es treated or untreated with GC-1. Mouse BFU-E cells were isolated from WT and *Ncoa4*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$*5b*$^{-/-}$ 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 TRβ 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$^+$ Erythroid Culture. The human CD34$^+$ 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 *Nan*/+ Mice Embryos. The *Nan*/+ mice were a gift from Luanne Peters, The Jackson Laboratory, Bar Harbor, ME. *Nan*/+ 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.

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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