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Differential GATA Factor Stabilities: Implications for Chromatin Occupancy by Structurally Similar Transcription Factors†

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ABSTRACT: Whereas the transcription factors GATA-1 and GATA-2 function both uniquely and redundantly to control blood cell development, the process termed hematopoiesis, mechanisms underlying their unique versus common functions are poorly understood. We used two independent assays to demonstrate that GATA-1 is considerably more stable than GATA-2 in multiple cellular contexts, even though both factors are subject to degradation via the ubiquitin−proteasome system. Studies with GATA factor mutants and novel chimeric GATA factors provided evidence that both GATA-1 and GATA-2 have highly unstable zinc finger core modules. The GATA-1 and GATA-2 N-termini both confer stabilization to their respective zinc finger core modules. In contrast, the GATA-1 and GATA-2 C-termini confer stabilization and destabilization, respectively. As GATA-2 stabilization via proteasome inhibition impairs the capacity of GATA-1 to displace GATA-2 from endogenous chromatin sites, we propose that differential GATA factor stability is an important determinant of chromatin target site occupancy and therefore the establishment of genetic networks that control hematopoiesis.

GATA-1 and GATA-2, members of the GATA transcription factor family, are crucial regulators of hematopoiesis (*1*, *2*). Despite having a nearly identical dual zinc finger DNA binding domain, these factors function uniquely to control distinct aspects of hematopoiesis (*3*). GATA-2 is expressed in hematopoietic stem cells and certain hematopoietic progenitors and uniquely promotes the proliferation, survival, and function of these cells (*4*−*7*). During erythropoiesis, GATA-1 and GATA-2 are expressed reciprocally, with GATA-1 levels rising as GATA-2 levels decline (*8*). GATA-1 uniquely stimulates erythropoiesis and the expression of genes essential for red blood cell function (*8*−*13*). In contrast to these unique activities, GATA-1 and GATA-2 can function redundantly to regulate the generation and/or survival of primitive or embryonic erythroblasts (*14*).

Efforts to determine whether GATA-1 and GATA-2 can substitute for each other in different systems have yielded variable results. Expression of GATA-2 from *Gata1* regulatory elements rescues lethality of a *Gata1*-null mutation in mice, but the embryos have reduced red blood cell counts, hemoglobin content, hematocrit level, and platelet numbers (*15*). Similarly, expression of GATA-2 from *Gata1* regulatory elements rescues lethality of a *Gata1* mutation, in which only 5% of the wild-type level of GATA-1 is expressed (*16*). However, again, the mice are abnormal, developing anemia and thrombocytopenia. In contrast to this apparent partial

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functional overlap, targeted disruption of *Gata1* results in upregulation of GATA-2, but erythropoiesis is blocked and canonical GATA-1 target genes are not activated (*8*). Whereas the biological actions attributed to GATA-1 and GATA-2 are well established, many questions remain unanswered regarding mechanisms underlying their overlapping and distinct functions.

Much of the regulatory potential of GATA factors resides within their conserved dual zinc finger domain, which mediates essential protein−DNA and protein−protein interactions. In both GATA-1 and GATA-2, the N-terminal zinc finger mediates binding to the coregulator Friend of GATA-1 (FOG-1)[1] (*17*−*20*), whereas the C-terminal finger binds DNA motifs with sequence specificity (*21*, *22*). On the basis of naked DNA binding studies, both GATA-1 and GATA-2 bind to WGATAR motifs, in which W represents A or T and R represents A or G (*23*, *24*). The N-terminal zinc finger can also stabilize DNA binding at certain palindromic motifs (*25*). Furthermore, the zinc fingers, either individually or collectively, mediate additional protein−protein interactions with the TRAP220 subunit of the mediator complex (*26*, *27*), the histone/protein acetyltransferases CBP/p300 (*28*, *29*), and the transcription factors Sp1 (*30*), EKLF (*30*), and PU.1 (*31*−*35*).

Important functional insights have emerged from studies of mechanisms underlying the reciprocal expression of GATA-1 and GATA-2 during erythropoiesis. This reciprocal expression is also apparent upon genetic complementation in GATA-1-null G1E cells (*36*, *37*). G1E cells were derived from GATA-1-targeted mouse ES cells, express endogenous GATA-2, and have a proerythroblast-like phenotype (*12*, *36*). G1E cell lines have been generated in which GATA-1 is stably expressed as an estrogen receptor ligand binding domain fusion protein (ER-GATA-1) (*38*, *39*), which allows for titration of GATA-1 activity with increasing concentrations of either tamoxifen or estradiol (*39*−*42*).

Analyses of the specificity of GATA factor chromatin occupancy in G1E cells and in additional systems provided evidence that GATA-1 and GATA-2 occupy only a small subset of the GATA motifs in chromatin (*37*, *40*, *42*−*47*). While GATA-1 and GATA-2 share most of the occupied regions, GATA-2 preferentially occupies the −1.8 kb region of the *Gata2* locus (*40*, *42*), indicating that differential occupancy can also occur. As GATA-1 levels rise, GATA-1 replaces GATA-2 at chromatin target sites (*37*, *40*, *42*, *44*, *48*, *49*). Such "GATA switches" have also been detected with endogenous GATA-1 and GATA-2 upon differentiation of mouse ES cells into hematopoietic precursors (*50*) and upon expression of FOG-1 in a FOG-1-null hematopoietic precursor cell line (*48*). Although FOG-1 facilitates GATA switches (*48*) and GATA-1 chromatin occupancy (*48*, *51*) at certain sites, other biochemical parameters governing GATA switches are not established. The absolute concentrations of GATA-1 and GATA-2 are important determinants, and other factors and mechanisms that modulate GATA factor DNA binding activity in the context of chromatin are also likely to be important. As residues near the DNA binding domain of GATA-1 are acetylated, and these residues are required for chromatin occupancy (*52*), GATA-1 acetylation might be an important determinant of GATA switches.

The GATA switch model assumes that GATA-1 and GATA-2 have certain intrinsic biochemical differences that translate into unique transcriptional outputs (*2*, *3*). Herein, we demonstrate that GATA-1 is considerably less susceptible to proteolysis than GATA-2 in cells expressing the factors individually, and in cells expressing both GATA-1 and GATA-2. Proteasome inhibition counteracted the GATA-2 destabilization mechanism. Despite not being recognized as an important GATA factor regulatory sequence, the divergent C-termini of GATA-1 and GATA-2 are important determinants of the differential GATA-1 and GATA-2 stabilities. These results suggest that as GATA-1 levels rise during erythropoiesis, the relatively high stability of GATA-1 allows it to efficiently replace the less stable GATA-2 at chromatin sites, including the *Gata2* locus itself, thereby altering the gene expression profile of erythroid precursors as an essential step in erythropoiesis.

EXPERIMENTAL PROCEDURES

Cell Culture. All cultures were grown in a humidified incubator at 37 °C with 5% carbon dioxide. Murine erythroleukemia (MEL) cells (*53*) were grown in DMEM (Cellgro) supplemented with 1% penicillin−streptomycin (Invitrogen), 5% FBS (Gibco), and 5% bovine serum (Gibco). GATA-1-null G1E cells were maintained in IMDM (Gibco) supplemented with 2% penicillin−streptomycin (Gibco), 2 units/mL erythropoietin, 120 nM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit-ligand-producing Chinese hamster ovary cell line, and 15% FBS (Gibco). G1E cells (*36*) stably expressing ER-GATA-1 (G1E-ER-GATA-1) were maintained as were G1E cells, with the addition of 1 μg/mL puromycin (Sigma). FOG-1-null cells (*45*, *48*, *54*) were grown in IMDM (Gibco) supplemented with 2% penicillin−streptomycin (Gibco), 10 ng/mL interleukin 3 (R & D Systems), and 15% FBS (Gibco). Human K562 erythroleukemia cells (*55*) were grown in IMEM (Cellgro) supplemented with 1% antibiotic−antimycotic (Gibco) and 10% FBS (Gibco).

Antibodies. Rabbit polyclonal antibodies against murine GATA-1 and GATA-2 were generated against amino acids 1−89 and 120−235, respectively (*43*). Goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz) was used for Western blot detection of GATA-1 and GATA-2. Mouse monoclonal anti-HA (HA.11, Covance) was used for immunoprecipitation of HA-tagged proteins. Goat anti-mouse IgG-HRP (sc-2005, Santa Cruz) was used for Western blot detection of the HA epitope.

Plasmid Construction. All coding sequences were inserted into the multiple cloning site of pcDNA3.1 (Invitrogen) between the KpnI and XbaI sites. HA−GATA-1 was a gift of John Crispino (Northwestern Univ.), and HA−GATA-2 was generated by PCR from GATA-2 cDNA in pBluescript, a gift from Doug Engel (Univ. of Michigan). The HA tag of each construct (GCCTATCCTTATGACGTGCCTGACTAT) was placed immediately behind the GCCGCCATG

[1] Abbreviations: CBP, CREB binding protein; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FBS, fetal bovine serum; FOG-1, Friend of GATA-1; G1E, GATA-1-null cell line; HA, hemagglutinin; IMDM, Iscove's Modified Dulbecco's Medium; IMEM, Iscove's Modified Eagle's Medium; MEL, mouse erythroleukemia; PI, preimmune; RT-PCR, reverse transcriptase polymerase chain reaction.

Kozak sequence. The GATA-1 and GATA-2 sequence deletion constructs, as well as the chimeras, were generated by PCR from HA−GATA-1 and HA−GATA-2 with the sequence endpoints described in the text.

Protein Analysis. Where applicable, MEL cells were differentiated with 1.5% DMSO (Sigma) for 4 days prior to analysis, and treatment with cycloheximide (Sigma) was at 0.2 mg/mL. MG132 pretreatment at 50 μM (Calbiochem) was 10 min before adding cycloheximide. Extracts were prepared by boiling cells for 10 min in SDS sample buffer [25 mM Tris (pH 6.8), 2% β-mercaptoethanol, 3% SDS, 0.1% bromophenol blue, 5% glycerol] at 1×10^7 cells/mL. For Western blotting, extracts from 1 to 2×10^5 cells were resolved by SDS-PAGE on 10% gels and transferred to Immobilon-P membranes (Millipore). Detection was with ECL-Plus (Amersham) or PicoWest (Pierce).

Immunohistochemistry. K562 cells were transfected with DMRIE-C (Invitrogen) (45 μg of plasmid and 135 μL of DMRIE-C per 1×10^7 cells) to express HA−GATA-1 and HA−GATA-2. Transfected cells were attached to microscope slides by cytospin at 1000*g*, fixed with 3.7% paraformaldehyde in PBS for 30 min, and blocked with 10% fish serum (Sigma) in PBS containing 0.1% Tween 20 (PBST) at 37 °C. Cells were stained with HA antibody in PBST containing 1% fish serum at 4 °C, washed in PBST, and incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) in PBST containing 1% fish serum at 37 °C. Cells were mounted with Vectashield Mounting Medium for Fluorescence with DAPI (Vector Labs) and analyzed with the α-plan-FLUAR 100× NA 1.45 oil immersion objective of an Axiovert 200M microscope (Zeiss). Images were captured with an AxioCam HR camera (Zeiss) and deconvoluted with Axiovision 4.5.

Pulse-Chase Assay. Pulse-chase analysis was conducted with endogenous GATA-1 and GATA-2 in FOG-1-null cells or HA-tagged GATA-1 and GATA-2 transiently expressed in K562 cells. K562 cells were transfected with DMRIE-C (Invitrogen) at a ratio of 45 μg of plasmid and 135 μL of DMRIE-C per 1×10^7 cells. Cells were washed in DMEM lacking methionine, cysteine, and glutamine (DMEM−, Cellgro) and transferred at 1×10^7 cells/mL to DMEM− supplemented with L-glutamine (Cellgro), nonessential amino acids (Cellgro), and FBS dialyzed to remove amino acids. Pro-mix ^{35}S *in vitro* cell labeling mix (Amersham), containing ^{35}S-methionine and ^{35}S-cysteine, was added at 1.0 mCi per 1×10^7 cells, and biosynthetic labeling was allowed to proceed for 3 h at 37 °C (5% CO_2). Labeled cells were washed with culture medium and transferred to medium supplemented with L-methionine and L-cysteine to 150 mg/L each for chasing of the labeled proteins. As appropriate, cells were treated with 50 μM MG132 (Calbiochem). Cells were returned to 37 °C (5% CO_2), removed at the indicated times, washed with PBS, and pelleted. Cells were lysed in RIPA [50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM DTT] at 2×10^7 cells/mL and centrifuged at 100 000*g* for 1 h at 4 °C. Lysates (400 μg, as determined by Bradford assay) were precleared overnight with rabbit preimmune serum (Covance) and Protein A-Sepharose (Sigma) (for endogenous GATA-1 and GATA-2) or murine IgG (Sigma) and Protein G-Sepharose (Sigma) (for HA-tagged proteins). Endogenous GATA factors were immunoprecipitated with GATA-1 and GATA-2 antibodies and Protein A-Sepharose; HA-tagged proteins were immunoprecipitated with the HA antibody and Protein G-Sepharose. Immunoprecipitates were washed with RIPA, and proteins were eluted by boiling for 10 min in SDS sample buffer [50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol] and analyzed by SDS-PAGE. Gels were enhanced with 2,5-diphenyloxazole (Sigma) and dried, and labeled proteins were detected with a Storm 840 phosphorimager (Molecular Dynamics). Densitometric quantitation was performed with ImageQuant (Molecular Dynamics). Exposure times ranged from <1 week for wild-type proteins to ~3 weeks for certain mutants.

Quantitative Chromatin Immunoprecipitation Assay. G1E-ER-GATA-1 cells were treated with 50 μM MG132 (Calbiochem) for 3.5 h and/or 1 μM 4-hydroxytamoxifen (Sigma) or β-estradiol (Steraloids) for 3 h prior to ChIP analysis. Protein−DNA cross-linking was achieved with 0.4% formaldehyde, and ChIP was conducted as described (*56*). Identical results were obtained with 1% formaldehyde. Immunoprecipitated DNA fragments were quantitated by real-time PCR.

Primers for Quantitative ChIP Assay. Primers were as follows:

Gata2 (−3.9 kb), GAGATGAGCTAATCCCGCTGTA (forward) and AAGGCTGTATTTTTCCAGGCC (reverse);

Gata2 (−2.8 kb), GCCCTGTACAACCCCATTCTC (forward) and TTGTTCCCGGCGAAGATAAT (reverse); *Gata2* (−1.8 kb), GCATGGCCCTGGTAATAGCA (forward) and CAGCCGCACCTTCCCTAA (reverse); *Gata1* HS1, ATGAAGGGTGCCTCTAAGGACA (forward) and TCCCTTATCTATGCCTTCCCAG (reverse); α-globin HS-26, AGCAGACCACTGTGGGATCTATG (forward) and TGGCCTCTAGAAACTCTGAGTCATG (reverse); necdin promoter, GGTCCTGCTCTGATCCGAAG (forward) and GGGTCGCTCAGGTCCTTACTT (reverse).

Quantitative RT-PCR. cDNA was synthesized from total RNA extracted from G1E-ER-GATA-1 cells with Trizol (Invitrogen). RNA (1 μg) was annealed with 250 ng of a 1:4 mixture of random (Promega) and oligo dT (Promega) primers at 68 °C for 10 min. First-strand synthesis was with 50 units of M-MLV reverse transcriptase (Invitrogen) and 0.5 mM dNTPs in First Strand Buffer (Invitrogen) containing 10 mM DTT and 20 units of RNasin (Promega) at 42 °C for 1 h. The cDNA was diluted to 150 μL and inactivated at 97 °C for 5 min. Each RT-PCR reaction (15 μL) contained 2.0 μL of cDNA, 7.5 μL of SYBR Green PCR Master Mix (Applied Biosystems), and the appropriate primers. Measured cDNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Primers for Quantitative RT-PCR. Primers were as follows:

mouse GATA-2, GCAGAGAAGCAAGGCTCGC (forward) and CAGTTGACACACTCCCGGC (reverse); mouse GAPDH, TGCCCCCATGTTTGTGATG (forward) and TGTGGTCATGAGCCCTTCC (reverse).

RESULTS AND DISCUSSION

Differential Stabilities of Endogenous GATA-1 and GATA-2. The FOG-1 activity to facilitate GATA switches (*48*), in which GATA-1 replaces GATA-2 at chromatin sites (*37*),

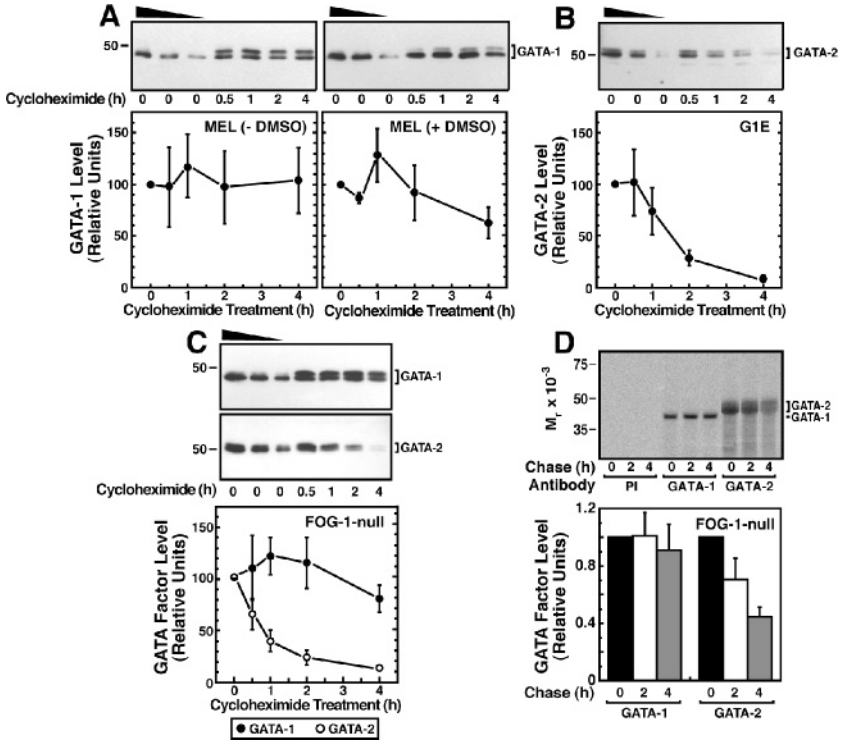

FIGURE 1: GATA-1 is more stable than GATA-2, independent of cellular environment and FOG-1 binding. (A) The stabilities of endogenous GATA-1 in undifferentiated and DMSO-induced (4 days) MEL cells were determined by treating cells with cycloheximide and analyzing GATA-1 protein levels in whole cell samples by semiquantitative Western blotting. Titrations of samples from cells not treated with cycloheximide indicate a linear relationship between signal intensity and protein concentration. Western blots were analyzed by densitometry, and the quantitative results are shown (mean ± SE, three independent experiments). (B) The stability of endogenous GATA-2 in G1E cells was determined by treating cells with cycloheximide and analyzing GATA-2 levels by semiquantitative Western blotting and densitometric quantitation (mean ± SE, three independent experiments). (C) The stabilities of endogenous GATA-1 and GATA-2 in FOG-1-null cells were determined by treating cells with cycloheximide and analyzing GATA factor levels by semiquantitative Western blotting and densitometric quantitation (mean ± SE, four independent experiments). (D) The stabilities of endogenous GATA-1 and GATA-2 in FOG-1-null cells were determined by biosynthetic labeling with ^{35}S-methionine and ^{35}S-cysteine, pulse-chase analysis, and immuoprecipitation of the labeled GATA factors. GATA factor levels were quantitated by phosphorimager analysis (mean ± SE, four independent experiments). PI, preimmune.

indicates that relative GATA-1 and GATA-2 concentrations are not the sole determinants of their chromatin target site occupancy. On the basis of the low sequence homology of the N- and C-termini of GATA-1 and GATA-2 (*3*), we reasoned that unique sequences within these regions might underlie intrinsic biochemical differences that influence target site occupancy. Such differences might include unique protein−protein interactions, distinct regulation via post-translational modifications, or differential susceptibility to regulated proteolysis.

To investigate the potential relevance of proteolytic mechanisms to GATA factor-specific functions, we measured the relative stabilities of GATA-1 and GATA-2 in undifferentiated (Figure 1A, left) and differentiated (DMSO-induced) (Figure 1A, right) MEL cells that only express endogenous GATA-1, in G1E erythroid precursor cells that only express endogenous GATA-2 (Figure 1B), and in a FOG-1-null multipotential hematopoietic cell line that expresses both endogenous GATA-1 and GATA-2 (Figure 1C). Cells were treated with cycloheximide to block protein synthesis, and GATA-1 and GATA-2 protein levels were quantitated by Western blotting at various times post-cycloheximide treatment. The apparent GATA-1 half-life exceeded the 4 h treatment, regardless of whether or not the MEL cells were induced to differentiate with DMSO (Figure 1A). Cycloheximide treatments could not be conducted for considerably longer times due to cytotoxicity (data not shown). Although cycloheximide treatment revealed a distinct GATA-1 isoform with slower mobility, both isoforms were similarly stable. In contrast, a cycloheximide treatment of 1 to 2 h sufficed to reduce the GATA-2 level by 50%, and only a small fraction of GATA-2 persisted at 4 h (Figure 1B). Similarly, in FOG-1-null cells, GATA-1 had a half-life of >4 h, whereas that of GATA-2 was ~1 h (Figure 1C). Titrations of protein extracts isolated from the various cell types (0 h cycloheximide condition) verified that Western blot signals increased proportionally to the protein level.

The results with FOG-1-null cells (Figure 1C) indicate that the differential GATA-1 and GATA-2 stabilities do not merely reflect the distinct cellular environments of MEL and G1E cells. Rather, the different stabilities likely represent an intrinsic biochemical difference between GATA-1 and GATA-2. On the basis of the absence of FOG-1 in the FOG-1-null cells, the differential stability cannot be explained by quantitative or qualitative differences in FOG-1 binding.

To confirm the cycloheximide results, a pulse-chase assay was established to measure the half-lives of endogenous GATA-1 and GATA-2 in FOG-1-null cells cultured with ^{35}S-methionine and ^{35}S-cysteine. Neither the half-lives of endogenous GATA-1 and GATA-2, nor the use of pulse-chase analysis to measure these factors, has been described previously. Although the assay requires a considerable amount of labeled cells to yield sufficient extract for a single immunoprecipitation (see Materials and Methods), both

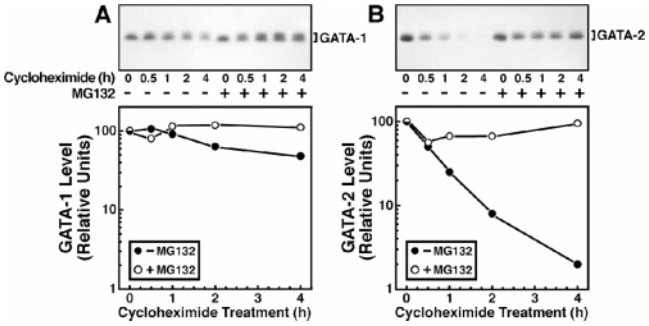

FIGURE 2: Endogenous GATA-1 and GATA-2 are both stabilized by MG132, an inhibitor of the ubiquitin−proteasome system. (A) FOG-1-null cells were pretreated with vehicle or MG132 for 10 min and then incubated with cycloheximide for the indicated times. GATA-1 levels were analyzed by semiquantitative Western blotting and densitometric analysis (mean ± SE, three independent experiments). (B) GATA-2 stability in FOG-1-null cells was quantitated as described in panel A (mean ± SE, three independent experiments).

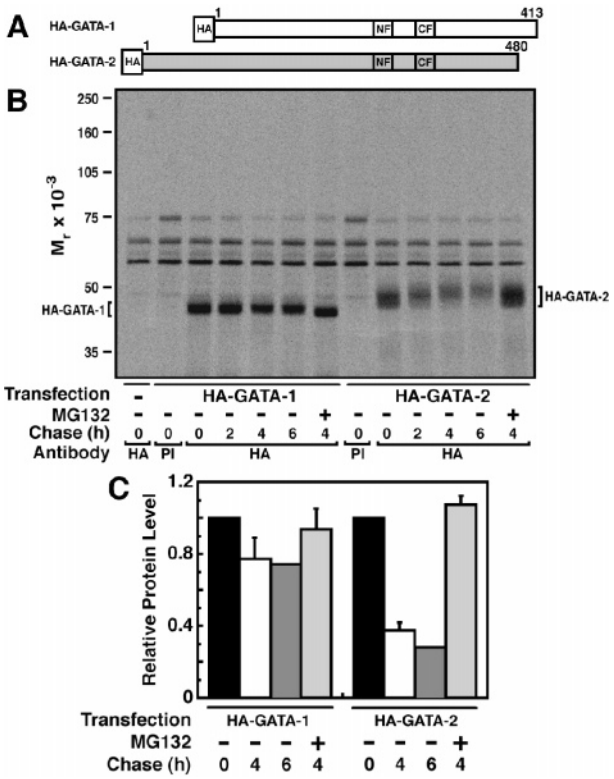

FIGURE 3: Transiently expressed HA−GATA-1 and HA−GATA-2 recapitulate the differential stabilities of endogenous GATA-1 and GATA-2. (A) HA−GATA-1 and HA−GATA-2 proteins. (B) K562 cells were transiently transfected with expression vectors encoding HA−GATA-1 and HA−GATA-2. The stabilities of HA−GATA-1 and HA−GATA-2 were determined by biosynthetic labeling with ^{35}S-methionine and ^{35}S-cysteine, and pulse-chase analysis with and without MG132. Labeled GATA factors were immunoprecipitated and analyzed by SDS-PAGE. PI, preimmune. (C) GATA factor levels were quantitated by phosphorimager analysis (mean ± SE, five and eight independent experiments for HA−GATA-1 and HA−GATA-2, respectively).

GATA-1 and GATA-2 could be reproducibly detected with phosphorimager exposure times of one week or less. Analyses conducted in the FOG-1-null cells revealed a homogeneous band for GATA-1, which was completely unaffected by the 4 h chase (Figure 1D). Labeled GATA-2 was detected as a doublet, with the faster migrating band more intensely labeled (Figure 1D). The signal of the major high mobility band decreased as a function of the chase. Thus, the metabolic labeling confirmed the GATA-2 half-life is considerably less than that of GATA-1. The half-life of GATA-2 measured by metabolic labeling was greater than that measured with the cycloheximide protocol, which might reflect pleiotropic actions of cycloheximide or the broader linear range of the Phosphorimager-based quantitative analysis of the metabolic labeling data.

The longer half-life of GATA-1 vs GATA-2, based on quantitative analyses with two independent assays in multiple cell lines, might either reflect distinct degradation mechanisms or the differential utilization of a common mechanism. As regulated protein degradation via the ubiquitin−proteasome system is a major pathway of controlling protein levels in eukaryotic cells (*57*, *58*), and both GATA-1 and GATA-2 can be ubiquitinated (*59*, *60*), at least when exogenously expressed in cells, we used inhibitors of the ubiquitin−proteasome system to assess the importance of this system for establishing the characteristic half-lives of GATA-1 and GATA-2. FOG-1-null cells were treated with cycloheximide for up to 4 h, and each treatment was followed by the addition of either MG132, an inhibitor of the 26S proteasome, or vehicle after 10 min. Western blot analysis under semiquantitative conditions revealed that MG132 prevented the small decrease in GATA-1 (Figure 2A) and the almost complete GATA-2 loss (Figure 2B) 4 h post-cycloheximide treatment. Similar results were obtained with the additional 26S proteasome inhibitor *clasto*-lactacystin β-lactone (data not shown). The MG132- and *clasto*-lactacystin β-lactone-mediated stabilization of GATA-1 and GATA-2, the reports of exogenous GATA-1 (*60*) and GATA-2 (*59*) ubiquitination, and our confirmation that coexpression of GATA-1 or GATA-2 with HA−ubiquitin results in their ubiquitination in 293 cells (data not shown) indicate a role for the ubiquitin−proteasome system in mediating degradation of both GATA-1 and GATA-2. While Minegishi et al. described the low stability of GATA-2 in cycloheximide-treated P815 cancer cells, which was counteracted by proteasome inhibition, GATA-2 stability in erythroid cells was not analyzed, GATA-1 and GATA-2 stabilities were not compared within the same cell, and biosynthetic labeling was not used (*59*). Intriguingly, despite being degraded by a common proteolytic system, GATA-1 and GATA-2 have quite different stabilities, suggestive of intrinsic biochemical differences that dictate their susceptibilities to proteasome-mediated degradation.

If both GATA-1 and GATA-2 are degraded by at least one common proteolytic pathway, what accounts for the greater GATA-1 stability? To dissect the underlying mechanisms, we tested whether the differential stabilities can be recapitulated in a transient expression assay in human K562 erythroleukemia cells, which express endogenous GATA-1 (*61*) and low levels of endogenous GATA-2 (*62*). HA-tagged GATA-1 and GATA-2 (Figure 3A) were transiently expressed in K562 cells, and their half-lives were measured by pulse-chase analysis. This analysis revealed that HA−GATA-1 persisted during a chase of up to 6 h (Figure 3B,C), whereas HA−GATA-2 levels decreased considerably (Figure 3C), resembling the endogenous proteins in MEL, G1E, and FOG-1-null cells (Figure 1).

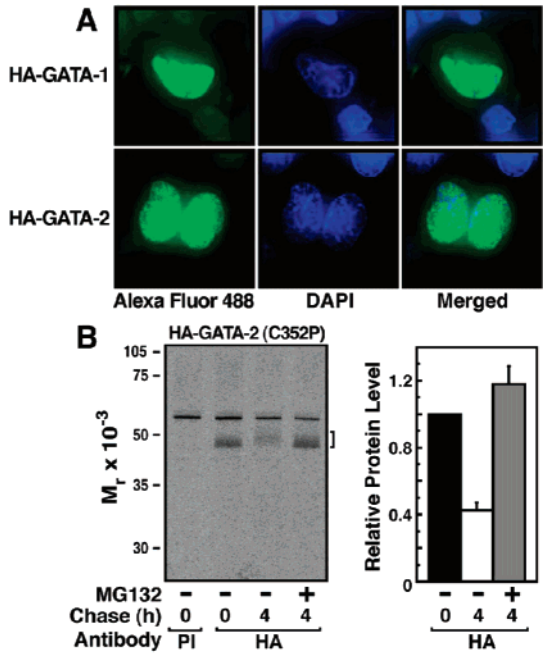

FIGURE 4: Differential stabilities of GATA-1 and GATA-2 are not attributable to distinct subcellular localizations, and the low stability of GATA-2 does not require a structurally intact C-terminal zinc finger. (A) Immunofluorescence analysis of HA−GATA-1 and HA−GATA-2 subcellular localization upon transient expression in K562 cells. The HA epitope was detected with anti-HA antibody and Alexa Fluor 488, while DNA was stained with DAPI. The results are representative of ~40 cells analyzed. (B) The stability of HA−GATA-2(C352P) transiently expressed in K562 cells was determined by biosynthetic labeling with ^{35}S-methionine and ^{35}S-cysteine, and pulse-chase analysis with and without MG132. Labeled HA−GATA-2(C352P) was immunoprecipitated, analyzed by SDS-PAGE, and quantitated by phosphorimager analysis (mean ± SE, three independent experiments). PI, preimmune. The graph on the right depicts the quantitative analysis.

Molecular Determinants of Differential GATA Factor Stabilities: Importance of C-Terminal Sequences. Whereas both GATA-1 and GATA-2 function in the nucleus to regulate target gene ensembles (*3*), differences in the subcellular localizations of newly synthesized GATA-1 and GATA-2 might be an important determinant of their distinct susceptibilities to proteasome-mediated degradation. FOG-1 has been reported to interact with the transforming acidic coiled-coil 3 protein (TACC3), thereby retaining FOG-1 in the cytoplasm (*63*, *64*), but whether this mechanism affects the subcellular localizations of GATA-1 and GATA-2 is unclear. Furthermore, in multipotent skin stem cells lacking GATA-3, FOG-1 exhibits a cytoplasmic localization (*65*). Nevertheless, FOG-1 is not a determinant of GATA-2 instability, based on the FOG-1-null cell results. To test whether GATA-1 and GATA-2 have distinct subcellular localizations, HA−GATA-1 and HA−GATA-2 were separately transfected into K562 cells, and the cells were immunostained with Alexa Fluor 488 to detect the HA epitope and DAPI to delineate the nucleus. Analysis of ~40 transfected cells revealed that both HA−GATA-1 and HA−GATA-2 consistently colocalized with DAPI (Figure 4A). The distinct GATA factor stabilities are therefore not attributable to gross differences in subcellular localization.

As certain transcription factors are proteolyzed at their cognate chromatin sites (*66*, *67*), we tested whether the relative instability of GATA-2 requires DNA binding activity. Though both GATA-1 and GATA-2 are nuclear factors degraded by the ubiquitin−proteasome system, the proteasome might preferentially degrade chromatin-bound GATA-2. We therefore investigated whether disabling GATA-2 DNA binding activity counteracts the destabilization mechanism. An HA-tagged GATA-2 mutant was generated containing a C352P point mutation, which was designed to destroy the structural integrity of the DNA-binding C-terminal zinc finger. As predicted from the structural perturbation, a GATA-1 mutant containing the homologous C261P mutation lacks DNA binding activity (*36*). Pulse-chase analysis revealed a nearly identical loss of HA−GATA-2(C352P) (Figure 4B) vs HA−GATA-2 (Figure 3B,C). Furthermore, MG132 prevented the decay of both HA−GATA-2(C352P) and HA−GATA-2. Thus, C352, an essential structural component of the C-terminal zinc finger that mediates DNA binding, is not required for low stability.

Given the irrelevance of C352 to GATA-2 instability and the high sequence homology of the zinc finger DNA binding domains of GATA-1 and GATA-2, we reasoned that the differential stability determinants might reside within the considerably less conserved N- and C-terminal sequences. We tested whether deleting the N- and C-terminal sequences of HA−GATA-2 abrogates the destabilization mechanism and whether the corresponding sequences of HA−GATA-1 are required for its characteristic high stability.

Deletion of the N-terminal 251 amino acids from HA−GATA-2 did not stabilize HA−GATA-2 but rather further destabilized HA−GATA-2 (compare Figure 5A, right, and Figure 3B). Similarly, removal of the corresponding 176 N-terminal amino acids of HA−GATA-1 destabilized HA−GATA-1 by approximately 50% (compare Figure 5A, left, with Figure 3B). Interestingly, MG132 negated the reduction in HA−GATA-2(ΔN) levels (Figure 5A, right), comparable to the activity of MG132 to stabilize HA−GATA-2 (Figure 3B). In contrast, MG132 only partially stabilized HA−GATA-1(ΔN) (Figure 5A, left).

Though the N-terminal deletions influenced HA−GATA-1 and HA−GATA-2 stabilities similarly, the C-terminal deletions differentially affected their stabilities. Deletion of the C-terminal 72 amino acids of HA−GATA-2 was stabilizing (compare Figure 5B, right, to Figure 3B). However, deletion of the corresponding 96 C-terminal amino acids of HA−GATA-1 was destabilizing (compare Figure 5B, left, to Figure 3B). Similar to the N-terminal deletions, MG132 maintained HA−GATA-2(ΔC) at its 0 h level after a 4 h chase, but MG132 did not fully prevent HA−GATA-1(ΔC) degradation. Removal of both N- and C-termini from either HA−GATA-1 or HA−GATA-2 similarly resulted in high instability of the resulting core DNA binding domain [HA−GATA-1(ΔN,C) and HA−GATA-2(ΔN,C)], and MG132 fully stabilized these proteins (Figure 5C).

The results of Figure 5 indicate that GATA-1 and GATA-2 are both constructed from highly unstable zinc-finger core modules. The N-termini of both factors similarly stabilize the core modules, whereas the fundamental differences in GATA-1 and GATA-2 stabilities are attributable to their C-termini. The GATA-1 C-terminus provides additional stability to the remaining sequences of GATA-1, whereas the GATA-2 C-terminus increases the susceptibility of GATA-2 to proteasomal degradation. The activity of MG132

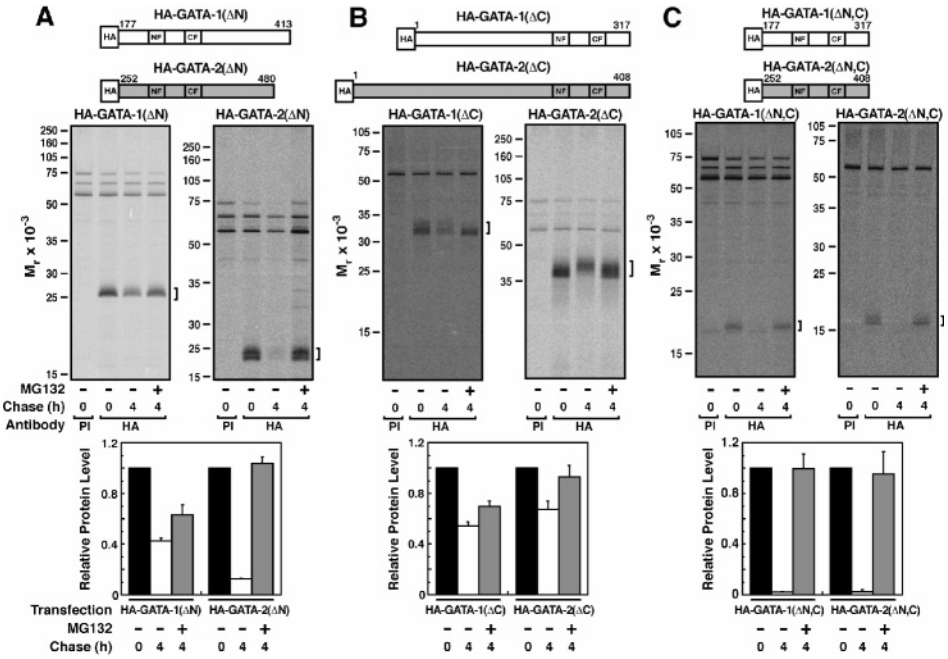

FIGURE 5: The N- and C-termini of GATA-1 and GATA-2 are important stability determinants. The stabilities of HA−GATA-1(ΔN) (A), HA−GATA-2(ΔN) (A), HA−GATA-1(ΔC) (B), HA−GATA-2(ΔC) (B), HA−GATA-1(ΔN,C) (C), and HA−GATA-2(ΔN,C) (C) transiently expressed in K562 cells were determined by biosynthetic labeling with ^{35}S-methionine and ^{35}S-cysteine, and pulse-chase analysis with and without MG132. Labeled GATA factors were immunoprecipitated, analyzed by SDS-PAGE, and quantitated by phosphorimager analysis (mean ± SE, three to four independent experiments). The graphs on the bottom depict the quantitative analysis. PI, preimmune.

to fully prevent decay of both HA−GATA-1(ΔN,C) and HA−GATA-2(ΔN,C) strongly supports a role for the ubiquitin−proteasome system in GATA factor degradation. That the N- and C-terminal deletion mutants of GATA-2, but not of GATA-1, are also fully stabilized by MG132 suggests that the ubiquitin−proteasome system is the predominant mediator of GATA-2 degradation, while additional proteolytic pathways might converge on GATA-1, though it is the more stable of the two proteins.

Molecular Determinants of Differential GATA Factor Stabilities: Testing Predictions with Novel Chimeric GATA Factors. As the GATA-1 and GATA-2 zinc-finger cores are equally unstable, and C-terminal sequences uniquely confer GATA-2 instability, we reasoned that substituting C-terminal, but not N-terminal, GATA-1 sequences for those of GATA-2 would stabilize GATA-2. To test this prediction, we generated three chimeric HA-tagged GATA factors and analyzed their expression and stabilities in K562 cells. Chimera 1 is GATA-2 with the GATA-1 N-terminus (GATA-1 residues 1−176 and GATA-2 residues 252−480), chimera 2 is GATA-2 with the GATA-1 C-terminus (GATA-2 residues 1−408 and GATA-1 residues 318−413), and chimera 3 is the GATA-2 zinc-finger core with both GATA-1 termini (GATA-1 residues 1−176 and 318−413 and GATA-2 residues 252−408). Pulse-chase analysis revealed that chimera 1 and GATA-2 are similarly unstable (compare Figure 6B with Figure 3B,C), consistent with our results that deleting the N-termini of GATA-1 and GATA-2 similarly reduced their stabilities (Figure 5A). MG132 prevented the decay of chimera 1 (Figure 6B), resembling the complete stabilization of GATA-2 by MG132 (Figure 3B,C). In contrast, substituting the GATA-1 C-terminus for that of GATA-2 (chimera 2) increased GATA-2 stability by ~50% (compare Figure 6C with Figure 3C). Moreover, MG132 did not fully stabilize chimera 2 (Figure 6C), similar to the

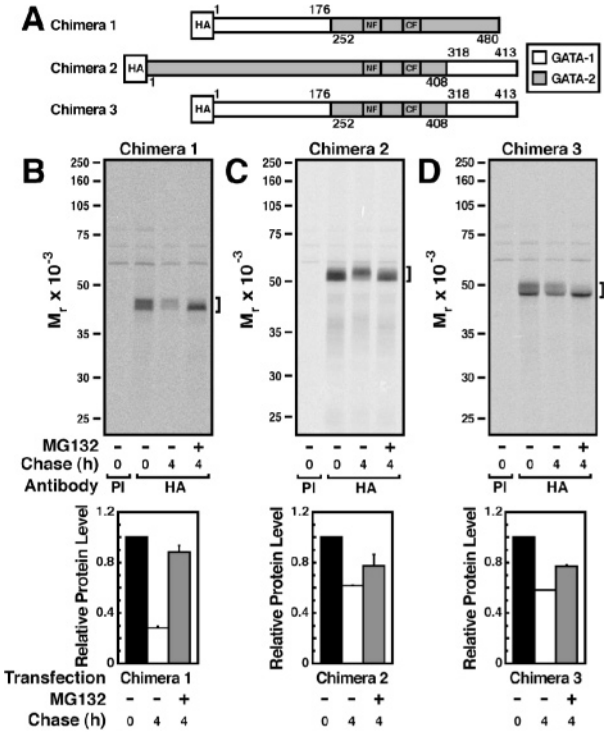

FIGURE 6: Evidence that the GATA-1 and GATA-2 C-termini differentially influence GATA factor stability. (A) Chimeric proteins. (B−D) The stabilities of chimeras transiently expressed in K562 cells were determined by biosynthetic labeling with ^{35}S-methionine and ^{35}S-cysteine and pulse-chase analysis with and without MG132. Labeled GATA factors were immunoprecipitated, analyzed by SDS-PAGE, and quantitated by phosphorimager analysis (mean ± SE, three independent experiments). The graphs on the bottom depict the quantitative analysis. PI, preimmune.

behavior of GATA-1, but not GATA-2, deletion mutants (Figure 5). The increased stability and altered MG132 response conferred on GATA-2 by the C-terminus of

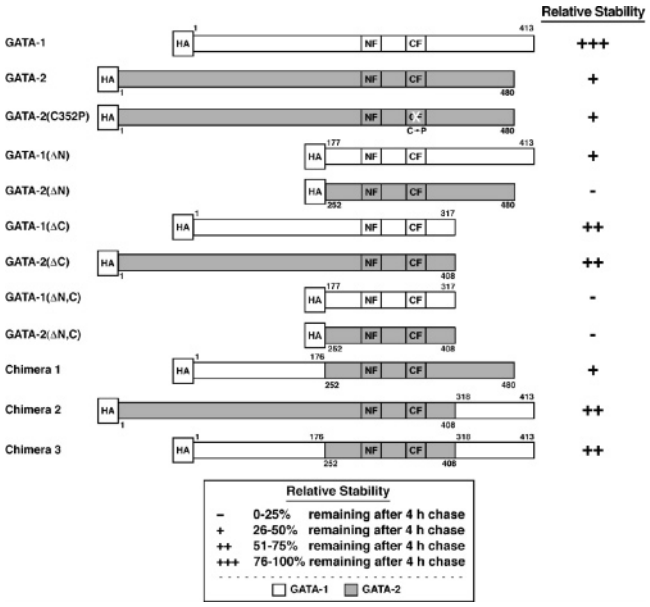

FIGURE 7: Summary of protein stabilities. The pulse-chase results of Figures 3−6 are summarized according to the scale depicted at the bottom.

GATA-1 implicate C-terminal sequences as an important determinant of the differential GATA-1 and GATA-2 stabilities. Chimera 3 exhibited the same stability and extent of MG132 stabilization (Figure 6D) as did chimera 2 (Figure 6C). Thus, substituting both the N- and C-termini of GATA-1 for those of GATA-2 yielded the same result as did replacing only the GATA-2 C-terminus with that of GATA-1. The analysis of chimera 3 therefore provides additional evidence that the N-termini of GATA-1 and GATA-2 confer similar stabilities to their unstable zinc-finger core modules, and differences in the C-termini dictate the greater stability of GATA-1 than of GATA-2. The relative stabilities of HA−GATA-1, HA−GATA-2, the N- and C-terminal deletion proteins, and chimeras, as determined by pulse-chase, are summarized in Figure 7.

In the pulse-chase analyses, HA−GATA-2, HA−GATA-2(ΔN), HA−GATA-1(ΔC), HA−GATA-2(ΔC), chimera 1, chimera 2, and chimera 3 were detected as bands consisting of upper and lower sub-bands. For the densitometric quantitations of Figures 3, 5, and 6, the upper and lower sub-bands were quantitated collectively to provide measurements of the total amount of immunoreactive proteins. The stabilities and MG132 stabilization of the upper and lower sub-bands were also quantitated separately (Supporting Information Figure 1). The greatest differences between the upper and lower sub-bands were observed with HA−GATA-1(ΔC), chimera 1, and chimera 3. In these cases, the stabilities of the upper and lower sub-bands were similar, but MG132 had little to no effect on the level of the upper sub-band after the 4 h chase. In each of the three cases, MG132 significantly increased the level of the lower sub-band. HA−GATA-1(ΔC), chimera 1, and chimera 3 all contain the GATA-1 N-terminus. Thus, it is possible that the GATA-1 N-terminus has distinct post-translational states that establish the band heterogeneity, with the upper and lower sub-bands being MG132-insensitive and -sensitive, respectively.

Implications of GATA-2 Instability for GATA Factor Chromatin Target Site Occupancy. As GATA-1 replaces GATA-2 at certain chromatin sites, including *Gata2* regulatory sequences, and the concentrations of these factors are almost certainly important determinants of GATA switches, the enhanced stability of GATA-1 vs GATA-2 could favor GATA-1 occupancy of GATA-2-bound chromatin sites. If this assumption is valid, one would predict that MG132-dependent stabilization of GATA-2 would create a potentially insurmountable obstacle that hinders GATA-1-mediated displacement of GATA-2 from chromatin.

We tested the possibility described above using the inducible G1E-ER-GATA-1 cell system. Tamoxifen or estradiol treatment of G1E-ER-GATA-1 cells activates ER-GATA-1, resulting in chromatin access and GATA switches, which are tightly coupled to *Gata2* repression. GATA switches occur at five sites of the *Gata2* locus: the −77, −3.9, −2.8, and −1.8 kb upstream sites, as well as the +9.5 kb intronic site (*37*, *40*, *42*). Furthermore, GATA switches have been detected at additional loci, including the upstream hypersensitive site 1 (HS1) of *Gata1* and hypersensitive site −26 (HS-26) of the α-globin locus (*2*, *48*).

To determine if MG132-dependent stabilization of GATA-2 affects the capacity of ER-GATA-1 to displace GATA-2 from chromatin, G1E-ER-GATA-1 cells were pretreated with MG132 and then treated with tamoxifen to activate ER-GATA-1. In this system, GATA switches require several hours post-tamoxifen or estradiol treatment to achieve a maximal response. However, the MG132 treatment time needed to be minimized, as prolonged exposure of cells to MG132 is cytotoxic. Thus, the tamoxifen treatment was conducted for 3 h, which induced a partial GATA switch without cytotoxicity. ER-GATA-1 activation resulted in loss of endogenous GATA-2 from *Gata2* locus sites, *Gata1* HS1, and α-globin HS-26 (Figure 8A). No GATA-2 occupancy was detected at the necdin promoter, which is not regulated by GATA factors. Intriguingly, under conditions in which MG132 stabilized GATA-2, ER-GATA-1-mediated displacement of GATA-2 from chromatin was severely impaired (Figure 8A). This result is consistent with the hypothesis that GATA-2 instability facilitates GATA-1-mediated displacement of GATA-2 from chromatin. Although we favor this possibility, given the broad effects of MG132-dependent proteasome inhibition, one cannot rule out potential indirect mechanisms.

As GATA switches at the *Gata2* locus correlate with transcriptional repression (*37*, *40*, *42*), one would predict that MG132-dependent impairment of GATA switches would be accompanied by a reduced magnitude of *Gata2* transcriptional repression. Consistent with this prediction, quantitative RT-PCR analysis revealed that the extent of ER-GATA-1-mediated repression of *Gata2* mRNA transcripts was significantly reduced (Figure 8B) ($p < 0.001$) under conditions in which MG132 stabilized GATA-2 and blocked GATA switches.

In aggregate, the results described herein provide evidence that GATA-1 is intrinsically more stable than GATA-2 in multiple cell systems, even though both factors are down-regulated by the ubiquitin−proteasome system. Whereas this differential stability is independent of differences in cellular environment among the diverse cells studied, and also independent of FOG-1 binding, it remains possible that the stability difference is not a characteristic of the full spectrum of GATA factor target cells. Indeed, it would be particularly important if cell signaling mechanisms, which differ in

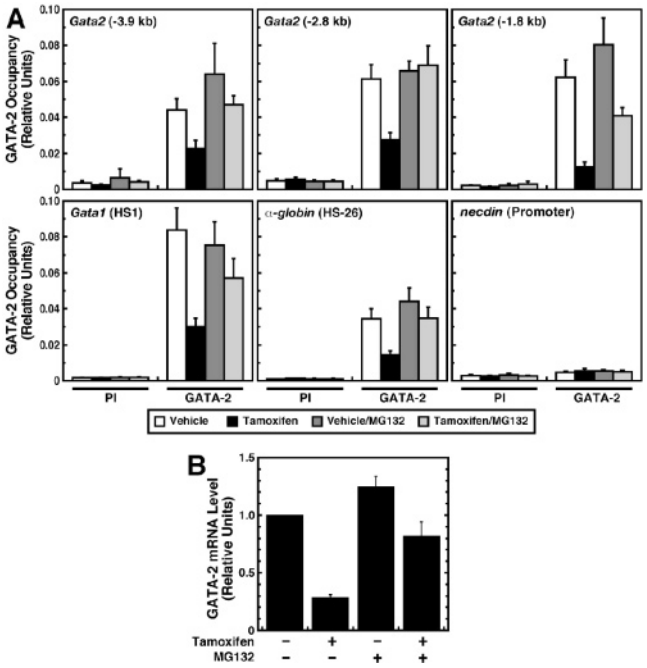

FIGURE 8: Under conditions in which MG132 stabilizes GATA-2, GATA switches are blocked. (A) Quantitative ChIP analysis of GATA-2 occupancy at the −3.9 kb, −2.8 kb, and −1.8 kb GATA switch sites of the *Gata2* locus in G1E-ER-GATA-1 cells. G1E-ER-GATA-1 cells were pretreated for 30 min with vehicle or MG132 and then cultured with or without tamoxifen for 3 h. In the most recent experiments, β-estradiol was used instead of tamoxifen, and the results were indistinguishable. The neuronal-specific necdin promoter, which is not regulated by GATA factors, was used as a negative control (mean ± SE, seven independent experiments). PI, preimmune. (B) Real-time RT-PCR analysis of *Gata2* expression. RNA was isolated from the G1E-ER-GATA-1 cultures analyzed by ChIP, and *Gata2* expression was quantitated by real-time RT-PCR (mean ± SE, eight independent experiments).

diverse cellular contexts, target GATA factors and/or associated components, thereby dynamically regulating stability. It is attractive to propose that GATA-2 instability is an important determinant of GATA switches, and signal-dependent modulation of instability could be an important determinant of GATA factor chromatin target site occupancy in a physiological context. Whereas GATA-1 is subject to multisite phosphorylation (*68*−*70*), the precise functions of GATA-1 phosphorylation have remained elusive, as a triple phosphorylation mutant can substitute for GATA-1 *in vivo* (*71*). Intriguingly, as the C-termini of GATA-1 and GATA-2 differ in their capacities to stabilize the respective DNA binding core modules, it will be important to determine if cell signals converge upon this region, thereby regulating GATA factor stability and chromatin occupancy as a fundamental step in establishing GATA factor-dependent transcriptional networks that control hematopoiesis.

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SUPPORTING INFORMATION AVAILABLE

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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