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GATA-1-Mediated Proliferation Arrest during Erythroid Maturation

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Transcription factor GATA-1 is essential for erythroid and megakaryocytic maturation. GATA-1 mutations are associated with hematopoietic precursor proliferation and leukemogenesis, suggesting a role in cell cycle control. While numerous GATA-1 target genes specifying mature hematopoietic phenotypes have been identified, how GATA-1 regulates proliferation remains unknown. We used a complementation assay based on synchronous inducible rescue of GATA-1$^-$ erythroblasts to show that GATA-1 promotes both erythroid maturation and G_1 cell cycle arrest. Molecular studies combined with microarray transcriptome analysis revealed an extensive GATA-1-regulated program of cell cycle control in which numerous growth inhibitors were upregulated and mitogenic genes were repressed. GATA-1 inhibited expression of cyclin-dependent kinase (Cdk) 6 and cyclin D2 and induced the Cdk inhibitors p18^{INK4C} and p27^{Kip1} with associated inactivation of all G_1 Cdks. These effects were dependent on GATA-1-mediated repression of the c-myc (*Myc*) proto-oncogene. GATA-1 inhibited *Myc* expression within 3 h, and chromatin immunoprecipitation studies indicated that GATA-1 occupies the *Myc* promoter in vivo, suggesting a direct mechanism for gene repression. Surprisingly, enforced expression of *Myc* prevented GATA-1-induced cell cycle arrest but had minimal effects on erythroid maturation. Our results illustrate how GATA-1, a lineage-determining transcription factor, coordinates proliferation arrest with cellular maturation through distinct, interrelated genetic programs.

The development of mature blood cells from multipotential progenitors is coordinated by lineage-specific transcription factors and their downstream effectors. Establishment and maintenance of the differentiated state involves the acquisition of tissue-specific functions and progressive restriction of proliferative potential, usually culminating in G_1 arrest. Both processes appear to be controlled by the hematopoietic transcription factor GATA-1 during erythrocyte and megakaryocyte development.

GATA-1 is a zinc-finger DNA binding protein that transactivates numerous hematopoietic-specific genes through cognate elements present in their promoters and enhancers (99). Loss-of-function studies demonstrate that GATA-1 is critical for the formation of early eosinophil precursors and for differentiation of committed erythroid precursors and megakaryocytes (39, 66, 81, 98, 104). GATA-1 appears to inhibit cell division during terminal hematopoietic differentiation. Mice and humans with GATA-1 mutations accumulate dysplastic megakaryocytes, and GATA-1$^-$ megakaryocytes proliferate excessively in a cell-autonomous fashion (61, 92, 95). Moreover, somatic mutations in GATA-1 are associated with the development of megakaryocytic leukemia (97). Primary GATA-1$^-$ erythroblasts undergo apoptosis, a common response to lesions that deregulate cell proliferation (20, 100). Female mice heterozygous for a hypomorphic mutation in the X-linked GATA-1 gene accumulate immature GATA-1-deficient erythroblasts, which could reflect their increased proliferative capacity (82, 92). How GATA-1 regulates the cell cycle during hematopoietic differentiation is not understood.

Cell cycle withdrawal during tissue maturation usually occurs in G_1 phase. In general, cell cycle progression requires the activity of regulatory cyclins and their catalytic partners, the cyclin-dependent kinases (Cdks). Specifically, passage through G_1 requires the activities of D-type cyclins (D1, D2, D3) associated with Cdk4 or Cdk6, followed by activation of the cyclin E- and A-dependent kinase, Cdk2, as cells near the G_1-S transition (79). G_1 Cdks function in part by phosphorylating pRb and related proteins, leading to the activation of E2F transcription factors, which are important for S-phase entry. G_1 arrest from antimitogenic signals can be achieved through regulation of cyclin synthesis or degradation, by specific posttranslational modifications of Cdk subunits, or via association of Cdks with protein inhibitors (CKIs). These include the Cip/Kip family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) and the INK4 family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d}), whose expression is also regulated at multiple levels (80). Lineage-specific nuclear proteins can cause proliferation arrest by directly interacting with core cell cycle components or, indirectly, by controlling transcription of cell cycle regulators (107).

Appropriate model systems are required to study how GATA-1 controls cell division, since these effects are context dependent. For example, murine erythroleukemia (MEL) cells express GATA-1 yet divide actively, indicating that oncogenic transformation may override GATA-1-mediated antiproliferative signals in erythroid cells (88). In this regard, antagonism of GATA-1 function by the oncoprotein PU.1 is believed to be critical for the development of virally induced murine erythroleukemia (73). Reports regarding overexpression of GATA-1

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in MEL cells vary; one group found that GATA-1 induced differentiation and proliferation arrest (73), while another showed that it blocked chemically induced maturation and cell cycle arrest (102). The effects of GATA-1 expression in nonerythroid cells are also variable. GATA-1 slowed cell proliferation and prolonged S phase in NIH 3T3 fibroblasts and FDCP-1 hematopoietic cells (16). In myeloid FDCW2 cells, GATA-1 inhibited S phase entry after cytokine stimulation (78). In contrast, GATA-1 blocked interleukin 6-induced proliferation arrest in the myeloid cell line M1 (84). Thus, GATA-1 produces different effects on cell proliferation, which probably depend on both expression levels and the cellular environment. Of particular importance, the cell cycle effects of GATA-1 appear to be hematopoietic stage specific, given that enforced expression of GATA-1 in immature progenitors does not arrest division but rather influences cell fate commitment decisions toward erythroid, eosinophil, and megakaryocytic lineages (38, 39, 49). In the present study, we investigated how expression of GATA-1 alters the cell cycle in committed erythroid precursors.

To examine further the actions of GATA-1 in an erythroid context, we created G1E (for GATA-1$^-$ erythroid) cells, an immortalized GATA-1 null erythroid line derived from in vitro differentiation of gene-targeted embryonic stem cells (101). G1E cells proliferate continuously in culture as immature erythroblasts and undergo terminal maturation when GATA-1 function is restored. G1E cells have proven to be a convenient and physiologically relevant system for studying various aspects of erythroid biology (46, 47, 89, 106). An important feature of G1E cells is that terminal erythropoiesis is regulated by a specific genetic alteration that complements a defined loss-of-function mutation.

Here we show that restoration of GATA-1 not only stimulates G1E cell maturation but also causes rapid and synchronous cell cycle arrest associated with inactivation of Cdks. Biochemical studies and RNA transcript profiling verified that GATA-1 induced known erythroid GATA-1 target genes and identified a simultaneous and distinct GATA-1-regulated program of gene expression related to cell cycle control. GATA-1-mediated alterations in gene expression included upregulation of candidate tumor suppressors and repression of proto-oncogenes including *Myc*. Remarkably, GATA-1 repressed *Myc* RNA within 3 h, and enforced Myc expression blocked GATA-1-induced cell cycle arrest, but had minimal effects on morphological changes associated with erythroid maturation or globin gene expression. Thus, GATA-1 is a pleiotropic regulator of gene expression that links cell cycle withdrawal to phenotypic maturation during hematopoietic development. In addition, our studies highlight a potential role for GATA-1 in transcriptional repression and identify several functionally relevant candidate genes, including *Myc*, for future mechanistic studies.

MATERIALS AND METHODS

Cell culture. G1E cells were cultured and transduced with retrovirus as described previously (101).

Plasmids. The MSCV-based retroviral vector MIGR1 (obtained from Warren Pear) was used to express wild-type GATA-1 in G1E cells. The retroviral vector pGD was used to stably express Myc fused to the ligand binding domain of the estrogen receptor (Myc-ER) (12).

Microarray experiments. For each sample, after induction with 10^{-7} M β-estradiol, RNA from 5×10^7 G1E-ER4 cells was extracted using the Trizol reagent (Invitrogen, Carlsbad, Calif.), prepared and analyzed as described previously (31) using an Affymetrix MG-U74Av2 gene chip. The average signal intensity for each chip was normalized to a value of 150, and comparative values were computed versus the corresponding time zero sample using MAS 5.0 software (Affymetrix). Signal values were then averaged across the three replicates. Fold change is expressed as the signal log(2) ratio of a time point versus time zero; averages were computed prior to log transformation. Annotations were extracted from the U96Mm version of Unigene and from the GO database (2).

Northern blot analysis. Total RNA was isolated using Trizol reagent as described above and fractionated on a 1.2% agarose-formaldehyde gel. RNA was transferred by capillary action to Hybond N+ membranes (Amersham, Arlington Heights, Ill.) according to the manufacturer's directions and fixed by UV irradiation. ^{32}P-dCTP-labeled probe DNA was produced using the High Prime labeling kit. Blots were washed at a final stringency of 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1× sodium dodecyl sulfate (SDS) at 65°C.

Flow cytometry. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). DNA content analysis was performed as described previously (40, 76).

Antibodies. Antibodies from Santa Cruz Biotechnology (Santa Cruz, Calif.) were the following: p15 (K-18; 1:200), p16 (M-156; 1:200), p18 (M-20-G; used for immunoprecipitation and Western blotting after Cdk4 immunoprecipitation), p19 (M-167; 1:200), p27 (F-8; 1:200), cyclin A (C-19; 1:200), cyclin D2 (M-20; 1:200), cyclin D3 (D-7; 1:100), Cdk2 (M2; 1:200), Cdk4 (C-22; 1:200), Cdk6 (C-21; 1:200; used for Western blotting), GATA-1 (N-6; 1:10000), Myc (N-262; 1:200), and Myc (C-33; 1:100). The following were from Upstate Biotechnology (Lake Placid, N.Y.): p18 (catalog no. 06-555; 1:1,000) and cyclin E (catalog no. 06-459; 1:150). The following were from BD Biosciences PharMingen (San Diego, Calif.): p21 (catalog no. 556430; 1:250), pRb (catalog no. 554136; 1:250), phycoerythrin-conjugated TER119 (catalog no. 09085B; 5 μl/2 × 10^6 cells/assay). Cdk6 (catalog no. RB-017-P; used for immunoprecipitation) was from NeoMarkers, Inc. (Fremont, Calif.). Anti-cyclin D1 (D1-72-13G) (91) was also used. N-262 anti-Myc antibody (Santa Cruz Biotechnology) was used in the Western blot (see Fig. 7).

Western blotting, immunoprecipitation, and kinase assays. Immunoblotting, immunoprecipitation, and kinase activity analyses were performed as previously described (40, 55). For Myc immunoprecipitation, cells were lysed in RIPA buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 10% glycerol, 1% (octylphenoxy)-polyethoxyethanol (Igepal), 0.1% SDS, 0.5% deoxycholate, 2 mM EDTA, including freshly added 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg (each) of leupeptin, aprotinin, pepstatin A, and trypsin inhibitor/ml).

ChIP. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (51). Antibodies against the ER moiety of GATA-1–ER (AB-10; NeoMarkers) were used for immunoprecipitation and gave results similar to those obtained with anti-GATA-1 antibodies (N6 [Santa Cruz]; data not shown). The following primer pairs, encompassing a 286-bp region beginning 406 bp upstream of the first *Myc* transcription initiation site, P1, were used in the PCR to detect *Myc* DNA sequences: 5′ TCC AGG GTA CAT GGC GTA TTG 3′ and 5′ TCT GCT TTG GGA ACT CGG GA 3′. Thirty-one PCR cycles were performed, each consisting of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s.

RESULTS

GATA-1 induces proliferation arrest of G1E cells. Expression of physiologic levels of normal GATA-1 in G1E cells via retroviral transfer triggers concurrent G_1 cell cycle arrest and erythroid maturation (data not shown) (101). To activate GATA-1 synchronously and homogeneously for gene expression and biochemical studies, we engineered G1E cells to stably express a conditional, estrogen-activated form of GATA-1 (GATA-1 fused to the ligand binding domain of the estrogen receptor [GATA-1–ER]) (32, 89). Addition of β-estradiol to GATA-1–ER-expressing G1E cell lines recapitulated the actions of native GATA-1, triggering proliferation arrest and terminal maturation (Fig. 1 and data not shown). Experiments presented below were performed using the clone designated G1E-ER4; similar results were obtained with other clonal lines (data not shown). Cell cycle arrest began between 12 and 24 h

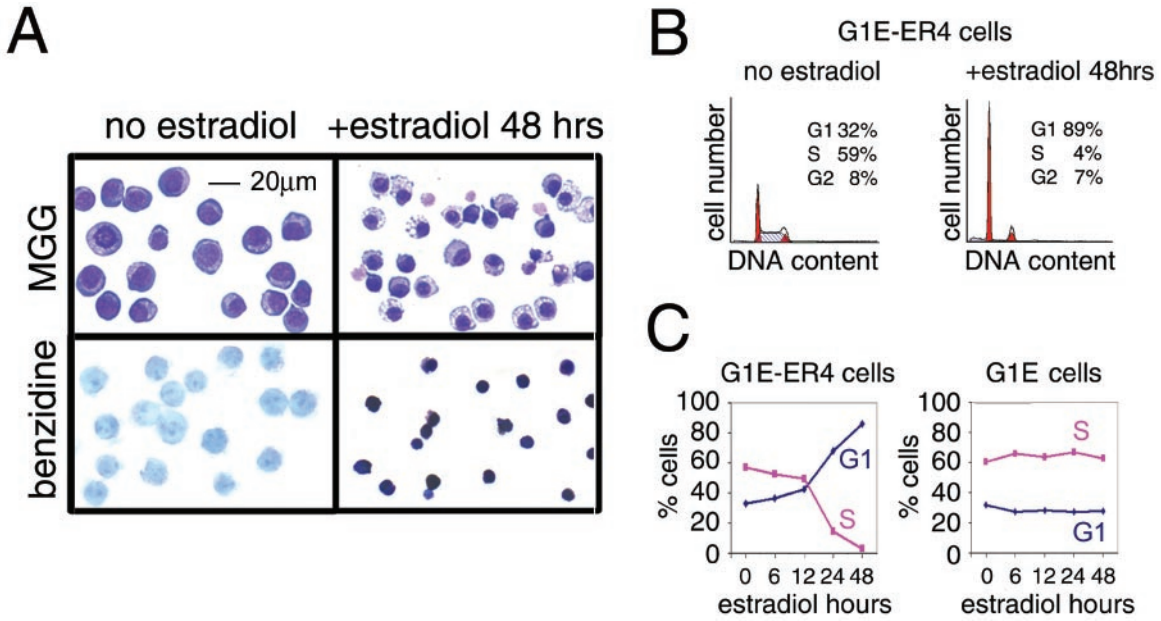

FIG. 1. Activation of conditional GATA-1 (GATA-1–ER) recapitulates the actions of wild-type GATA-1, inducing synchronous maturation and G_1 arrest in G1E cells. G1E-ER4 is a G1E subclone stably expressing GATA-1–ER. (A) May-Grunwald Giemsa (MGG) and benzidine staining of G1E-ER4 cells before and after β-estradiol-induced activation of GATA-1. The scale is maintained in all panels. (B) Cell cycle analysis before and after activation of GATA-1 in G1E-ER4 cells. (C) Kinetics of cell cycle withdrawal induced by GATA-1. G1E is the parental line which does not express GATA-1–ER.

after addition of β-estradiol, and by 48 h, ~90% of the cells accumulated in G_1 and acquired phenotypic characteristics of terminal maturation. No discernible changes occurred after β-estradiol treatment of G1E cells not expressing GATA-1–ER (Fig. 1C; also data not shown). Also of note, cell cycle arrest and terminal maturation were triggered by tamoxifen, an estrogen analog that activates chimeric ER fusion proteins but, unlike β-estradiol, does not induce interaction of the ER ligand-binding domain with its cellular coactivators (data not shown) (34, 36). These control experiments indicate that the biological activities of GATA-1–ER are attributable to the GATA-1 moiety of the fusion protein.

GATA-1-induced inactivation of Cdks. To investigate the mechanism of GATA-1-mediated cell cycle arrest during terminal erythropoiesis, we performed in vitro kinase assays on immunoprecipitated Cdks after β-estradiol treatment (Fig. 2A). Activities of all G_1 Cdks (Cdk4, Cdk6, and Cdk2) decreased markedly 12 to 24 h after GATA-1 activation, and hypophosphorylated forms of pRb accumulated by 24 h, consistent with the kinetics of G_1 arrest (Fig. 2C).

Western blotting showed that during the onset of cell cycle arrest and loss of G_1 Cdk activity, the level of Cdk4 remained relatively constant, Cdk2 decreased slightly, and Cdk6 declined more markedly (Fig. 2B). These results suggest that GATA-1 induces G_1 arrest by reducing Cdk protein levels and also by regulating molecules that govern Cdk activity.

GATA-1-induced alterations in G_1 phase cyclins and CKIs. Cdk activities are subject to a variety of positive and negative regulatory influences, including cyclin availability, activating and inactivating phosphorylation, and CKI proteins (79). GATA-1 could influence the cell cycle through one or more of these mechanisms. We determined that a non-DNA-binding version of GATA-1 (C261P, a missense mutation disrupting the C-terminal zinc finger) did not trigger G_1 arrest (data not shown). Therefore, GATA-1-dependent cell cycle arrest is mediated by either induction or repression of GATA-1-regulated genes, protein interactions through the DNA binding domain, or both. To investigate the first possibility, we assessed GATA-1-induced alterations in the expression of cell cycle proteins that influence Cdk activity.

Western blotting demonstrated that cyclin D3, E, and A levels were relatively stable up to 24 h after GATA-1 activation

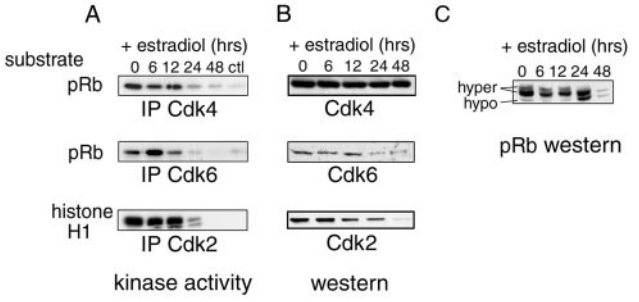

FIG. 2. Loss of Cdk activity after GATA-1 activation. (A) IP-kinase assays for Cdks after GATA-1 activation in G1E-ER4 cells. Cdks were immunoprecipitated from whole-cell extracts and assayed for kinase activities. Equivalent amounts of protein were used for each time point within a given IP experiment (Cdk2, 100 μg; Cdk4, 200 μg; Cdk6, 400 μg). For the control lanes (ctl), an irrelevant isotype-specific antibody was used. pRb, retinoblastoma protein. (B) Western blots for Cdks from whole-cell lysates. Cdk4, 25 μg of protein/lane; Cdk6, 100 μg of protein/lane; Cdk2, 25 μg of protein/lane. (C) Western blot for retinoblastoma protein (pRb) indicating hypo- and hyperphosphorylated forms (100 μg of protein/lane).

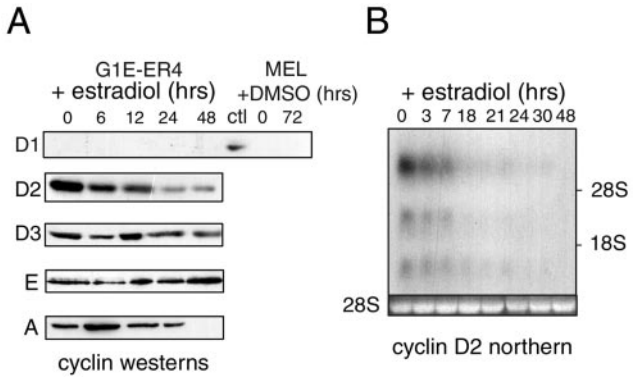

FIG. 3. Expression of G_1 cyclins during maturation of G1E-ER4 cells. (A) Western blot using whole-cell lysates. Each panel contains equivalent amounts of protein per lane. Cyclin D1, 50 μg of protein/lane, not detected; control (ctl), NIH 3T3 nuclear extract; cyclin D2, 25 μg of protein/lane; D3, 100 μg of protein/lane; E, 100 μg of protein/lane; A, 50 μg of protein/lane. (B) Northern blot for cyclin D2 RNA: μg of total RNA/lane.

when G_1 Cdk activities declined most sharply (Fig. 3A). In contrast, cyclin D2 protein and RNA declined substantially between 12 and 24 h (Fig. 3A and B). Cyclin D1 was not detected in G1E-ER4 or MEL cells. Since cyclin A is typically expressed in S phase, its decline at 48 h is consistent with the onset of G_1 arrest. Together, these data suggest that loss of cyclin D2 could contribute to GATA-1-induced G_1 arrest but that additional mechanisms must occur as cyclin D3 and E protein are available for their respective Cdks when maximal catalytic activity is lost.

We next examined the expression of cell cycle inhibitor proteins during GATA-1-induced maturation. Among the INK4 family inhibitors, which bind Cdk4 and Cdk6, p19 protein remained relatively constant up to 24 h, while p18 increased substantially (Fig. 4A). Transcription of *p18* yields 2.4- and 1.2-kb mRNA species (67). The longer transcript is generally expressed in proliferating undifferentiated cells, while the shorter correlates with terminal differentiation of numerous

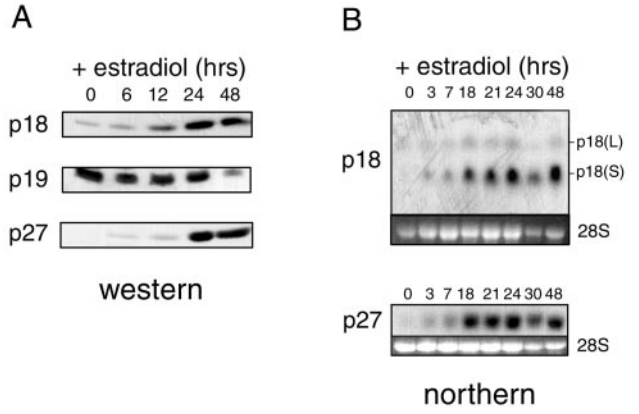

FIG. 4. CKIs p18 and p27 are induced by GATA-1 in G1E-ER4 cells. (A) Western blots for p18, p19, and p27, 50 μg of protein/lane. The CKIs p15, p16, p21, and p57 were not detected in G1E-ER4 cells by Western blotting (data not shown). (B) p18 and p27 Northern blots: 20 μg of total RNA/lane. L, long transcripts; S, short transcripts.

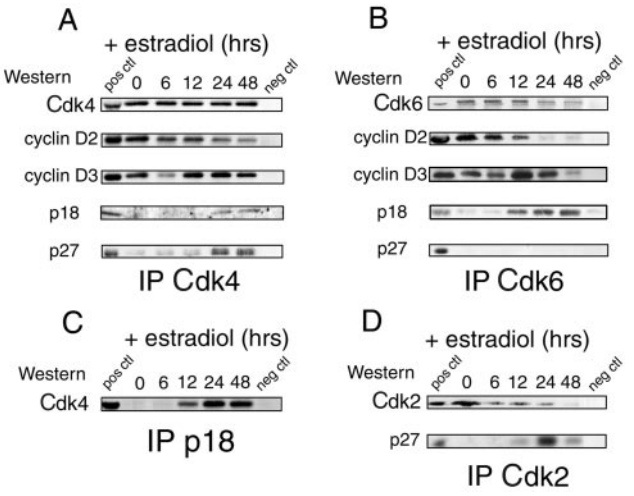

FIG. 5. GATA-1-regulated Cdk protein associations. G1E-ER4 whole-cell lysates were immunoprecipitated using antibodies indicated at the bottom of each panel and analyzed by Western blotting. (A) Cdk4 IP. Positive control (pos ctl): 20% input protein; 0-h samples for Cdk4, cyclins D2 and D3; 24-h samples for p18, p19, and p27. Negative control (neg ctl): IP using isotype-specific antibody (anti-rabbit IgG) against 0 h samples for Cdk4, cyclins D2 and D3 and 24-h samples for p18, p19, and p27. (B) Cdk6 IP. Controls were the same as for panel A, except that pos ctl represents 10% input. (C) p18 IP. Controls were the same as for panel A, except that pos ctl represents 5% input. (D) Cdk2 IP. Controls were the same as for panel B.

tissues. Accordingly, the 1.2-kb *p18* transcript was markedly upregulated during GATA-1-induced maturation of G1E-ER4 cells (Fig. 4B). The INK4 CKI proteins p15 and p16 were not detected at any time points (not shown).

Of the Cip/Kip inhibitors, p27 protein increased markedly between 12 and 24 h, coincident with the onset of G_1 arrest (Fig. 4A). p27 levels are regulated by transcription, translation, and protein degradation (80). Northern blotting demonstrated that GATA-1 induced marked accumulation of p27 mRNA, beginning between 3 and 7 h, suggesting that in G1E-ER4 cells accumulation of p27 protein is predominantly regulated by enhanced transcription and/or RNA stability. The Cip/Kip CKI proteins p21 and p57 were not detected at any time points (not shown).

Cell cycle subunit rearrangements during erythroid maturation. To investigate further the mechanisms of Cdk inactivation, we examined protein associations by immunoprecipitation (IP)-Western blot studies (Fig. 5). Coincident with declining Cdk activities (hours 12 to 24, Fig. 2C), binding of cyclin D2 to Cdk4 was reduced, consistent with the overall loss of cyclin D2 protein seen in Fig. 3A. At the same time, p18-Cdk4 and p27-Cdk4 complexes accumulated (Fig. 5A and C). Together, these data indicate that Cdk4 activity was inhibited by decreased availability of cyclin D2, along with increased binding of Cdk4 to p18 and possibly p27.

Concurrently, cyclin D2-Cdk6 complexes decreased, while Cdk6-p18 complexes accumulated (Fig. 5B). Cdk6-cyclin D2 complex decreased more markedly than Cdk4-cyclin D2, probably resulting from a more pronounced loss of Cdk6 protein (Fig. 2B and 5B). Cdk6 did not bind p27 at any time. Thus, loss of Cdk6 activity may be attributed to association with p18 and loss of Cdk6/cyclin D2 and Cdk6/cyclin D3 complexes.

Total Cdk2 protein decreased modestly between 12 and 24 h (Fig. 2B and 5D), and p27-Cdk2 complexes accumulated (Fig. 5D). These findings indicate that reduced Cdk2 levels and increased binding of Cdk2 to the strongly upregulated inhibitor p27 contribute to loss of Cdk2 activity.

In summary, GATA-1 inhibition of G_1 Cdk activities is associated with an increase in p18 and p27 and a decrease in cyclin D2 proteins. These changes are reflected at the mRNA level, so direct or indirect transcriptional regulation by GATA-1 is likely.

Gene expression profiling. To define more comprehensively GATA-1-regulated programs of erythroid gene expression, we used oligonucleotide microarrays to profile transcripts during induced maturation of G1E cells. Total RNA from G1E-ER4 cells was analyzed at 0, 3, 7, 14, 21, and 30 h after activation of GATA-1. We chose these time points to define alterations in gene expression surrounding the onsets of Cdk inactivation, G_1 arrest, and terminal maturation (Fig. 1) and to help distinguish early versus late GATA-1 effects. To minimize artifactual differences in gene expression caused by intersample variation, we analyzed data for each time point using RNA from three independent experiments, each sample on its own chip. The microarray allows for the simultaneous interrogation of 12,450 murine sequences. Numerous known erythroid GATA-1 target genes were observed to be upregulated, validating our experimental approach (Table 1). These included globins, heme biosynthetic enzymes, erythroid transcription factors, membrane proteins, and others. For the present study, we focused on genes that participate in cell cycle control, especially ones regulating the G_1-to-S transition (Table 1).

Electronic annotation and keyword searches identified 180 genes on the array associated with cell cycle functions; 82 of these genes were detected above background level and underwent at least a twofold change after activation of GATA-1 (Table 1 and data not shown). In general, expression of mRNAs encoding core cell cycle components were in agreement with results in our molecular studies, although RNA encoding the Cdk inhibitor p21 was predicted to be present at all time points and upregulated at 30 h, while the corresponding protein was not detected by Western blotting (not shown).

Several antiproliferative genes were induced by GATA-1 (Table 1). These include the genes for Btg2, a nuclear protein that is upregulated during tissue maturation and inhibits the G_1-to-S transition (17), Hipk2 (homeodomain-interacting protein kinase 2), a transcriptional corepressor which inhibits adipocyte cell division (68), JunB, an AP-1 family transcription factor which negatively regulates cell cycle progression in MEL cells (43), and Creg (cellular repressor of E1A-stimulated genes), which represses E2F-mediated activation of proliferation-related genes (94).

Genes encoding the replication licensing proteins Mcm2-7, Cdt1, and Cdc6, which prevent DNA from being replicated more than once per cell cycle, were repressed at late time points. These proteins are generally downregulated during tissue maturation, possibly through loss of E2F-dependent transcriptional activation (5).

Several genes that promote cell division were repressed by GATA-1 at early time points that preceded Cdk inactivation and cell cycle arrest. These included the proto-oncogenes *Myc* and *Myb*, whose products inhibit cell cycle withdrawal and maturation of MEL cells (9, 56, 70), and *Nab2*, which encodes a transcriptional repressor previously noted to inhibit proliferation arrest of pheochromocytoma cells (72).

In summary, microarray studies of GATA-1-rescued G1E cells verified the induction of numerous target genes related to erythroid maturation and also identified a program of gene expression related to proliferation arrest. Some of the latter changes occurred as early as 3 h, indicating potentially direct regulation by GATA-1, and included both activation and repression of cell cycle control genes. The role of GATA-1 in gene repression is implicated in prior studies but poorly understood (see Discussion). Our data suggest that transcriptional repression by GATA-1 may be important for its antiproliferative effects.

GATA-1 represses *Myc* during erythroid maturation. Microarray data indicated that *Myc* was repressed by GATA-1 in G1E-ER4 cells. In addition, *Mad1*, *Mad4*, and *Mxi1*, whose products oppose Myc actions (54), were upregulated (Table 1). We confirmed that *Myc* RNA and the protein were rapidly downregulated (Fig. 6). These findings are of potential significance for several reasons. First, *Myc* repression began within 3 h and was nearly complete by 14 h, highlighting an early step in the regulatory pathway that links GATA-1 to cell cycle arrest. Second, *Myc* is a critical cell cycle-regulatory gene that is controlled directly by numerous regulators of tissue differentiation (see below). Third, putative *Myc*-regulated genes that promote cell cycle progression, including *Cdc25a* and those for prothymosin alpha (*Ptma*) and protein kinase B (*Akt*), were also shown to be downregulated in the microarray experiments. Of particular note, genes for cyclin D2 and p27, potential effectors for GATA-1-mediated cell cycle arrest in G1E-ER4 cells (see above), are reported to be regulated directly by Myc. Specifically, Myc activates *cyclin D2* transcription and represses *p27* transcription through interactions with the respective gene promoters (54, 103). Consistent with our results, primary fetal liver erythroblasts that are induced to differentiate in culture accumulate GATA-1 and subsequently undergo G_1 arrest with downregulation of Myc, induction of *p27*, and repression of *cyclin D2*, *Cdc25a*, *Ptma*, and *Akt* (14).

Forced Myc expression inhibits GATA-1-induced cell cycle arrest but not maturation. If GATA-1 alters expression of cell cycle regulators by repressing *Myc*, then enforced Myc expression should override GATA-1-induced cell cycle effects. To test this, we engineered G1E-ER4 cells to express an estrogen-activated form of Myc (Myc-ER, Myc fused to the ligand binding domain of the estrogen receptor), which has been demonstrated previously to recapitulate the actions of native Myc (18). Treatment of G1E-ER4/Myc-ER cells with β-estradiol is predicted to switch on both Myc and GATA-1 activities simultaneously, allowing for examination of GATA-1 actions when *Myc* is derepressed. Three randomly selected clones of G1E-ER4 cells expressing Myc-ER were analyzed. In each clone, Myc-ER protein was moderately overexpressed compared to endogenous Myc in parental G1E-ER4 cells (Fig. 7A). In this regard, a recent report indicated that moderate overexpression of Myc-ER produced transcriptional and biological responses similar to those with physiological Myc levels (62). Expression of endogenous Myc was decreased in the Myc-ER-expressing lines from that in the parental G1E-ER4 line (Fig. 7A). This probably reflects low-level activity of Myc-ER in the absence of

TABLE 1. Microarray expression data

Category or designation and description[a]	Signal $\log_2$ ratio versus time zero					Comment (reference[s])
	3 h	7 h	14 h	21 h	30 h	
GATA-1 targets						
Alad, aminolevulinate, delta, dehydratase	0.4	0.7	1.6	1.8	1.6	Heme synthetic enzyme (4)
Alas2, aminolevulinic acid synthase 2, erythroid	0.9	1.9	4.3	6.0	6.7	Heme synthetic enzyme (48)
Ank1, ankyrin 1, erythroid	0.2	1.0	1.6	2.0	2.0	Erythroid-specific membrane protein (26)
Gypa, glycophorin A	−0.1	1.0	2.8	3.5	3.4	Erythroid-specific membrane protein (85)
Hbb-a1, hemoglobin, alpha, adult chain 1	0.4	0.7	1.8	2.4	2.6	Hemoglobin component (58)
Hbb-b1, hemoglobin, beta adult major chain	0.0	−0.1	1.6	2.1	2.2	Hemoglobin component (58)
Klf1, Kruppel-like factor 1 (erythroid)	0.6	1.0	1.3	1.4	1.1	Erythroid transcription factor (11)
Nfe2, nuclear factor, erythroid derived 2, 45 kDa	−0.4	0.0	0.6	1.2	1.2	Erythroid transcription factor (86)
Slc4a1, band 3 anion exchanger	−0.5	−0.6	1.9	4.6	5.1	Erythroid-specific membrane protein (74)
Uros, uroporphyrinogen III synthase	0.0	0.8	1.8	1.8	0.9	Heme synthetic enzyme (1)
Core cell cycle components						
Cdc2a, murine homolog of cdc2 (cdk1)+	0.2	−0.2	−0.3	−1.1	−3.9	G_1/S and G_2/M, complex with cyclin B (6, 15)
Cdk2, cyclin-dependent kinase 2*+	−0.1	−0.2	−0.4	−1.1	−1.6	G_1/S, bound by Cdk2, cyclins A&E, inhib[b] by p21/p27 (50, 79)
Cdk4, cyclin-dependent kinase 4+	−0.1	−0.2	−0.6	−1.0	−1.6	G_1/S, bound by Cdk4/6, cyclins D, inhib by p16 (37, 79)
Cks1, CDC28 protein kinase 1	−0.1	0.2	0.2	0.0	−2.0	Binds catalytic subunit of Cdks, essential for their function (28)
Cena2, cyclin A2+	0.7	0.7	0.9	0.7	−1.0	G_1/S, G_2/M, binds/activates Cdc2 and Cdk2 (44, 79)
Ccnd1, cyclin D1*+	0.1	0.3	−0.2	−0.2	0.3	G_1/S, bound by Cdk4/6 (64)
Ccnd2, cyclin D2+	−0.2	−0.6	−1.1	−1.3	−1.4	G_1/S, bound by Cdk4/6 (8, 79)
Cend3, cyclin D3+	−0.6	0.3	1.5	1.6	1.3	G_1/S, bound by Cdk4/6 (57, 79)
Ccne1, cyclin E1+	0.3	0.7	1.0	0.6	0.5	Late G_1/S, bound by Cdk2 (50, 79)
Ccne2, cyclin E2	0.2	0.3	−0.3	0.0	−0.3	Late G_1/S, bound by Cdk2 (79)
Cdkn1a, cyclin-dependent kinase inhibitor 1A (p21)+	−0.5	0.1	0.0	0.3	1.2	Inhibits activation of cyclin D/Cdk4 (8, 79)
Cdkn1b, cyclin-dependent kinase inhibitor 1B (p27)*+	2.0	1.8	2.8	3.3	3.1	Inhibits activation of cyclin E/Cdk2 and cyclin D/Cdk4 (79, 103)
Cdkn1c, cyclin-dependent kinase inhibitor 1C (p57)*	0.1	0.4	−0.1	−0.2	0.1	Inhibits activation of cyclin G/Cdk complexes (79)
Cdkn2b, cyclin-dependent kinase inhibitor 2B (p15)*	−0.2	−0.1	0.2	0.8	0.2	Inhibits Cdk4/6 activation, G_1 progression (79)
Cdkn2c, cyclin-dependent kinase inhibitor 2C (p18)	0.4	0.6	1.1	1.7	2.0	Inhibits Cdk4/6 activation, G_1 progression (79)
Cdkn2d, cyclin-dependent kinase inhibitor 2D (p19)	−0.3	0.1	0.1	0.6	0.2	Inhibits Cdk4/6 activation, G_1 progression (79)
Tfdp1, transcription factor Dp 1	−0.1	0.1	0.7	0.6	0.6	Rb dimerization partner (59)
Rb1, retinoblastoma 1	0.3	0.1	0.3	−0.7	−2.3	Pocket protein; binds E2F; represses transcription (79)
Rbl1, retinoblastoma-like 1 (p107)	0.1	−0.3	−0.5	−1.8	−2.6	Rb analogue (42)
Cdc25a, cell division cycle 25 homolog A+	−0.1	−0.3	−0.6	−1.2	−1.7	Dephosphorylates/activates Cdk2 (3, 25)
Chek1, checkpoint kinase 1 homolog	−0.1	−0.3	−0.5	−1.2	−1.9	Phosphorylates/inactivates Cdc25a, S-phase checkpoint (75)
Rpa1, replication protein A2	0.0	−0.2	−0.1	−1.1	−1.8	ssDNA bp required for replication (90)
Proteins related to proliferation						
Induced						
Btg2, B-cell translocation gene 2	0.7	1.6	2.2	2.7	2.9	Inhibits cyclin D1 transcription, pRb function (33)
Hipk2, homeodomain-interacting protein kinase 2	1.2	1.6	2.0	1.7	2.1	Antiproliferative at G_2/M (68)
JunB, JunB oncogene	0.3	0.6	0.9	1.0	0.9	Antiproliferative in erythroid cells (43)
Creg, cellular repressor of E1A-stimulated genes	−0.5	−0.5	0.1	0.8	1.7	Represses E2F-dependent proliferative genes (94)
Repressed						
Akt, thymoma viral proto-oncogene+	−0.1	−0.4	−1.0	−1.6	−1.3	Modulation of p27; c-myc stability (29, 63, 77)
Gata2, GATA-binding protein 2+	−1.9	−2.2	−3.2	−3.4	−3.2	Proliferation/survival of hematopoietic progenitors (87)
Kit, kit oncogene+	−0.4	−0.9	−2.6	−3.4	−4.0	Proliferation/survival of early erythroid progenitors (69, 83)
Myb, myeloblastosis oncogene (c-myb)	0.0	−0.9	−3.3	−6.1	−7.3	Required G_1/S transition of hematopoietic cells (60)
Nab2, Ngfi-A binding protein 2	−3.1	−2.5	−3.7	−4.1	−4.0	Overexpression associated with proliferation (72)
Ptma, prothymosin alpha+	0.3	0.2	0.3	−0.6	−1.3	Promotes proliferation (19, 93)
Tmk, thymidylate kinase+	0.0	0.0	−0.1	−1.1	−2.2	Essential for DNA synthesis (41, 71)
Vav, Vav oncogene	−0.9	−1.1	−1.1	−1.2	−1.8	Cyclin D2 induction, hematopoietic proliferation (30)
Myc/Max partners						
Myc, myelocytomatosis oncogene (c-myc)+	−0.7	−1.1	−2.2	−3.8	−4.5	Transactivates genes related to proliferation (54)
Max, Max protein	0.0	0.2	0.2	0.5	0.9	Common partner of Myc and Mad proteins (54)
Mad1, Max dimerization protein	1.5	3.0	3.6	4.4	3.7	Form inhibitory complexes with Max, oppose Myc (54)
Mad4, Max dimerization protein 4	−0.9	0.1	0.3	0.8	1.0	Form inhibitory complexes with Max, oppose Myc (54)
Mxil, Max interacting protein 1 (Mad2)	−0.5	0.6	0.6	1.2	1.4	Form inhibitor complexes with Max, oppose Myc (54)

[a] +, putative c-myc target; *, signal < 100 at all time points.
[b] inhib, inhibited.

β-estradiol, since Myc is known to repress its own transcription (62, 65). In all clones tested, G1E-ER4 cells expressing Myc-ER failed to undergo G_1 arrest upon activation of GATA-1 and Myc with either β-estradiol (Fig. 7B) or tamoxifen (not shown). Between 24 and 48 h after addition of estradiol, the G1E-ER4 cell number was unchanged while all Myc-ER-expressing lines approximately doubled in cell number (not shown).

Our findings demonstrate that enforced Myc expression overrides GATA-1-induced cell cycle arrest. To investigate the molecular basis for this effect, we examined the expression of cell cycle components previously identified to be altered by

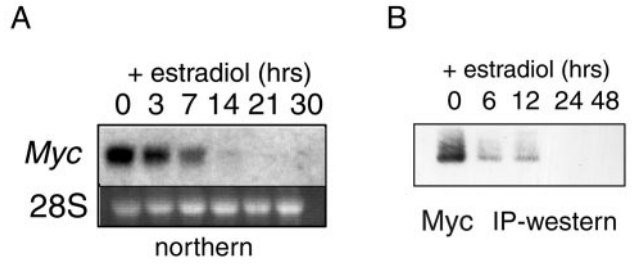

FIG. 6. GATA-1 represses *Myc* in G1E-ER4 cells. (A) Northern blot, 20 μg of total RNA/lane. (B) IP-Western blot analysis for Myc protein. One milligram of total protein from whole cell lysates was immunoprecipitated with rabbit anti-Myc antibody, fractionated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting with mouse anti-Myc antibody.

GATA-1. Induction of *p27* and *p18* mRNAs by GATA-1 was inhibited by enforced Myc (Fig. 7C). Failure to induce *p27* is consistent with the observation that Myc inhibits *p27* transcription by binding to the initiator element in its promoter (103). How Myc regulates *p18* expression is not known. Overall, our findings are consistent with a regulatory hierarchy in which GATA-1-mediated repression of Myc permits induction of *p18* and *p27*.

The effects of Myc on GATA-1-induced changes in cyclin D2 expression were more complex (Fig. 7D). The basal level of cyclin D2 mRNA was approximately two- to threefold elevated before addition of estradiol, possibly due to leakiness of Myc-ER as noted above. At 12 h after addition of β-estradiol, there was a rise in *cyclin D2* gene expression in G1E-ER4 cells

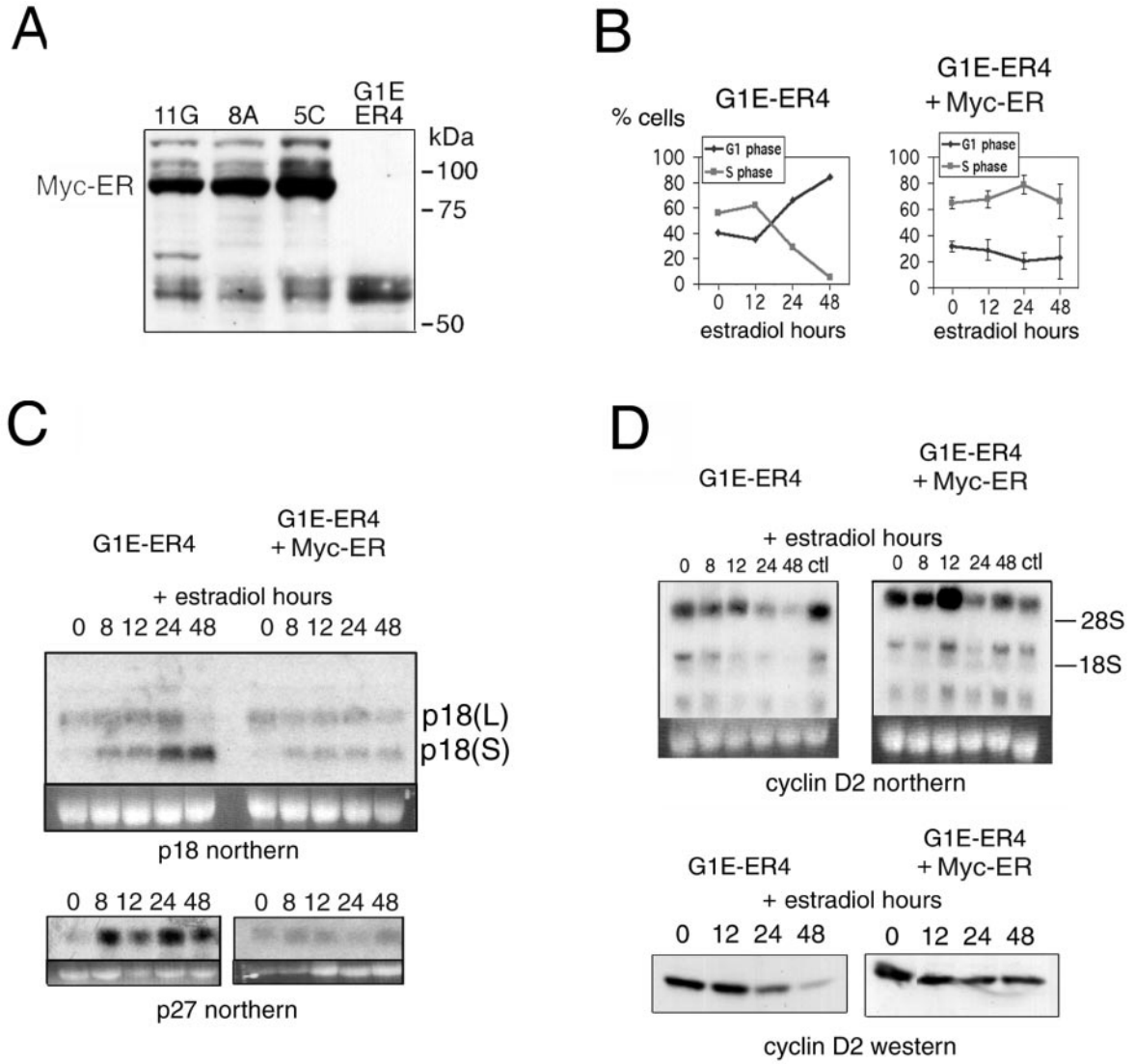

FIG. 7. Enforced Myc blocks GATA-1-induced cell cycle withdrawal. (A) Western blot for Myc protein in whole-cell lysates of G1E-ER4 cells and three separate clones expressing Myc-ER; 15 μg of protein/lane. (B) Cell cycle status by flow cytometry after GATA-1 activation in G1E-ER4 cells and three separate clones of G1E-ER4 cells expressing Myc-ER. (C) Effects of enforced Myc-ER expression on *p18* and *p27* mRNA levels in a single clone of G1E cells expressing Myc-ER (clone 8A); similar data were obtained with two additional clones (not shown). Northern blot with 20 μg of total RNA/lane is shown. (D) Effect of enforced Myc expression on cyclin D2 mRNA and protein levels. A Northern blot from clone 8a, 20 μg of total RNA/lane (left panel), and a Western blot, 50 μg of total protein/lane (right panel) are shown. Similar data were obtained using two additional clones (not shown). ctl, 20 ng of RNA from uninduced G1E-ER4 cells.

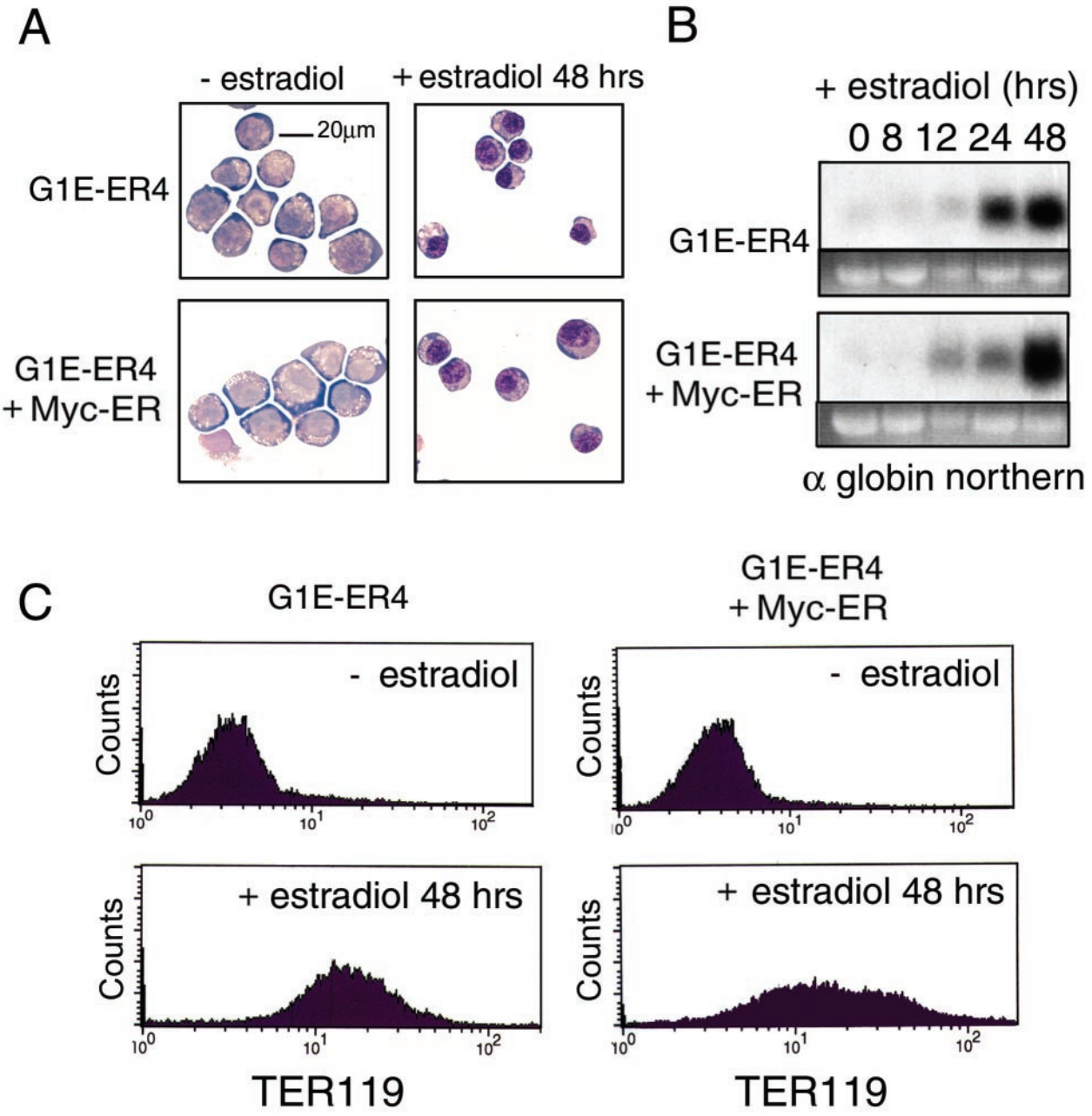

FIG. 8. Enforced Myc-ER expression does not block GATA-1-induced erythroid maturation in G1E-ER4 cells. Similar results were obtained with two other clones (data not shown). (A) May-Gruwald Giemsa staining of cytocentrifuge preparations; the scale is maintained in all panels. (B) Northern blot for α-globin expression. Ethidium bromide-stained 28S rRNA is shown in the bottom panels. Twenty micrograms of total RNA/lane was used. (C) Flow cytometry showing expression of the erythroid differentiation marker, TER119.

expressing Myc-ER compared to controls, consistent with previous findings that Myc activates *cyclin D2* transcription directly. *cyclin D2* mRNA expression diminished at 24 and 48 h in the Myc-ER-expressing cells, similar to the trend in control cells. However, at corresponding time points, *cyclin D2* levels were always higher in cells expressing Myc-ER than in controls (compare left and right panels in Fig. 7D). Thus, GATA-1 and Myc appear to have opposing effects on *cyclin D2* expression. The net effect of enforced Myc was to attenuate the GATA-1-mediated decline in cyclin D2 protein levels at later time points (Fig. 7D, far right panel).

Surprisingly, while enforced Myc expression blocked GATA-1-mediated cell cycle withdrawal in G1E-ER4 cells, the ability of GATA-1 to orchestrate other aspects of erythroid maturation was relatively spared. Upon treatment with β-estradiol for 48 h, Myc-expressing G1E-ER4 cells produced globin mRNAs and exhibited nuclear condensation, cytoplasmic hemoglobinization, and cell surface TER119 (Fig. 8). Morphological features of cellular maturation and induction of TER119 occurred homogeneously in Myc-ER-expressing cells (Fig. 8A and C), indicating that proliferation arrest became uncoupled from phenotypic differentiation in individual cells. Of note,

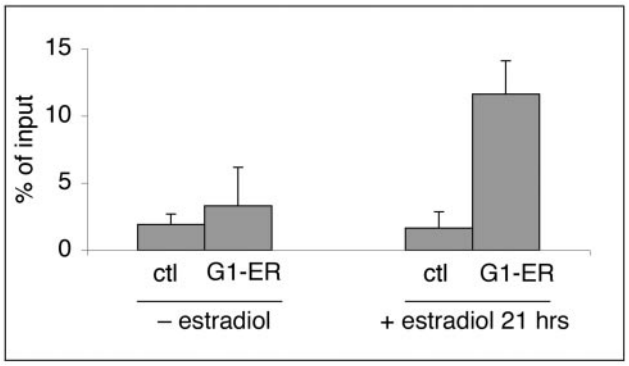

FIG. 9. GATA-1 associates with the *Myc* gene in vivo. ChIP assay using antibodies against GATA-1–ER (G1-ER) or isotype-matched control antibodies (ctl) to immunoprecipitate chromatin from G1E-ER4 cells left untreated or treated with estradiol for 21 h. The average results of three separate experiments are shown.

Myc-ER cells were slightly larger and exhibited a broader TER 119 profile, indicating that enforced Myc affected the maturation program to a minor extent (Fig. 8A and C). At 72 h following estradiol treatment, a fraction of cells in all clones died (not shown). Remarkably, cells in clone #8A recovered rapidly and continued both to proliferate and to exhibit signs of terminal maturation. Removal of estradiol had little effect on cell proliferation but reversed the differentiation phenotype. Together, these findings indicate that Myc repression is required for erythroid GATA-1 actions related to cell cycle arrest. In contrast, functions of GATA-1 related to acquisition of the mature erythroid phenotype are largely Myc independent.

GATA-1 occupies the *Myc* promoter during erythroid maturation. To investigate whether GATA-1 interacts directly with the *Myc* gene in vivo, we performed ChIP experiments. In three independent experiments, chromatin prepared from estradiol-stimulated G1E-ER4 cells immunoprecipitated with antibodies against GATA-1–ER was enriched for *Myc* promoter sequences by two to threefold compared to levels for negative controls (Fig. 9). Together with the rapid loss of *Myc* transcripts during induced G1E-ER4 cell maturation (Fig. 6), these results support a model in which GATA-1 inhibits *Myc* transcription by direct association with its promoter.

DISCUSSION

GATA-1 is a lineage-instructive transcription factor that regulates both differentiation and proliferation arrest. Cell cycle-inhibitory effects of GATA-1 during terminal erythropoiesis were implicated by gene targeting studies but have been difficult to elucidate for two reasons. First, primary GATA-1-null erythroid precursors undergo apoptosis, which precludes their isolation for biochemical studies and masks potential cell cycle defects (100). Second, the phenotypic effects of enforced GATA-1 expression are gene dosage- and context-dependent. G1E cells are an immortalized GATA-1$^-$ erythroblast line; they no longer undergo apoptosis but retain their ability to terminally differentiate in response to physiologic levels of GATA-1. This system allowed us to examine GATA-1 actions in an erythroid environment without confounding apoptosis. GATA-1 induces known erythroid target genes in G1E cells, validating this experimental system and indicating that the transcriptional network controlled by GATA-1 reflects physiologic events occurring during normal erythropoiesis. Moreover, the changes in gene expression that occur during GATA-1-induced G1E cell maturation are similar to those observed as GATA-1 accumulates during terminal maturation of cultured primary fetal liver erythroblasts (14). Here we demonstrate that GATA-1 induces G_1 arrest during erythroid maturation.

As observed with other differentiation-promoting nuclear proteins, GATA-1 could block cell proliferation through transcriptional effects or through direct physical interactions with core cell cycle components. For example, non-DNA-binding versions of MyoD and c/EBPα block cell cycle progression by inhibiting Cdks directly (96, 105). MyoD also activates p21 transcription (35). GATA-1 was reported to bind pRb, although it is not known whether this interaction is critical for inducing cell cycle arrest (102). Of note, an intact DNA binding domain was required for GATA-1-induced cell cycle arrest, and we were unable to detect direct interactions between GATA-1 and G_1 Cdks in IP-Western blot experiments. While our data do not exclude the possibility that GATA-1 inhibits cell cycle components directly, they implicate a requirement for its transcriptional activity. Through DNA microarray studies, we identified an extensive GATA-1-regulated network of gene activation and repression related to cell cycle control.

The kinetics of GATA-1-induced changes in gene expression in G1E-ER4 cells delineate a regulatory hierarchy through which proliferation arrest is initiated and maintained during erythroid maturation. Cell cycle regulators whose expression was significantly altered at early time points are more likely to be controlled directly by GATA-1 (Table 1). The growth inhibitors *Btg2*, *Hipk2*, *JunB*, and *Crep* were rapidly induced and therefore represent potential new targets for GATA-1-mediated transcriptional activation. In addition, several genes with mitogenic properties, including *Myc*, *Nab2*, and *Myb*, were rapidly downregulated, indicating that they could be directly repressed by GATA-1. Thus, it is likely that GATA-1 triggers cell cycle arrest by simultaneously and directly activating growth inhibitor genes and repressing mitogenic ones. Here we identified several candidate effector genes from both classes.

Alterations in expression of core cell cycle proteins, including repression of cyclin D2 and Cdk 6, and induction of the CKIs p18 and p27 occur at relatively late time points and are therefore likely to be indirectly regulated by GATA-1. Nonetheless, our findings link these events to GATA-1 activity and are consistent with prior studies on the mechanisms of erythroid cell cycle arrest. For example, upregulation of p27 associated with loss of Cdk2 activity is observed consistently during erythroid maturation (14, 40, 55, 83). Gene targeting in mice demonstrated that loss of *p27* alone or in combination with loss of *p18* causes accumulation of erythroid precursors in the bone marrow and spleen, although terminal maturation did not seem to be impaired (21, 24). Together, these data support our observations that p18 and p27 limit proliferation of erythroid precursors. However, additional independent mechanisms must exist to ensure cell cycle withdrawal during terminal erythropoiesis in vivo.

Repression of *Myc* appears to be critical for induction of CKIs and subsequent G_1 arrest mediated by GATA-1 in G1E-ER4 cells. *Myc* regulates cell cycle progression at multiple

levels and appears to be a nodal point for integrating tissue-specific signals involved in differentiation. Our ChIP experiments support a model in which GATA-1 inhibits *Myc* transcription by direct interaction with the gene. Several mechanisms for repression are possible within this context. For example, GATA-1 could inhibit transcription by associating with a GATA element in the *Myc* promoter, similar to the mechanism for *Myc* repression by the B-cell transcription factor Blimp-1 (53). In this regard, GATA-1 consensus motifs are present in the murine *Myc*-proximal promoter (data not shown). Alternatively, GATA-1 could block *Myc* expression by binding and inhibiting a promoter-associated transcriptional activator, similar to the mechanism proposed for C/EBPα-mediated *Myc* repression during myeloid development (45). Additionally, indirect mechanisms might contribute to shutting off Myc. For example, downregulation of Myb, which activates the *Myc* promoter, could contribute to loss of Myc expression in erythroid cells (7).

GATA-1 is traditionally viewed as an activator of transcription; relatively little evidence exists to support a direct role in gene repression. GATA-1 is believed to inhibit human epsilon globin transcription by binding to its proximal promoter (52). Moreover, the GATA-1-associated cofactor FOG-1 can repress GATA-1 activity within specific cell and promoter contexts (22, 23) and is required for GATA-1 repression of selected genes during erythroid maturation (10). Our transcriptome analysis points to a broad role for GATA-1 in gene repression. At early time points, the number of genes upregulated is similar to the number downregulated (J. J. Welch and M. J. Weiss, unpublished data). Many of the genes inhibited at early time points encode mitogenic proteins (Table 1), indicating that transcriptional repressor functions of GATA-1 may be especially important for cell cycle withdrawal during hematopoietic differentiation.

In G1E cells, enforced Myc expression blocks GATA-1-induced proliferation arrest but spares many aspects of cellular maturation, consistent with findings that Myc does not always interfere directly with the expression of differentiation-related genes (27). These findings distinguish the GATA-1-regulated genetic programs controlling erythroid maturation and cell cycle arrest in that the latter is selectively blocked by enforced expression of Myc. The effects of Myc in G1E cells differ from those of the proto-oncogene PU.1, which blocks both erythroid maturation and cell cycle arrest by GATA-1 (106). Consistent with these observations, PU.1 inhibits GATA-1 function through physical interaction (73). In comparison, the present findings indicate that Myc does not interfere with transcriptional activation by GATA-1 directly, highlighting potential differences in the mechanisms of malignant transformation by Myc and PU.1.

Despite apparent functional antagonism detected in our studies, GATA-1 and Myc probably cooperate at specific stages of erythropoiesis. During terminal maturation, primary erythroblasts undergo four to five specialized divisions characterized by induction of erythroid markers, shortened G_1 phase, and reduction of cell volume (13, 14). Presumably, both GATA-1 and *Myc* are required during this process, and these cell divisions may be a prerequisite for early steps of differentiation. How GATA-1 ultimately becomes competent to extinguish *Myc* and arrest cell division during terminal erythropoiesis is not clear. One possibility is that *Myc* becomes repressed when GATA-1 levels reach a critical threshold. This could explain why G1E cells divide only once or twice during their terminal maturation, which is triggered by a burst of high-level GATA-1 activity, rather than a more gradual accumulation as occurs in primary cells.

In summary, our studies define one regulatory hierarchy through which GATA-1 inhibits the cell cycle to cause G_1 arrest during terminal maturation. Repression of *Myc* appears to be an early and essential event for GATA-1-mediated cell cycle arrest, but it is largely dispensable for other aspects of G1E cell maturation. Future investigations into the mechanisms of *Myc* repression and the functions of additional growth regulators identified to be induced or repressed by GATA-1 in the present study should provide further insight into how this essential multifaceted nuclear protein participates in normal hematopoiesis and leukemogenesis.

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M.B.R. and J.J.W. contributed equally to this work.

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