THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 46, Issue of November 16, pp. 43428–43434, 2001
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Thyroid Hormone Receptor-interacting Protein 1 Modulates Cytokine and Nuclear Hormone Signaling in Erythroid Cells*

Received for publication, July 16, 2001, and in revised form, August 15, 2001
Published, JBC Papers in Press, September 5, 2001, DOI 10.1074/jbc.M106645200

Evan Ingley‡, David Chappell‡§, Sally Y. K. Poon‡, Mohinda K. Sarna‡, Jennifer G. Beaumont‡¶, James H. Williams‡, Justin P. Stillitano‡, Schickwann Tsai‖, Peter J. Leedman‡, Peta A. Tilbrook‡, and S. Peter Klinken‡

From the ‡Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Royal Perth Hospital and the Department of Biochemistry, University of Western Australia, Perth, Western Australia 6000, Australia and ‖The Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, New York, New York 10030

Erythropoietin (Epo) and thyroid hormone (T_3) are key molecules in the development of red blood cells. We have shown previously that the tyrosine kinase Lyn is involved in differentiation signals emanating from an activated erythropoietin receptor. Here we demonstrate that Lyn interacts with thyroid hormone receptor-interacting protein 1 (Trip-1), a transcriptional regulator associated with the T_3 receptor, providing a link between the Epo and T_3 signaling pathways. Trip-1 co-localized with Lyn and the T_3 receptor α in the cytoplasm/plasma membrane of erythroid cells but translocated to discrete nuclear foci shortly after Epo-induced differentiation. Our data reveal that T_3 stimulated the proliferation of immature erythroid cells, and inhibited maturation promoted by erythropoietin. Removal of T_3 reduced cell division and enhanced terminal differentiation. This was accompanied by large increases in the cell cycle inhibitor $p27^{Kip1}$ and by increasing expression of erythroid transcription factors GATA-1, EKLF, and NF-E2. Strikingly, a truncated Trip-1 inhibited both erythropoietin-induced maturation and T_3-initiated cell division. This mutant Trip-1 acted in a dominant negative fashion by eliminating endogenous Lyn, elevating $p27^{Kip1}$, and blocking T_3 response elements. These data demonstrate that Trip-1 can simultaneously modulate responses involving both cytokine and nuclear receptors.

Erythropoiesis, the process of generating red blood cells, is controlled by hormones that bind to cytokine receptor and nuclear hormone receptor families (1, 2). Two well characterized molecules that strongly influence erythropoiesis are erythropoietin (Epo)[1] and thyroid hormone (T_3). Epo binds to a cell surface receptor of the cytokine receptor family (3), initiating an intracellular signaling cascade that has been deciphered gradually over the past decade (reviewed in Refs. 4–7). Numerous signaling proteins are activated by Epo, including JAK2, STAT5, Ras, phosphatidylinositol 3-kinase, phospholipase Cγ, and MAP kinase (5, 7); activation of negative regulators such as SHP1, SOCS1, and CIS also occurs after receptor engagement (8–10).

T_3 binds to the intracellular thyroid hormone receptor (TR), a member of the nuclear hormone receptor family, which regulates gene expression (11). T_3 has a potent effect on erythropoiesis, especially in hypothyroid patients who are often anemic; however, erythroid hyperplasia can occur in individuals with hyperthyroidism (12, 13). It is noteworthy that the TRα isoform is expressed preferentially in differentiating erythroid cells (14) and that the v-*erb*A oncogene involved in avian erythroleukemia represents a mutated form of TRα (15, 16). Studies with whole animals have indicated that T_3 stimulates erythropoiesis (17), whereas *in vitro* assays have shown that T_3 inhibits colony formation by erythroid progenitors (18, 19). The elegant studies of Beug and colleagues (19–24) have demonstrated that the balance between proliferation and differentiation can be altered by the introduction of exogenous TRα (c-*erb*A) or v-*erb*A into immature avian red blood cells.

We have examined Epo-initiated signaling in J2E erythroid cells as they proliferate, remain viable, produce hemoglobin, and undergo morphological maturation in response to Epo (25–27). Following Epo stimulation of these cells, phosphorylation changes to the Epo receptor, JAK2, STAT5, Ras-GAP, phosphatidylinositol 3-kinase, phospholipase Cγ, and MAP kinase are identical to the kinetics reported in other cell systems (28). The tyrosine kinase Lyn is crucial for Epo-induced differentiation of immature J2E and R11 cell lines (27). Lyn associates with the Epo receptor and can phosphorylate the receptor and STAT5 *in vitro* (28, 29). As the most abundant Src family kinase in red blood cells (30), Lyn also phosphorylates key erythrocyte membrane proteins (31). Our recent data indicate JAK2 is the primary kinase that initiates Epo signaling and that Lyn acts as a secondary kinase to promote differentiation (32). Significantly, the erythroid progenitor compartment is altered in $Lyn^{-/-}$ mice.[2]

* This work was supported by Grants 980610, 990596, and 139008 from the National Health and Medical Research Council, Australia and by grants from the Medical Research Foundation of Royal Perth Hospital. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, FL 33612-4799.

¶ Present address: Molecular Genetics of Cancer Division, Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Melbourne, Victoria 3050, Australia.

** To whom correspondence should be addressed: Laboratory for Cancer Medicine, University of Western Australia, Level 6, Medical Research Foundation Bldg., Rear 50 Murray St., Perth WA6000, Australia. Tel.: 61-8-92240334; Fax: 61-8-92240322; E-mail: pklinken@cyllene.uwa.edu.au.

[1] The abbreviations used are: Epo, erythropoietin; T_3, thyroid hormone; TR, thyroid hormone receptor; Trip-1, thyroid hormone receptor-interacting protein 1; tTrip-1, truncated Trip-1; SH2 and -3, Src homology 2 and 3; CFU-E, erythroid colony-forming units; BFU-E, erythroid burst-forming units; GST, glutathione *S*-transferase; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAP, mitogen-activated protein; NF-E2, nuclear factor E2; COUP-TFII, chicken ovalbumin upstream promoter transcription factor-II; EKLF, erythroid Kruppel-like factor; ORCA, orphan receptor coactivator.

[2] M. Hibbs, personal communication.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

In this study we attempted to identify downstream effectors of Lyn in erythroid cells using a yeast two-hybrid screen. HS1, a known target for Src family kinases including Lyn, was identified in this screen and its effects on erythroid differentiation demonstrated (33). Here, we report on the interaction between Lyn and thyroid hormone receptor-interacting protein 1 (Trip-1), a transcriptional regulator that associates with TRα (34–36). The Lyn/Trip-1 association, therefore, provides a link between the Epo and T_3 signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were grown in Dulbecco's modified Eagle's medium, 5% fetal calf serum, or serum depleted of T_3 (24). T_3 could not be detected in depleted sera by radioimmunoassay. The Epo-responsive J2E (25) and R11 (37) cell lines were derived from murine fetal liver cells transduced with retroviruses expressing v-Raf/v-Myc (J2E) or v-Raf (R11). Differentiation of J2E and R11 cell lines was initiated with Epo (5 units/ml). Nuclear hormones were used at a final concentration of 1 μM. Viability was determined by eosin dye exclusion and hemoglobin synthesis by benzidine staining (28). Cell morphology was examined following cytocentrifugation onto glass slides and Wright-Giemsa staining (26). Proliferation was assayed by [^{3}H]thymidine incorporation (26). Fetal liver cells were plated in methylcellulose for CFU-E and BFU-E assays as described previously (38) before benzidine-positive colonies were enumerated. All graphical data are represented as the mean ± S.D. ($n \geq 3$).

Yeast Two-hybrid Analysis—The yeast two-hybrid system (33, 39) utilized the *S. cerevisiae* L40 strain. Wild-type Lyn (Lyn) and a dominant negative Lyn (Y397F) cDNAs were used to screen a yeast two-hybrid library derived from the lymphohemopoietic progenitor cell line EML C.1 (40) as described previously (33). The plasmids expressing VP16 fusions of full-length Trip-1 (pVP16-Trip-1), amino acids 1–150 (pVP16-Trip-1-CC-A), and the coiled-coil domain, amino acids 50–100 (pVP16-Trip-1-CC) were generated by ligating polymerase chain reaction fragments into pVP16.

In Vitro Binding Assay—Plasmids expressing Glutathione *S*-transferase (GST) fusion proteins of Lyn (pGEX-Lyn) and Trip-1 (amino acids 1–171) (pGEX-Trip-1–171) were generated by ligating polymerase chain reaction fragments into pGEX-2T. GST fusion proteins were purified as described previously (33). Binding experiments were performed by the addition of purified soluble Trip-1 (100 ng) to GST, GST-Lyn, GST-LynY397F, or GST-LynUn (500 ng) attached to glutathione-agarose beads in buffer (33) and then incubated at 4 °C for 2 h. Bound Trip-1 was detected by SDS-polyacrylamide electrophoresis and immunoblotting using an anti-Trip-1 antibody (36).

Immunoprecipitation and Immunoblotting—Cells were lysed as described previously (33), and proteins were co-immunoprecipitated with antibodies (anti-Trip-1 (41), anti-EpoR (no. 187), anti-Lyn; SC-15G, anti-STAT5; SC-1081, anti-TRα; SC-772, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 h at 4 °C and then collected with protein A-Sepharose beads for 16 h before analysis by immunoblotting. Additional antibodies used in immunoblotting were directed against Lyn, Lck, Src, Hck, Fyn, Syk, MAPK, v-Raf, phosphotyrosine and GATA-1 (SC-15, SC-13, SC-19, SC-72, SC-16, SC-573, SC-154, SC-133, SC-7020, and SC-265, Santa Cruz). Antibodies to EKLF, NF-E2, globin (catalog no. 55447, Cappel Research, Organon Technika, Boxtel, The Netherlands), and p27^{Kip1} and p57^{Kip2} (catalog no. 13231A and 65021A, respectively, PharMingen, San Diego, CA) were also used for immunoblotting. Secondary antibodies were coupled to horseradish peroxidase and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

Indirect Immunofluorescent Microscopy—Cells were cytocentrifuged onto slides, fixed in 50% methanol, 50% acetone, and then stained for indirect immunofluorescence using anti-Lyn, anti-Trip-1 (41), or anti-TRα antibodies and AlexaFluor-conjugated anti-rabbit and anti-mouse secondary antibodies (Molecular Probes, Eugene, OR). DNA was counterstained with Hoechst 33258. Slides were mounted in 50 mM Tris-HCl, pH 8.0, 50% glycerol, 2.5% 1,4-diazabicyclo-[2.2.2]octane and visualized using a Bio-Rad MRC-1000/1024 UV laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Retroviral Infection of Cells—Sense (Cs) and antisense (Cα) tTrip-1 cDNAs encoding amino acids 1–171 were generated by polymerase chain reaction, and the fragments were subcloned into the pMSCV2.2neo vector (42). Amphotropic and ecotropic retroviruses expressing the Cs and Cα constructs were used to infect erythroid cells as described previously (33). The efficiency of fetal liver cell infection was at least 50% (43). Numerous independent clones were isolated, and representative clones are shown.

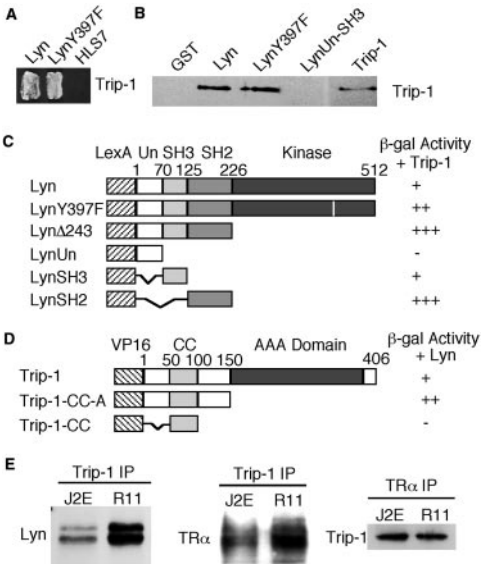

FIG. 1. **Lyn associates with Trip-1.** *A*, Lyn and Trip-1 interact in the yeast two-hybrid system. His^3 assays of yeast co-expressing LexA fusions of Lyn, LynY397F (kinase inactive Lyn), or HLS7 (negative control) (43) with a VP16 fusion of Trip-1. *B*, purified Lyn and Trip-1 interact *in vitro*. Immunoblot analysis of a binding assay with purified Trip-1 (amino acids 1–171) and GST fusions of Lyn, LynY397F, or the Unique plus SH3 domain (*LynUN-SH3*). *C*, the SH2 and SH3 domains of Lyn bind Trip-1. Yeast co-expressing LexA fusions of Lyn, LynY397F, LynΔ243, LynUn (Unique), LynSH3, or LynSH2 and a VP16 fusion of Trip-1 were assayed for β-galactosidase (*β-gal*) activity. *D*, the coiled-coil (*CC*) containing amino-terminal domain of Trip-1 binds Lyn. Yeast co-expressing pVP16 fusions of Trip-1, Trip-1-CC-A, or Trip-1-CC and Lyn were assayed for β-galactosidase activity. *E*, Lyn, Trip-1, and TRα interact *in vivo*. J2E and R11 cells were lysed, and then Trip-1 (or TRα) was immunoprecipitated, and co-immunoprecipitation of Lyn, Trip-1, or TRα was detected by immunoblotting.

Transient Transfection Assays—Cells (10^7) were electroporated with 10 μg of the TR reporter constructs pF2-Luc or pDR4-Luc (44) and 1 μg of pRL-SV40 (Promega, Madison, WI) at 300 V/1000 microfarad using a Gene Pulser II (Bio-Rad). Transfected cells were harvested after 48 h of culture in the presence or absence of T_3, and dual luciferase reporter assays were performed (Promega) on an Autolumat LB953 (EG&G Berthold, Oak Ridge, TN).

RESULTS

Trip-1 Associates with Lyn and TRα—To identify specific binding partners of Lyn, a yeast two-hybrid screen was conducted using wild-type Lyn and the kinase-inactive Y397F mutant (33); Fig. 1*A* shows the association of Lyn with Trip-1. In addition to transcriptionally regulating TRα (34–36), Trip-1 also possesses intrinsic helicase activity, and independently it has also been described as SUG1, a component of the 26-S proteasome (41, 45).

In vitro studies with purified proteins revealed that Lyn and Trip-1 interacted directly (Fig. 1*B*) in a phosphotyrosine-independent manner (Fig. 1, *A–C*). Trip-1 also bound the kinase-inactive Y397F mutant of Lyn (Fig. 1, *A* and *B*), indicating that the enzymatic activity of Lyn was not required for this association. Deletion analyses showed that the SH2 and, to a lesser extent, the SH3 domains of Lyn were responsible for Trip-1 binding *in vitro* (Fig. 1, *B* and *C*); these observations were confirmed using lysates from erythroid cells (data not shown). The regions of Trip-1 that bound Lyn were then analyzed. Fig. 1*D* shows that the amino-terminal of Trip-1 but not the coiled-coil domain alone was required for Lyn binding. This region is distinct from the highly conserved ATPase/DNA helicase AAA (ATPase associate with a variety of cellular activities) domain of Trip-1 needed to bind the TRα (41).

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

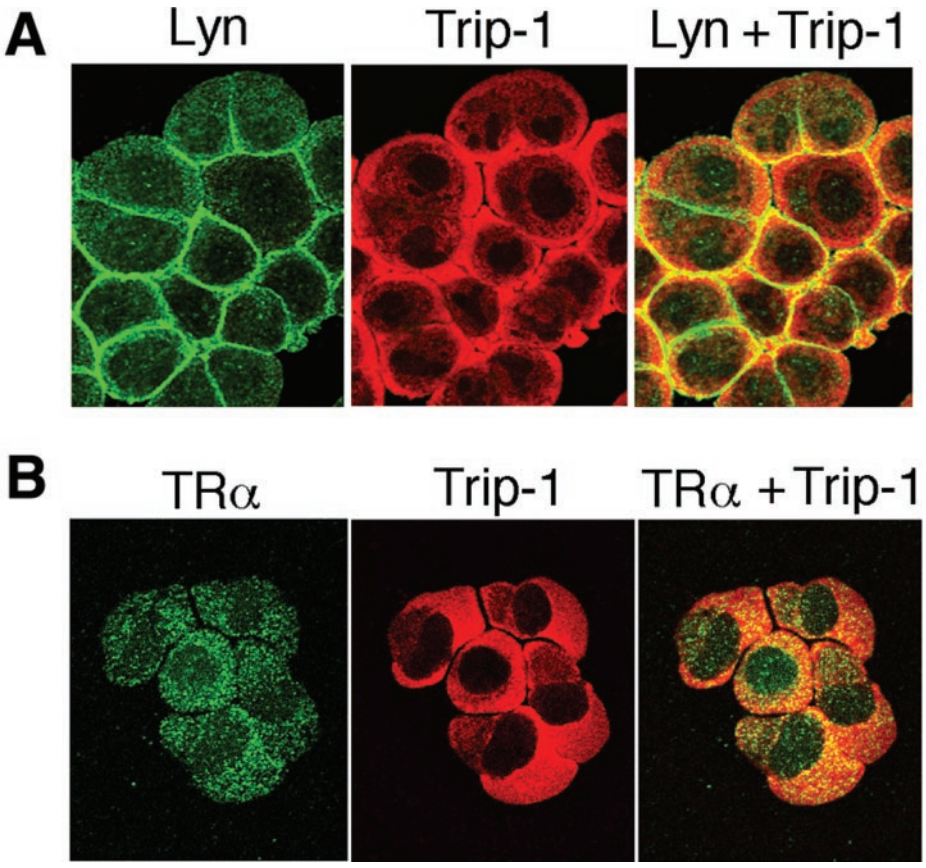

FIG. 2. **Cytoplasmic co-localization of Trip-1 with Lyn and TRα.** *A*, Lyn and Trip-1 can localize in the cytoplasm and plasma membrane. Erythroid cells were fixed on slides and analyzed by confocal microscopy utilizing antibodies to Lyn and Trip-1. Areas of Lyn (*green*) and Trip-1 (*red*) co-localization are *yellow* (Lyn + Trip-1). *B*, TRα and Trip-1 co-localize in the cytoplasm of erythroid cells. Cells were analyzed as above with antibodies to TRα and Trip-1. Areas of TRα (*green*) and Trip-1 (*red*) co-localization are *yellow* (*TRα + Trip-1*).

Co-immunoprecipitation experiments performed on lysates from erythroid cell lines show that Lyn does indeed associate with Trip-1 *in vivo* (Fig. 1*E*). Similarly, Trip-1 and TRα co-immunoprecipitated in these cells. These studies show that Trip-1 is able to bind both Lyn and TRα in erythroid cells, thereby connecting discrete signaling pathways involving Epo and T_3.

Trip-1 Co-localizes with Lyn and TRα in the Cytoplasm of Erythroid Cells—Having established a biochemical interaction between Lyn and Trip-1, the subcellular localization of these proteins was ascertained in uninduced erythroid cells. Lyn was found primarily in the cytoplasm of erythroid cells, with significant concentration at the plasma membrane (Fig. 2*A*). As Trip-1 was also distributed in the cytosol and plasma membrane, appreciable co-localization between Lyn and Trip-1 was observed (Fig. 2*A*). As described by Zhu *et al.* (46), TRα was detected in both the cytoplasm and nucleus. Consequently, Trip-1 co-localized with TRα in the cytoplasm, but not the nucleus, of these unstimulated erythroid cells (Fig. 2*B*). Comparable results were obtained with other erythroid cells (data not shown). These experiments demonstrate that the association of Trip-1 with Lyn and TRα occurs in the cytoplasm/plasma membrane of erythroid cells.

T_3 Inhibits Epo-induced Differentiation—As both Epo and T_3 affect erythropoiesis (1, 2), and Trip-1 was shown to associate with Lyn and TRα (Figs. 1 and 2), biological evidence for interplay between these pathways was sought. To this end, T_3 and Epo concentrations were manipulated and cellular responses monitored. Fig. 3*A* shows that the removal of T_3 enhanced Epo-induced hemoglobin production, whereas the addition of T_3 severely impeded synthesis of the oxygen carrier. These effects were T_3-specific, as reverse T_3 (Fig. 3*A*) and a variety of nuclear hormones (data not shown) had no effect on differentiation. The inhibitory effects of T_3 on hemoglobin synthesis were concentration-dependent with an IC_{50} of 100 pM. Furthermore, morphological maturation was severely retarded in the presence of T_3; nuclear condensation, cytoplasmic acidophilia, reduced cell size, and enucleation were all restricted by T_3 (Fig. 3*B*). Conversely, removal of T_3 accelerated the appearance of erythroid cells with a more mature phenotype, in particular orthochromatic erythroblasts and reticulocytes. Identical results were obtained with other Epo-responsive cell lines (data not shown).

In marked contrast with the inhibition of Epo-initiated differentiation, T_3 promoted [^{3}H]thymidine uptake (Fig. 3*C*). When T_3 was removed from cultures, DNA synthesis almost ceased; however, replication resumed upon re-introduction of T_3. This observation was supported by monitoring cell numbers in these cultures (data not shown).

To extend the analysis of erythroid cells beyond cell lines, the Epo/T_3 axis was examined in red cell progenitors from murine fetal livers. Fig. 4*A* shows that the inhibitory effects of T_3 on differentiation were not restricted to immortalized cells, as increasing the T_3 concentration reduced Epo-induced colonies from normal progenitors. However, the effect was more pronounced on erythroid colony-forming units (CFU-E) than erythroid burst-forming units (BFU-E). These data indicate that T_3 also had an inhibitory effect on Epo-induced differentiation of normal erythroid cells.

To determine whether T_3 affected fetal liver erythroid progenitors *before* exposure to Epo, cells were pre-incubated with T_3 and then treated with Epo. Intriguingly, when T_3 was added prior to Epo, both the BFU-E and CFU-E numbers rose (Fig. 4*B*). This expansion of the erythroid progenitor compartment by pre-incubation with T_3 indicates that the effects of T_3 are stage-specific. Taken together these results show that T_3 promotes proliferation and the expansion of immature erythroid

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

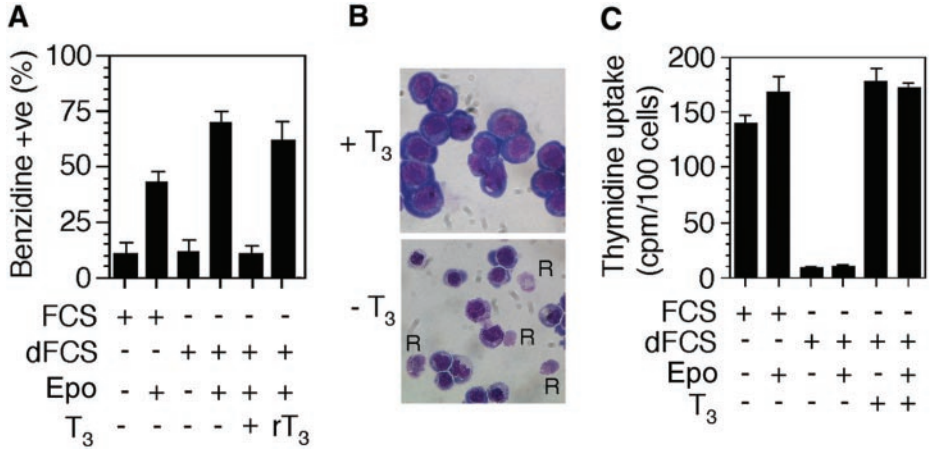

FIG. 3. **T_3 inhibits hemoglobin production and promotes erythroid proliferation.** *A*, J2E cells were cultured as indicated for 48 h then assayed for hemoglobin content by benzidine staining. *FCS*, fetal calf serum; *dFCS*, fetal calf serum-depleted of T_3; *rT_3*, reverse T_3. *B*, J2E cells were cultured as indicated for 48 h before morphological changes were analyzed. *R*, reticulocytes. *C*, J2E cells were cultured as indicated and then assayed for DNA synthesis by [^{3}H]thymidine uptake.

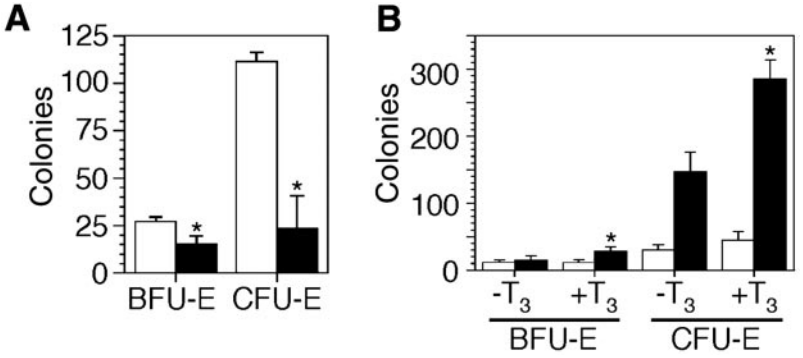

FIG. 4. **T_3 affects erythroid colony formation.** *A*, fetal liver cells were plated in methylcellulose (colony numbers/5,000 fetal liver cells plated are shown) in the presence of Epo with (*filled bars*) or without (*open bars*) T_3. An *asterisk* indicates a significant decrease in colony number in the presence of T_3 ($p < 0.01$, $n = 3$). *B*, fetal liver cells were incubated in the presence or absence of T_3 for 3 days before being plated in methylcellulose with Epo (*filled bars*) or without Epo (*open bars*). An *asterisk* indicates a significant increase in colony number with T_3 pre-incubation ($p < 0.01$, $n = 3$).

cells at the expense of maturation, whereas Epo favors terminal differentiation toward a nonreplicating state.

T_3 Affects Erythroid Transcription Factors and $p27^{Kip1}$—To identify the biochemical mechanism for the effects of T_3 on erythroid proliferation and differentiation, an immunoblot analysis was performed on key transcription factors and cell cycle regulators. The effects of T_3 were quite striking as the levels of erythroid-restricted transcription factors EKLF, NF-E2, and GATA-1 rose 3–10-fold when T_3 concentrations were reduced (Fig. 5, *left*). In addition, withdrawal of T_3 resulted in a 20-fold increase in $p27^{Kip1}$, a cell cycle inhibitor important for the maturation of erythroid cells (47, 48). In contrast, no change was observed in $p57^{Kip2}$ (Fig. 5, *right*). Interestingly, the Lyn and TRα content doubled when cells were cultured in T_3-depleted media. These observations provide biochemical explanations for enhanced differentiation and restricted replication after T_3 withdrawal *i.e.* raised GATA-1, EKLF, and NF-E2 facilitate red cell maturation, whereas elevated $p27^{Kip1}$ enables cells to exit the cell cycle and enter the terminally differentiated state. At this stage it is unclear whether the addition of T_3 increases the frequency of immature cells containing less EKLF, NF-E2, and GATA-1 or directly reduces the expression of these factors in erythroid cells.

Trip-1 Affects Responsiveness to Epo and T_3—To determine whether Trip-1 could simultaneously regulate differentiation signaling by Epo and proliferation promoted by T_3, a truncated Trip-1 (tTrip-1) encompassing the Lyn-binding domain (Fig. 1*D*) was introduced into J2E cells. Numerous independently isolated transfectants were termed JCs cells, whereas the antisense controls were labeled JCα. Significantly, tTrip-1 had a marked inhibitory effect on Epo-induced hemoglobin production and morphological maturation; very few hemoglobin-synthesizing cells were detected in JCs cultures, and the cells were incapable of proceeding beyond the proerythroblast/basophilic erythroblast boundary of maturation (Fig. 6, *A* and *B*). Moreover, T_3-induced proliferation was severely impeded (Fig. 6*C*). In contrast, the antisense controls behaved like control cells or those infected with the retroviral vector alone (data not shown). These data demonstrate that the truncated Trip-1 acted in a dominant negative manner and had a profound impact on *both* Epo-induced differentiation *and* T_3-promoted cell division.

The effect of tTrip-1 on normal erythroid progenitors was investigated by infecting fetal liver cells and observing colony formation. Consistent with the effects of tTrip-1 on cell lines, the truncated Trip-1 decreased the number of Epo-induced BFU-E and CFU-E (Fig. 7*A*). Furthermore, tTrip-1 greatly diminished the ability of T_3 pre-incubation to expand the progenitor compartment (Fig. 7*B*). It was concluded from these experiments that Trip-1 plays an important role in regulating the responses of normal as well as immortalized erythroid cells to Epo and T_3.

tTrip-1 Affects Lyn and $p27^{Kip1}$ Levels—The biochemical mechanisms by which tTrip-1 inhibited Epo and T_3 action were then investigated. The inhibition of Epo-induced differentiation by tTrip-1 coincided with the elimination of Lyn but not other tyrosine kinases such as Fyn, Src, Syk, Lck, and Hck (Fig. 8*A* and data not shown). Conversely, the elevated levels of $p27^{Kip1}$ in JCs cells correlated with reduced proliferation (Fig. 8*A*). However, the inability to differentiate was not caused by a reduction in GATA-1, EKLF, NF-E2, globins, or endogenous Trip-1 (Fig. 8*A*), nor was it due to restricted phosphorylation of the Epo receptor or STAT5 (data not shown). It is noteworthy that the antisense control did not affect Trip-1 protein levels, validating its use as an additional control (Figs. 6 and 7).

To determine whether tTrip-1 also interfered with T_3-induced transcription, activation of T_3 response elements was examined in JCs cells. The cells were transfected with either direct repeats or inverted palindromes of the T_3 response elements and were then exposed to T_3. Significantly tTrip-1 negated the T_3 responsiveness of both elements (Fig. 8*B* and data not shown). It was concluded from this series of experiments that tTrip-1 blocked erythroid differentiation and proliferation

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

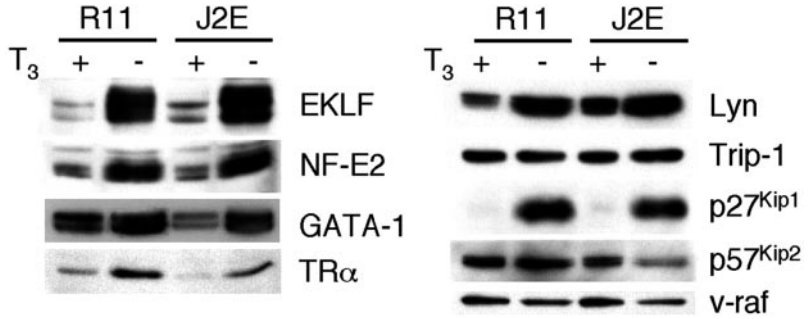

FIG. 5. **T_3 suppresses erythroid transcription factors and p^{27}.** J2E and R11 cells were cultured with or without T_3 for 48 h, and then proteins were extracted and analyzed by immunoblotting. Constitutively expressed v-Raf was used as a loading control.

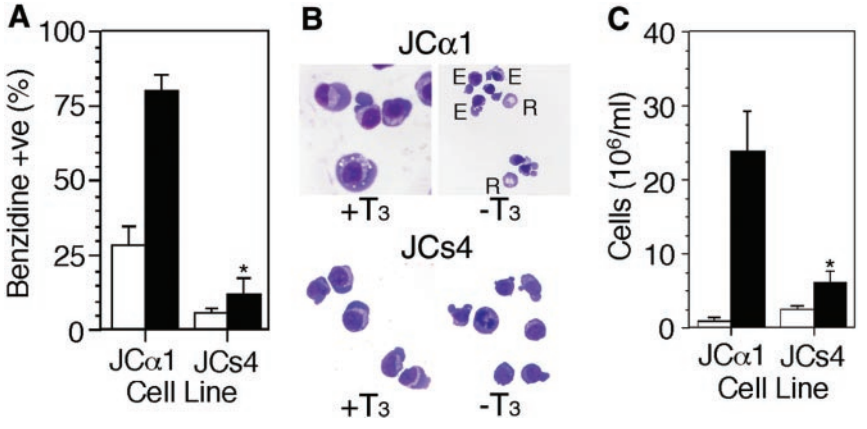

FIG. 6. **tTrip-1 inhibits Epo and T_3 responses.** *A*, JCα1 (antisense control cells) and JCs4 (tTrip-1 expressing cells) were cultured in the presence (*filled bars*) or absence (*open bars*) of Epo for 48 h and then assayed for hemoglobin content. The *asterisk* indicates a significant decrease in Epo-induced hemoglobin production ($p < 0.01$, $n = 3$). *B*, JCα1 and JCs4 cells were cultured in the presence or absence of T_3 for 48 h, and then morphological maturation was examined. Reticulocytes (*R*) and enucleating erythroblasts (*E*) are indicated. *C*, JCα1 and JCs4 cells were cultured in the presence (*filled bars*) or absence (*open bars*) of T_3 for 48 h before cell numbers were determined. The *asterisk* indicates a significant decrease in T_3-induced proliferation ($p < 0.01$, $n = 3$).

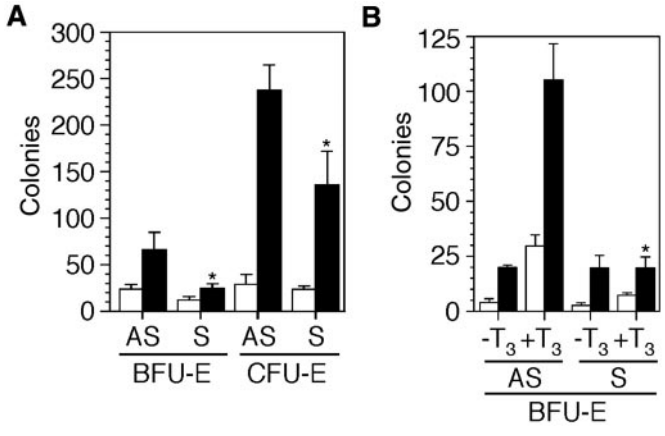

FIG. 7. **tTrip-1 affects erythroid colony formation.** *A*, erythroid progenitors in fetal liver were infected with retroviruses expressing tTrip-1 (*S*) or an antisense control (*AS*), and then colonies were determined in the presence (*filled bars*) or absence (*open bars*) of Epo. Cells were plated as described in Fig. 4*A*. An *asterisk* indicates a significant decrease in Epo-induced colony number ($p < 0.05$, $n = 3$). *B*, fetal liver cells were infected with retroviruses as described in *A* in the presence or absence of T_3 for 3 days of pre-incubation before BFU-E formation in the presence (*filled bars*) or absence (*open bars*) of Epo was ascertained. The *asterisk* indicates a significant decrease in T_3-induced enhanced colony formation ($p < 0.01$, $n = 3$).

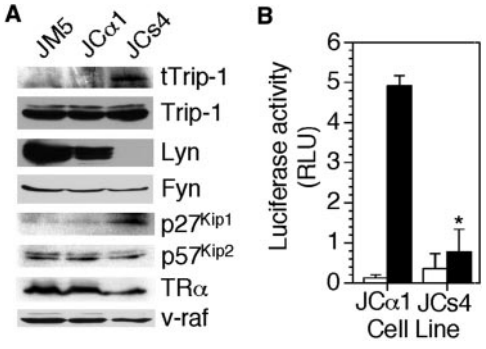

FIG. 8. **tTrip-1 induces biochemical changes.** *A*, JCα1 and JCs4 cell lysates were analyzed by immunoblotting for tTrip-1, Trip-1, Lyn, Fyn, $p57^{Kip2}$, $p27^{Kip1}$, TRα, and v-Raf (loading control). *B*, JCα1 and JCs4 cells were transiently transfected with the F2 (inverted palindrome T_3 response element) reporter plasmids (44). After 48 h in the presence (*filled bars*) or absence (*open bars*) of T_3, cells were analyzed for luciferase activity (*RLU*, relative light units; measured relative to control luciferase activity). The *asterisk* indicates a significant suppression in T_3-induced reporter activity ($p < 0.01$, $n = 3$).

by suppressing endogenous Lyn, increasing $p27^{Kip1}$ and interfering with the transcriptional activity of T_3 response elements.

Trip-1 Translocates to the Nucleus during Erythroid Differentiation—Trip-1 localized primarily in the cytoplasm of uninduced erythroid cells (Fig. 2). The subcellular localization of this protein was then examined in cells stimulated with Epo or when T_3 was withdrawn from culture. Strikingly, cytoplasmic Trip-1 translocated to discrete nuclear regions after 30 min of exposure to Epo or removal of T_3 (Fig. 9, *A* and *B*). Altering the compartmental balance of Trip-1, therefore, coincided with enhanced differentiation and reduced replication. Trip-1 also relocated to the nucleus in JCs cells (data not shown), indicating that the inhibitory effects of tTrip-1 were not due to impaired nuclear translocation.

DISCUSSION

In this article we have demonstrated that Trip-1, a transcriptional regulator of TRα (34, 36), associates with the tyrosine kinase Lyn in erythroid cells. The interaction between Lyn and Trip-1 was initially identified by a yeast two-hybrid screen and was confirmed by *in vitro* binding, co-immunoprecipitations, and intracellular co-localization (Figs. 1 and 2). Because Lyn is involved in the Epo signaling cascade (27, 29, 32), Trip-1 provides a link between pathways mediated by cytokine receptors and nuclear hormone receptors.

The parallels between the Trip-1 association with Lyn and TRα and the interaction of $p62^{ORCA}$ with Lck and COUP-TFII are striking. Like Trip-1 and Lyn, $p62^{ORCA}$ associates with Lck (the closest Src kinase family member to Lyn) through phos-

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

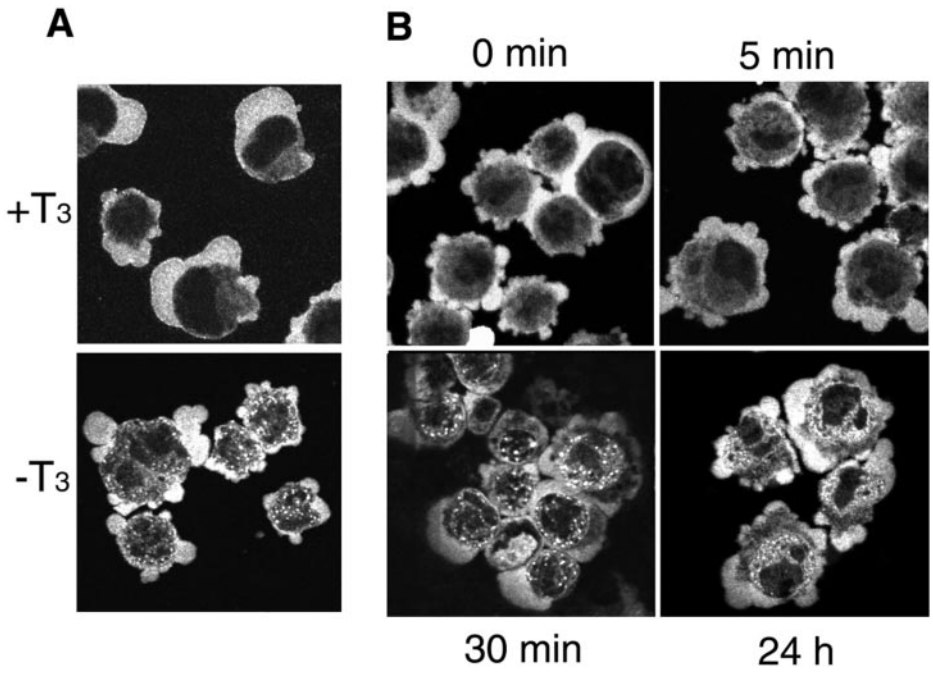

FIG. 9. **Trip-1 subcellular localization changes with differentiation.** *A*, J2E cells were incubated with or without T_3 (1 μM) before analysis of Trip-1 localization by confocal microscopy. *B*, J2E cells were incubated with Epo (5 units) for the indicated times before analysis of Trip-1 subcellular localization.

photyrosine-independent binding of $p62^{ORCA}$ to the SH2 domain of Lck (49). Furthermore, the Trip-1/TRα association (34, 36) is similar to $p62^{ORCA}$ interacting with the nuclear hormone receptor COUP-TFII (50), which has been implicated in the regulation of globin genes (51). Marcus *et al.* (50) proposed "a role for ORCA and related factors in mediating cross-talk among distinct signal transduction pathways important for cellular growth and differentiation." Here we have provided the biological and biochemical evidence to support this proposition and have shown that Trip-1 can modulate pathways activated by Epo and T_3.

The dominant negative effects of the truncated Trip-1 were quite profound, as it simultaneously inhibited Epo-induced differentiation in immortalized and normal erythroid cells and suppressed T_3-mediated proliferation. The introduction of tTrip-1 resulted in a complete loss of endogenous Lyn, increased $p27^{Kip1}$, and an inability to activate T_3 response elements. As Trip-1 is also a component of the 26-S proteasome (45), perhaps the truncated Trip-1 fosters proteasomal degradation of Lyn. The importance of Trip-1 to the T_3 pathway was illustrated by the suppression of T_3 response elements with the truncated Trip-1.

It is noteworthy that Trip-1 localized with Lyn and TRα in the cytoplasm/plasma membrane of uninduced erythroid cells and then translocated to discrete nuclear foci within 30 min of Epo stimulation or after withdrawal of T_3. To our knowledge this is the first description of Trip-1 relocation between subcellular compartments correlating with enhanced differentiation. Studies are currently under way to determine the nature of these nuclear structures and their function during erythroid maturation.

Epo and T_3 had major effects on erythroid maturation in both cell lines and normal progenitors. Whereas Epo promoted differentiation (manifest by hemoglobin synthesis and morphological maturation), T_3 stimulated proliferation at the expense of terminal differentiation. Similarly, it has been shown that T_3 prevents hemoglobin production by NFS-60 cells and increases the erythrocyte yield from erythroblasts (18, 20). Our data with normal erythroid progenitors confirm that T_3 inhibits colony formation (18). However, we also demonstrated that T_3 is able to expand the erythroid progenitor compartment prior to Epo stimulation, which supports the notion that the effects of T_3 may be stage-specific (18, 19, 24).

Withdrawal of T_3 from erythroid cells produced large increases in erythroid-restricted transcription factors GATA-1, EKLF, and NF-E2, which have been strongly implicated in the control of red cell maturation, especially in hemoglobin production (52–56). Increasing the concentration of these transcription factors, therefore, promotes differentiation. Cross-regulation of these nuclear proteins may also be important because TRα and COUP-TFII together suppress GATA-1 transcription (57), whereas TRα is able to associate directly with NF-E2 (58). Altering the concentration, activity, or combinations of these DNA proteins can have a major impact upon expression of genes required for red cell maturation. Removal of T_3 also caused a marked elevation in $p27^{Kip1}$, which is significant because entry of erythroid cells into the noncycling, terminally differentiated state involves increasing $p27^{Kip1}$ levels (48). Thus, terminal differentiation was enhanced by the combination of cell cycle exit and elevated transcription factor levels.

It is conceivable that the Epo/T_3 axis provides a complementary mechanism for expanding erythroid progenitors and increasing cell numbers before terminal differentiation to generate the correct number of functionally mature red blood cells. As TRα has been proposed to act as a switch between proliferation and differentiation (24), Trip-1 may be a vehicle for coordinating the biological responses initiated by T_3 and Epo.

Acknowledgments—Assistance with confocal microscopy was kindly provided by Dr. P. Rigby (University of Western Australia), and recombinant human Epo (Eprex) was donated by Drs. J. Adams and J. Patava (Jansen-Cilag). We are indebted to Drs. J. Adams and T. Metz (Walter and Eliza Hall Institute, Melbourne, Australia) for providing the cell lines and to Drs. J. Bieker, N. Andrews, S. Watowich, and P. Chambon for generous gifts of antibodies. The plasmids pDR4-Luc and pF2-Luc were kind gifts from Dr. P. M. Yen (Brigham and Women's Hospital, Boston, MA).

REFERENCES

1. Krantz, S. B. (1991) *Blood* **77,** 419–434
2. Dainiak, N., Sutter, D., and Kreczko, S. (1978) *Nature* **272,** 260–262
3. D'Andrea, A. D., Lodish, H. F., and Wong, G. G. (1989) *Cell* **57,** 277–285
4. Youssoufian, H., Longemore, G., Neumann, D., Yoshimura, A., and Lodish, H. F. (1993) *Blood* **81,** 2223–2236
5. Ihle, J. N., Quelle, F. W., and Miura, O. (1993) *Semin. Immunol.* **5,** 375–389
6. Wojchowski, D. M., Gregory, R. C., Miller, C. P., Pandit, A. K., and Pircher,

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

T. J. (1999) *Exp. Cell Res.* **253,** 143–156
7. Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999) *Trends Endocrinol. Metab.* **10,** 18–23
8. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) *Cell* **80,** 729–738
9. Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., and Hilton, D. J. (1997) *Nature* **387,** 917–921
10. Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T., and Miyajima, A. (1995) *EMBO J.* **14,** 2816–2826
11. Glass, C. K. (1994) *Endocr. Rev.* **15,** 391–407
12. Fein, H. G., and Rivlin, R. S. (1975) *Med. Clin. N. Am.* **59,** 1133–1145
13. Singh, V., and Catlett, J. P. (1998) *Endocrinologist* **8,** 87–91
14. Hentzen, D., Renucci, A., le Guellec, D., Benchaibi, M., Jurdic, P., Gandrillon, O., and Samarut, J. (1987) *Mol. Cell. Biol.* **7,** 2416–2424
15. Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom, B. (1986) *Nature* **324,** 635–640
16. Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J., and Evans, R. M. (1986) *Nature* **324,** 641–646
17. Malgor, L. A., Blanc, C. C., Klainer, E., Irizar, S. E., Torales, P. R., and Barrios, L. (1975) *Blood* **45,** 671–679
18. Perrin, M. C., Blanchet, J. P., and Mouchiroud, G. (1997) *Hematol. Cell Ther.* **39,** 19–26
19. Schroeder, C., Gibson, L., Zenke, M., and Beug, H. (1992) *Oncogene* **7,** 217–227
20. Zenke, M., Munoz, A., Sap, J., Vennstrom, B., and Beug, H. (1990) *Cell* **61,** 1035–1049
21. Disela, C., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., and Zenke, M. (1991) *Genes Dev.* **5,** 2033–2047
22. Bartunek, P., and Zenke, M. (1998) *Mol. Endocrinol.* **12,** 1269–1279
23. Bauer, A., Ulrich, E., Andersson, M., Beug, H., and von Lindern, M. (1997) *Oncogene* **15,** 701–715
24. Bauer, A., Mikulits, W., Lagger, G., Stengl, G., Brosch, G., and Beug, H. (1998) *EMBO J.* **17,** 4291–303
25. Klinken, S. P., Nicola, N. A., and Johnson, G. R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8506–8510
26. Busfield, S. J., and Klinken, S. P. (1992) *Blood* **80,** 412–419
27. Tilbrook, P. A., Ingley, E., Williams, J. H., Hibbs, M. L., and Klinken, S. P. (1997) *EMBO J.* **16,** 1610–1619
28. Tilbrook, P. A., Bittorf, T., Busfield, S. J., Chappell, D., and Klinken, S. P. (1996) *J. Biol. Chem.* **271,** 3453–3459
29. Chin, H., Arai, A., Wakao, H., Kamiyama, R., Miyasaka, N., and Miura, O. (1998) *Blood* **91,** 3734–3745
30. de Franceschi, L., Fumagalli, L., Olivieri, O., Corrocher, R., Lowell, C., and Berton, G. (1997) *J. Clin. Invest.* **99,** 220–227
31. Brunati, A. M., Bordin, L., Clari, G., James, P., Quadroni, M., Baritono, E., Pinna, L. A., and Donella-Deana, A. (2000) *Blood* **96,** 1550–1557
32. Tilbrook, P. A., Palmer, G. A., Bittorf, T., Wright, M. J., Sarna, M. K., Linnekin, D., V., C., Williams, J. H., Ingley, E., Schneider-Mergener, J., Krystal, G., and Klinken, S. P. (2001) *Cancer Res.* **61,** 2453–2458
33. Ingley, E., Sarna, M. K., Beaumont, J. G., Tilbrook, P. A., Tsai, S., Takemoto, Y., Williams, J. H., and Klinken, S. P. (2000) *J. Biol. Chem.* **275,** 7887–7893
34. Swaffield, J. C., Melcher, K., and Johnston, S. A. (1995) *Nature* **374,** 88–91
35. Lee, J. W., F., R., Swaffield, J. C., Johnston, S. A., and Moore, D. D. (1995) *Nature* **374,** 91–94
36. vom Baur, E., Zechel, C., Herry, D., Heine, M. J. S., Garnier, J. M., Vivat, V., Le Douarin, B., Gronemeyer, H., Chambon, P., and Losson, R. (1996) *EMBO J.* **15,** 110–124
37. Metz, T., Harris, A. W., and Adams, J. M. (1995) *Cell* **82,** 29–36
38. Klinken, S. P., Rapp, U. R., and Morse, H. C., III (1989) *J. Virol.* **63,** 1489–1492
39. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) *Cell* **74,** 205–214
40. Tsai, S., Bartelmez, S., Sitnicka, E., and Collins, S. (1994) *Genes Dev.* **8,** 2831–2841
41. Fraser, R. A., Rossignol, M., Heard, D. J., Egly, J. M., and Chambon, P. (1997) *J. Biol. Chem.* **272,** 7122–7126
42. Hawley, R. G., Lieu, F. H. L., Fong, A. Z. C., and Hawley, T. S. (1994) *Gene Ther.* **1,** 136–138
43. Williams, J. H., Daly, L. N., Ingley, E., Beaumont, J. G., Tilbrook, P. A., Lalonde, J. P., Stillitano, J. P., and Klinken, S. P. (1999) *EMBO J.* **18,** 5559–5566
44. Yen, P. M., Liu, Y., Sugawara, A., and Chin, W. W. (1996) *J. Biol. Chem.* **271,** 10910–10916
45. Rubin, D. M., Coux, O., Wefes, I., Hengartner, C., Young, R. A., Goldberg, A. L., and Finley, D. (1996) *Nature* **379,** 655–657
46. Zhu, X. G., Hanover, J. A., Hager, G. L., and Cheng, S. Y. (1998) *J. Biol. Chem.* **273,** 27058–27063
47. Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Tsai, L. H., Broudy, V., Perlmutter, R. M., Kaushansky, K., and Roberts, J. M. (1996) *Cell* **85,** 733–744
48. Hsieh, F. F., Barnett, L., Green, W. F., Freedman, K., Matushansky, I., Skoultchi, A. I., and Kelley, L. L. (2000) *Blood* **96,** 2746–2754
49. Park, I., Chung, J., Walsh, C. T., Yun, Y., Strominger, J. L., and Shin, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 12338–12342
50. Marcus, S. L., Winrow, C. J., Capone, J. P., and Rachubinski, R. A. (1996) *J. Biol. Chem.* **271,** 27197–27200
51. Filipe, A., Li, Q. L., Deveaux, S., Godin, I., Romeo, P. H., Stamatoyannopoulos, G., and Mignotte, V. (1999) *EMBO J.* **18,** 687–697
52. Miller, I. J., and Bieker, J. J. (1993) *Mol. Cell. Biol.* **13,** 2776–2786
53. Tsai, S., Martin, D. I. K., Zon, L. I., D'Andrea, A. D., Wong, G. G., and Orkin, S. H. (1989) *Nature* **339,** 446–451
54. Perkins, A. C., Sharpe, A. H., and Orkin, S. H. (1995) *Nature* **375,** 318–322
55. Nuez, B., Michalovich, D., Bygrave, A., Ploemacher, R., and Grosveld, F. (1995) *Nature* **375,** 316–318
56. Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P., and Orkin, S. H. (1993) *Nature* **362,** 722–728
57. Trainor, C. D., Evans, T., and Felsenfeld, G. (1995) *Mol. Endocrinol.* **9,** 1135–1146
58. Cheng, X., Reginato, M. J., Andrews, N. C., and Lazar, M. A. (1997) *Mol. Cell. Biol.* **17,** 1407–1416

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017

Thyroid Hormone Receptor-interacting Protein 1 Modulates Cytokine and Nuclear Hormone Signaling in Erythroid Cells

Evan Ingley, David Chappell, Sally Y. K. Poon, Mohinda K. Sarna, Jennifer G. Beaumont, James H. Williams, Justin P. Stillitano, Schickwann Tsai, Peter J. Leedman, Peta A. Tilbrook and S. Peter Klinken

J. Biol. Chem. 2001, 276:43428-43434.
doi: 10.1074/jbc.M106645200 originally published online September 5, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106645200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 26 of which can be accessed free at
http://www.jbc.org/content/276/46/43428.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at Murdoch University (CAUL) on November 26, 2017