

# Differential coregulator requirements for function of the hematopoietic transcription factor GATA-1 at endogenous loci

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## ABSTRACT

The critical regulator of hematopoiesis GATA-1 recruits diverse coregulators to chromatin, which mediate transcriptional activation and repression. These coregulators include the cell-type-specific multi-zinc finger protein Friend of GATA-1 (FOG-1), the histone acetyltransferase CREB binding protein (CBP), and the key component of the Mediator complex Med1. While FOG-1 is an established GATA-1 coregulator, the importance of interactions between GATA-1 and other coregulators is poorly understood. Furthermore, whether GATA-1 utilizes multiple coregulators at all loci, or if certain coregulators are dedicated to specific loci is unknown. We compared the capacity of GATA-1 to recruit and utilize FOG-1 and Med1 at activated and repressed target genes. Similar to FOG-1, GATA-1 recruited Med1 to activated genes, and the kinetics of FOG-1 and Med1 recruitment were similar. GATA-1 recruited Med1 in *Fog1*<sup>-/-</sup> cells, indicating that GATA-1-mediated Med1 recruitment is FOG-1-independent. In contrast to FOG-1, GATA-1 evicted Med1 during transcriptional repression. Whereas knocking-down FOG-1 had catastrophic effects on GATA-1-mediated activation and repression, knocking-down Med1 modestly impaired GATA-1 activity only at select loci. These results illustrate both similarities and differences between GATA-1-mediated recruitment of FOG-1 and Med1 to chromatin, with a fundamental difference being the quantitatively greater requirement for FOG-1.

## INTRODUCTION

A particularly intriguing question in understanding transcriptional mechanisms is how a specific transcription factor can mediate diverse transcriptional outcomes.

This context-dependent activity involves combinatorial action of the transcription factor with lineage and developmental stage-specific factors and coregulators, altered chromatin environment, and posttranslational modification of transcription factors and coregulators. How these mechanisms are integrated to yield positive or negative transcriptional outputs is poorly understood (1).

In the context of hematopoiesis, it is instructive to consider mechanisms underlying the activity of the cell-type-specific transcription factor GATA-1 (2,3). GATA-1 is a member of the GATA family of dual zinc-finger transcription factors (4–6), which controls the development of erythrocytes, mast cells, megakaryocytes and platelets (7–10). GATA-1 knockout mice die at embryonic day 10.5 of severe anemia (7), and human GATA-1 mutations are associated with leukemia and anemia (11). The C-terminal zinc finger binds the DNA sequence (A/T)GATA(A/G) *in vitro* (12–14), while the N-terminal finger interacts with the cell-type-specific coregulator Friend of GATA-1 (FOG-1) (15,16). Analysis of 5749 endogenous GATA-1 occupancy sites, corresponding to 4061 genes, revealed a highly significant position weight matrix with the consensus (C/G)(A/T)GATAA(G/A/C)(G/A/C) (17).

FOG-1 knockout mice die between E10.5 and E12.5 of severe anemia and defective megakaryopoiesis (18). Though FOG-1 plays a crucial role in GATA-1-mediated regulation of numerous genes, it appears to be dispensable at others (16,19). GATA-1 recruits FOG-1 to activated and repressed genes (20–23), suggesting that FOG-1 occupancy does not specify the precise GATA-1-mediated transcriptional output.

Additional coregulators have been implicated in GATA-1 function. GATA-1 directly interacts with the histone acetyltransferase CREB binding protein (CBP)/p300 (24), and FOG-1 interacts with the histone deacetylase complex NuRD (25). Presumably, the differential recruitment/regulation of coactivators and corepressors dictates activation versus repression of GATA-1 target genes. However, CBP persists at certain

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GATA-1 repressed genes (23), and NuRD occupies both GATA-1-repressed and -activated genes (26). Thus, additional coregulators might dictate the precise transcriptional output, such as the GATA-1-interacting coregulator Med1 (27), a core component of the Mediator complex (28,29). While knowledge of biochemical and molecular aspects of Mediator function is quite advanced, cell-type-specific mediator functions are poorly understood.

The Mediator complex is a 1–2 MDa complex crucial for most activated transcription in eukaryotes (30). Nuclear hormone receptors bind Mediator through a central 220 kDa component termed Med1 (30). *Med1*<sup>-/-</sup> mice die at E11.5 and have severe anemia, cardiac failure, and vascular defects (31–34). Certain phenotypes of the *Med1*<sup>-/-</sup> mice resemble those of GATA factor knockout mice. In addition, several members of the GATA factor family, including GATA-1, bind Med1 in a GST pull-down assay (32). These findings were subsequently extended in a study focusing solely on GATA-1-Med1 interactions (27).

Although GATA-1 binds Med1, and phenotypes suggest the importance of the GATA-1-Med1 interaction, whether Med1 controls hematopoiesis predominantly through GATA-1, interactions with other erythroid factors, or a non-cell autonomous mechanism is unknown. Hematopoietic precursors from Med1 knockout mice produce fewer erythroid burst forming units (BFU-E) (27), and erythroid and megakaryocyte maturation was reduced *in vivo*; myeloid cells were unaffected (33). An *in vivo* biotinylation assay revealed biotinylated GATA-1 binding to endogenous Med1 (27). Overexpressed GATA-1 and Med1 function collectively to activate a GATA-1 reporter plasmid, and GATA-1 and Med1 co-occupy three regulatory regions of the *Gata1* locus (27). Thus, diverse lines of evidence suggest a role for Med1 in GATA-1 function.

To establish whether Med1 is important for GATA-1 function at endogenous target genes, we conducted quantitative chromatin immunoprecipitation (ChIP) analysis in a GATA-1-null proerythroblast cell line expressing conditionally active GATA-1 (G1E-ER-GATA-1). We found that Med1 was recruited to all GATA-1-occupied loci tested and was evicted from repressed sites. Furthermore, endogenous Med1 and GATA-1 colocalized at these same sites. GATA-1 recruited Med1 and FOG-1 with similar kinetics, although studies in *Fog1*<sup>-/-</sup> cells indicated that Med1 recruitment is FOG-1-independent. Knocking-down Med1 and FOG-1 revealed that Med1 is crucial for maximal GATA-1 activity at endogenous target genes, although the contribution of Med1 towards GATA-1 function was considerably less than that of FOG-1.

## MATERIALS AND METHODS

### Cell culture

G1E cells expressing ER-GATA-1 (35,36) were cultured in Iscove's Modified Dulbecco's Medium (Gibco/Mediatech) containing 2% Antibiotic/Antimycotic

(Penicillin–Streptomycin–Fungizone) (Gibco), 2 U/ml erythropoietin, 120 nM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit ligand-producing CHO cell line, 15% FBS (Gemini Bioproducts), and 1 µg/ml puromycin (Sigma). MEL cells (37) were cultured in Dulbecco's Modified Eagle's Medium (Gibco/Mediatech) containing 5% Bovine Serum (Gibco) and 5% FBS (Gemini Bioproducts) or 10% FBS (38). *Fog1*<sup>-/-</sup> hematopoietic precursor cells (39) were maintained in Iscove's Modified Dulbecco's Medium (Gibco/Mediatech) containing 15% FBS (Gemini), 2% Antibiotic/Antimycotic (Penicillin–Streptomycin–Fungizone) (Gibco) and 10 ng/ml Interleukin-3 (R&D Systems). *Fog1*<sup>-/-</sup> cells expressing ER-GATA-1 were cultured under identical conditions, with the addition of 1 µg/ml puromycin (Sigma) to the media. All cells were cultured in a humidified incubator at 37°C with 5% carbon dioxide.

### Real-time RT-PCR

Cells for mRNA analysis of GATA-1 target genes were harvested from identical cultures as those used for ChIP. RNA was isolated using TRIzol (Invitrogen), and cDNA was prepared as described (40). cDNAs were diluted to 150 µl, and 1 µl of cDNA was amplified in a 15 µl reaction volume by real-time PCR using *Power SYBR Green PCR Master Mix* (Applied Biosystems) and the appropriate primers in the Prism 7900 (Applied Biosystems). The relative enrichment of specific cDNA sequences was compared with a genomic DNA standard using the comparative cycle threshold method. All data were normalized to *Gapdh* mRNA levels. RT-PCR primer sequences are available upon request.

### Quantitative ChIP assay

G1E-ER-GATA-1 cells for ChIP analysis were seeded at  $2 \times 10^5$  cells/ml and treated with 1 µM β-estradiol (Steraloids Inc) for 24 h. For kinetic studies, G1E-ER-GATA-1 cells were divided into five identical cultures and treated with 1 µM β-estradiol for 0, 6, 14, 24 or 48 h. MEL cells were induced to differentiate by culturing for 3 days in the presence of 1.5% dimethyl sulfoxide (DMSO) (Sigma). *Fog1*<sup>-/-</sup> cells were grown as described above, while *Fog1*<sup>-/-</sup> cells expressing ER-GATA-1 (19) were treated with 1 µM β-estradiol for 48 h. All cells were crosslinked with 1% formaldehyde (Sigma) immediately after harvest. Crosslinked cells were frozen and stored at -80°C until use. ChIP was conducted as described (41). Rabbit anti-GATA-1 and anti-FOG-1 polyclonal antibodies were described previously (42), and anti-Med1 antibody (M-255, sc-8998) was from Santa Cruz Biotechnology. DNA was quantitated by real-time PCR in the Prism 7900. Primers amplified 50- to 150-bp amplicons, specific product was measured by SYBR Green fluorescence, product was quantified relative to a standard curve of input chromatin, and dissociation curves showed that PCR yielded single products. ChIP primer sequences are available upon request.

## RNA interference

siRNA-dependent knockdown experiments were conducted in G1E-ER-GATA-1 cells using siGENOME SMARTpool siRNAs from Dharmacon Inc. SMARTpools targeting Med1 (Dharmacon Catalog # M-040964-02) and FOG-1 (M-045857-01) were compared to a non-targeting control pool (Non-Targeting siRNA Pool #1, D-001206-13-05) (Dharmacon). To ensure maximal transfection efficiency, siRNAs were electroporated into cells twice, allowing 24 h between transfections, using the Amaxa Nucleofector II (Lonza Cologne AG) program G-016 and Nucleofector Kit R (Lonza Cologne AG).  $3 \times 10^6$  G1E-ER-GATA-1 cells were resuspended in 100  $\mu$ l Nucleofector solution, electroporated in the provided cuvettes, and transferred to a 4 ml culture volume of G1E media (as described above, but lacking Antibiotic/Antimycotic) in 6-well plates (Fisher). Samples were treated with 1  $\mu$ M  $\beta$ -estradiol (Steraloids Inc) 6 h posttransfection for 18 h. Cells were isolated by centrifugation, transfected a second time, and then treated with 1  $\mu$ M  $\beta$ -estradiol for an additional 24 h. Cells were counted and used to prepare total RNA and protein samples.

## Protein analysis and western blotting

Whole cell lysates were prepared from  $1 \times 10^6$  cells boiled for 10 min in 100  $\mu$ l SDS Sample Buffer (50 mM Tris, pH 6.8, 2%  $\beta$ -Mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 5% glycerol). Proteins were resolved by SDS polyacrylamide gel electrophoresis on a 7.5% acrylamide gel and analyzed by western blotting with anti-Med1 (Santa Cruz, M-255, sc-8998), anti-FOG-1 (42) and anti- $\alpha$ -Tubulin (Calbiochem, Catalog #: CP06) antibodies using ECL+ (GE Healthcare).

## Microscopy and cell staining

siRNA-treated cells were cytocentrifuged onto slides at 1500g for 10 min at room temperature. Cells were fixed in methanol for 30 s and analyzed by Wright-Giemsa staining. Coverslips were mounted to slides with MicroMount media (Surgipath). Cells were imaged using an Olympus IX81 motorized inverted microscope equipped with an Olympus DP25 brightfield camera. Images were acquired and analyzed with the Olympus DP2-DSW software. Nuclear and cellular diameter was determined as an average of two measurements for each cell, and 50 cells were analyzed for each condition.

## RESULTS AND DISCUSSION

### Med1 is recruited to GATA-1-activated genes and evicted from GATA-1-repressed genes

Since the importance of Med1 as a GATA-1 coregulator at diverse endogenous loci had not been established, we tested whether Med1 resembles FOG-1 in being recruited to all GATA-1 target sites in chromatin. Using a genetic complementation assay in GATA-1-null G1E cells, in which ER-GATA-1 is activated by  $\beta$ -estradiol (35,36), we analyzed GATA-1 and Med1 occupancy at well

characterized GATA-1-activated genes (Figure 1).  $\beta$ -Estradiol treatment of G1E cells stably expressing ER-GATA-1 (G1E-ER-GATA-1) recapitulates a normal erythroid gene expression program and a window of erythropoiesis (43). Activated ER-GATA-1 rapidly induced or repressed endogenous target genes (Figure 1A and C) and occupied sites at and surrounding the respective genes (Figure 1B and D, top). ER-GATA-1 recruited Med1 to sites at and near activated genes (Figure 1B, bottom). In contrast, ER-GATA-1 evicted Med1 from GATA-1-repressed genes (Figure 1D, bottom). This differs from FOG-1, which is recruited to both activated and repressed genes (20–22) and also CBP, which can persist postrepression (23). The differential Med1 occupancy at activated versus repressed GATA-1 target genes highlights the potential importance of Med1 in GATA-1-mediated transcriptional regulation.

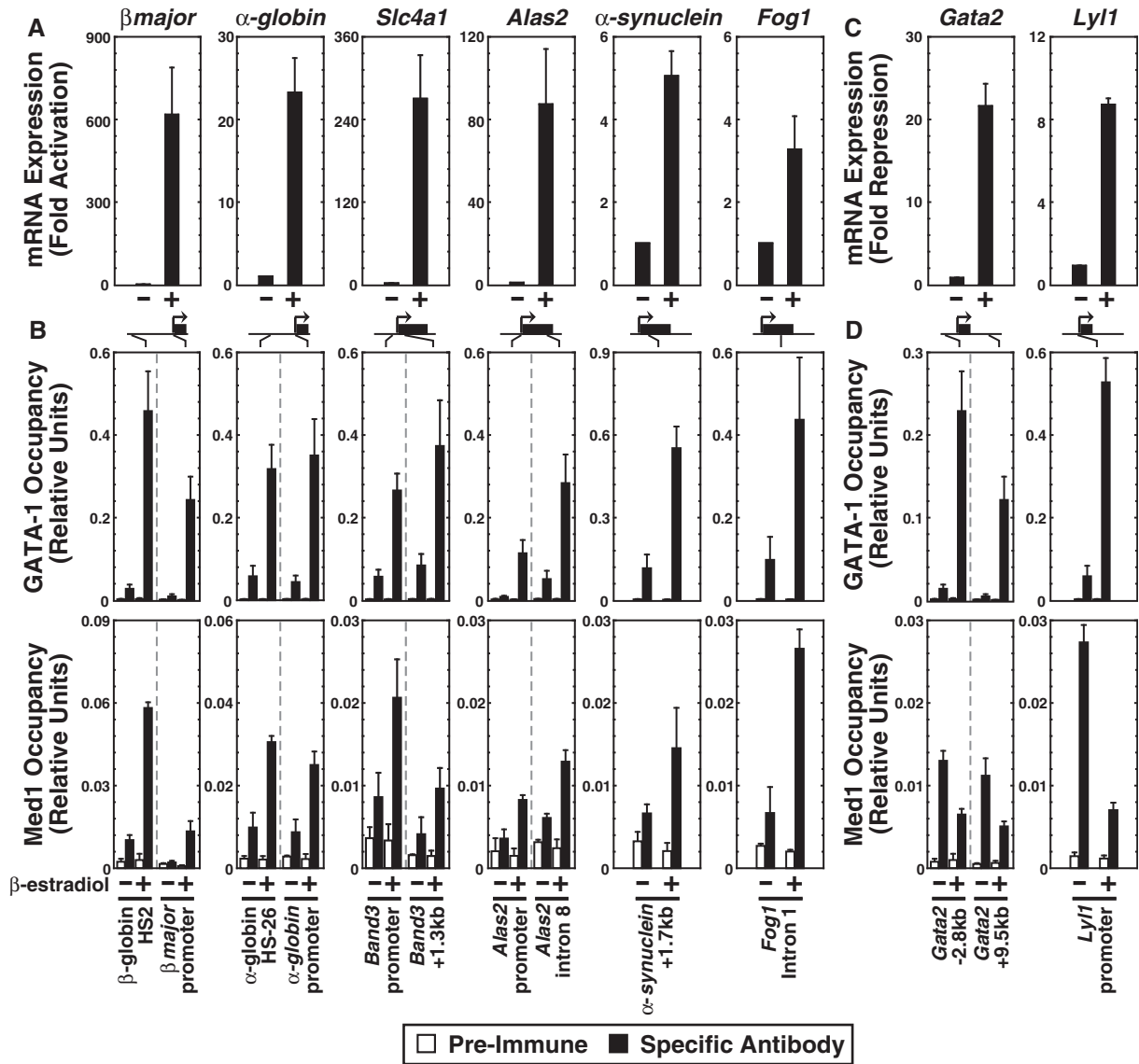
To determine whether endogenous GATA-1 recruits Med1, we analyzed Med1 and GATA-1 occupancy in Mouse Erythroleukemia (MEL) cells stimulated to differentiate for 3 days with 1.5% DMSO (Figure 2A and B). Upon MEL cell differentiation, endogenous GATA-1 target gene expression increased (Figure 2A) and GATA-1 occupancy at the respective genes increased (Figure 2B, top). Resembling G1E-ER-GATA-1 cells, Med1 was recruited to these sites (Figure 2B, bottom), indicating that both ER-GATA-1 and endogenous GATA-1 recruit Med1 to chromatin.

As Med1 is recruited to GATA-occupied sites at all GATA-1-activated genes tested, Med1 might be a crucial coactivator at most, if not all, GATA-1 target genes. Furthermore, in contrast to FOG-1 and CBP, the failure of Med1 to persist within repressive GATA-1 complexes at *Gata2* and *Lyl1* loci suggests that Med1 eviction might be uniquely important for GATA-1-dependent repression.

### Med1 and FOG-1 are recruited with similar kinetics, but Med1 recruitment is FOG-1-independent

Since Med1 is a component of the Mediator complex that binds Pol II, GATA-1-dependent Med1 recruitment might reflect Pol II recruitment as a late step in transcriptional activation. Alternatively, GATA-1 might directly and rapidly recruit Med1, similar to FOG-1. We compared the kinetics of GATA-1, FOG-1 and Med1 occupancy at 0, 6, 14, 24, and 48 h post-ER-GATA-1 activation. GATA-1 recruited Med1 and FOG-1 to chromatin with similar kinetics at the  $\beta$ -major promoter and upstream LCR region (HS2), as well as the  $\alpha$ -globin promoter and upstream enhancer region (HS-26) (Figure 3A). Med1 occupancy did not increase at control sites lacking ER-GATA-1, specifically the active *RPII215* promoter and the inactive *neccdin* promoter (Figure 3B). The direct GATA-1 binding to FOG-1 and Med1, and the similar kinetics of FOG-1 and Med1 recruitment suggest that Med1 recruitment involves the direct binding of Med1 to GATA-1, which was reported previously (27,32).

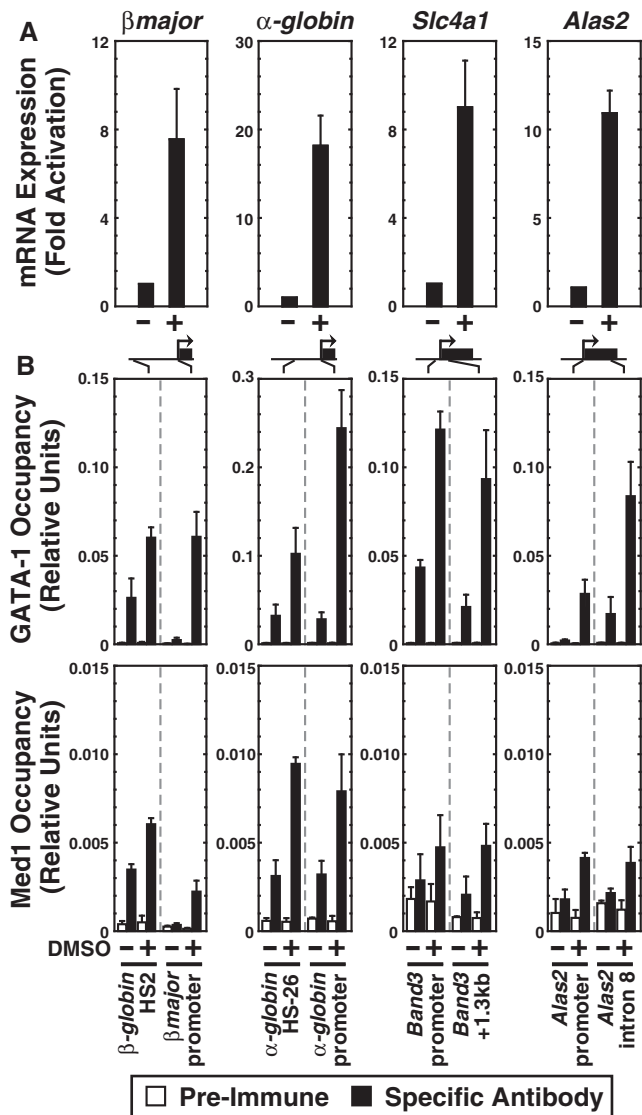
Although GATA-1 binds Med1, the precise molecular underpinnings of the GATA-1-Med1 interaction are not established. In principle, the similar kinetics of



**Figure 1.** Med1 is recruited to GATA-1 occupancy sites in GATA-1-activated genes upon induction, and evicted from GATA-1-repressed genes. (A) GATA-1-activated genes. Real-time RT-PCR quantitation of mRNA in G1E-ER-GATA-1 cells treated with 1  $\mu$ M  $\beta$ -estradiol for 24h (mean  $\pm$  standard error, three independent experiments). (B) Quantitative ChIP analysis of GATA-1 and Med1 occupancy at GATA-1-activated genes in G1E-ER-GATA-1 cells treated with 1  $\mu$ M  $\beta$ -estradiol for 24h (mean  $\pm$  standard error, three independent experiments). The locations of chromatin sites analyzed at the respective genes are shown at the top. (C) GATA-1-repressed genes. Real-time RT-PCR quantitation of mRNA in G1E-ER-GATA-1 cells treated with 1  $\mu$ M  $\beta$ -estradiol for 24h (mean  $\pm$  standard error, three independent experiments). (D) Quantitative ChIP analysis of GATA-1 and Med1 occupancy at GATA-1-repressed genes in G1E-ER-GATA-1 cells treated with 1  $\mu$ M  $\beta$ -estradiol for 24h (mean  $\pm$  standard error, three independent experiments). The locations of chromatin sites analyzed at the respective genes are shown at the top.

FOG-1 and Med1 recruitment could be explained by a FOG-1-dependent mechanism of Med1 recruitment (Figure 4A). To test this possibility, we conducted quantitative ChIP analysis in *Fog1*<sup>-/-</sup> cells. These cells express endogenous GATA-1, and GATA-1 occupies certain sites that do not critically require FOG-1 for chromatin occupancy, e.g. the  $\beta$ -globin LCR. Both GATA-1 and Med1 occupied  $\beta$ -globin HS2,  $\alpha$ -globin HS-26, and  $\alpha$ -globin promoter (Figure 4B), indicating that FOG-1 is not required for GATA-1 to recruit Med1 to chromatin. As

FOG-1 is required for GATA-1 occupancy at the  $\beta$ -major promoter, little to no GATA-1 occupied the promoter in *Fog1*-null cells, and accordingly Med1 did not occupy the promoter. We also tested whether FOG-1 mediates Med1 recruitment using *Fog1*<sup>-/-</sup> cells stably expressing ER-GATA-1, which allow for a more dynamic analysis than the *Fog1*<sup>-/-</sup> cells. After  $\beta$ -estradiol treatment, ER-GATA-1 occupancy increased at  $\beta$ -globin HS2,  $\alpha$ -globin HS-26 and to a lesser extent at  $\alpha$ -globin promoter (Figure 4C, top). Similarly, Med1 occupancy



**Figure 2.** Med1 is recruited to endogenous GATA-1-bound target sites. (A) Real-time RT-PCR quantitation of mRNA in MEL cells differentiated for 3 days in 1.5% DMSO (mean  $\pm$  standard error, three independent experiments). (B) Quantitative ChIP analysis of GATA-1 and Med1 occupancy in MEL cells differentiated for 3 days in 1.5% DMSO (mean  $\pm$  standard error, three independent experiments). The locations of chromatin sites analyzed at the respective genes are shown at the top.

increased at these sites (Figure 4C, bottom). Thus, GATA-1 recruits Med1 to chromatin via a FOG-1-independent mechanism.

#### Med1 facilitates GATA-1-dependent transcriptional regulation of select targets, but to a much lesser degree than FOG-1

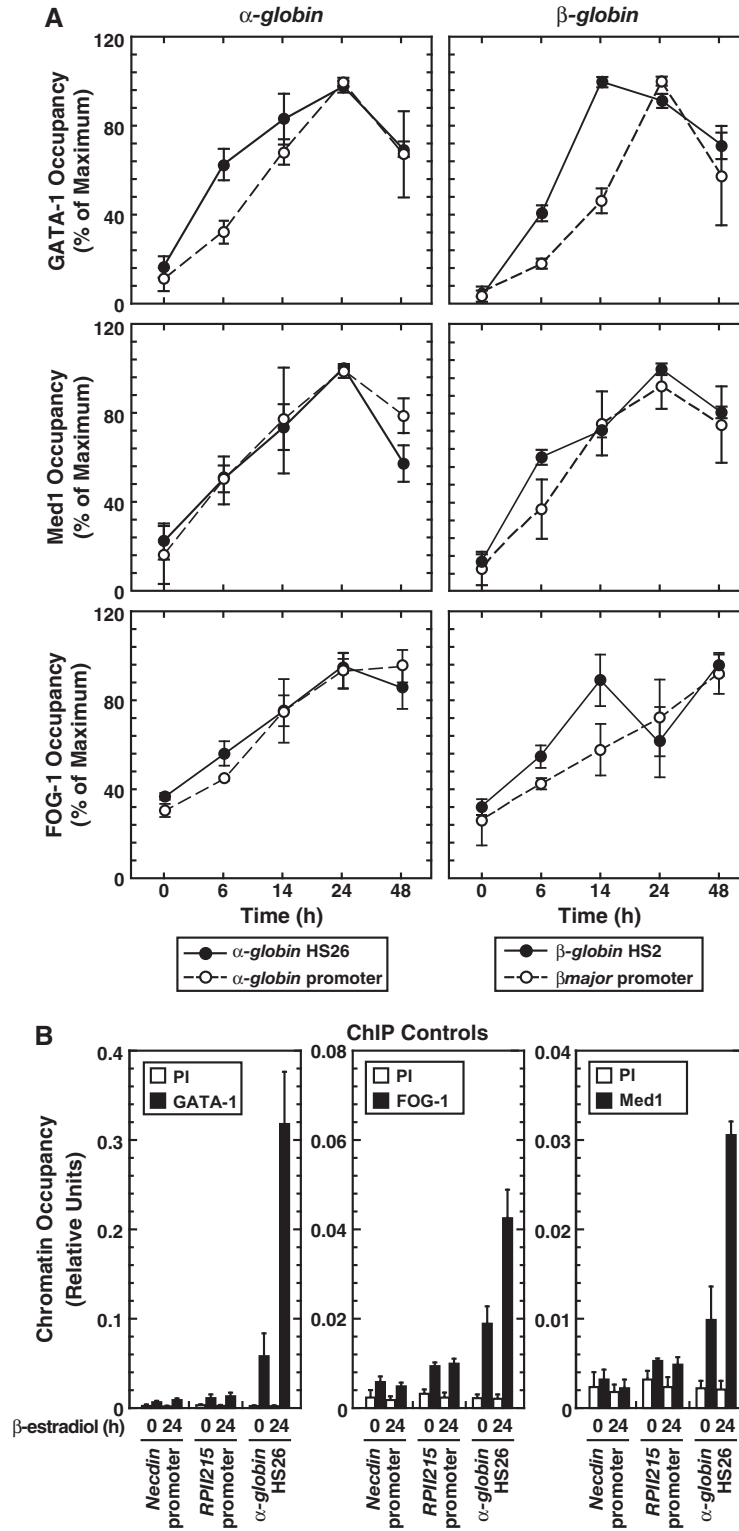
To establish whether Med1 mediates GATA-1 function, we used siRNA technology to knockdown Med1 in G1E-ER-GATA-1 cells. Six hours posttransfection, cells were treated with  $\beta$ -estradiol for 18 h, transfected again, and then treated with  $\beta$ -estradiol for an additional 24 h

(Figure 5A). The knockdown efficiency was assessed by real-time RT-PCR analysis of *Med1* mRNA, and semi-quantitative western blotting of Med1 protein (Figure 5B). Real-time RT-PCR was used to quantitate target gene expression in cells transfected with control and Med1 siRNAs. *Med1* mRNA levels were reduced  $\sim$ 70% relative to controls, and Med1 protein was reduced  $\sim$ 95% (Figure 5B).

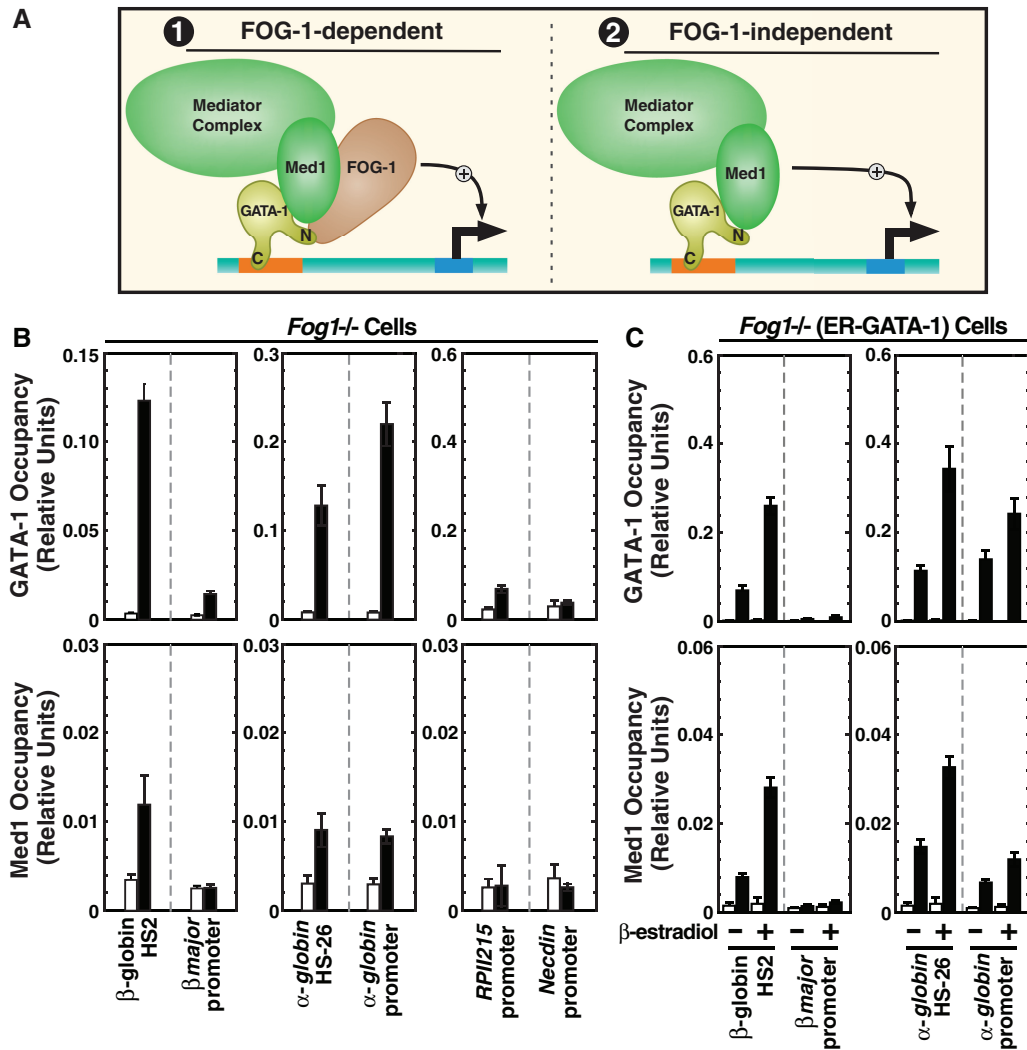
The Med1 knockdown induced a small, but statistically significant, defect in GATA-1-mediated gene activation at a subset of target genes (Figure 5C).  $\beta$ -Major ( $P = 0.008$ ), *Alas2* ( $P = 0.015$ ) and *Epb4.9* ( $P = 0.001$ ) expression decreased significantly, while  $\alpha$ -globin ( $P = 0.056$ ) expression decreased to a lesser extent. The Med1 requirement for maximal GATA-1 activity was not global, however, as expression of the highly responsive GATA-1 target gene, *Slc4a1* was insensitive to the knockdown (Figure 5C). The Med1 knockdown did not prevent GATA-1-dependent repression, as *Gata2* repression was not significantly altered ( $P = 0.104$ ) (Figure 5C). This is consistent with the eviction of Med1 from these genes upon  $\beta$ -estradiol treatment.

To compare the relative importance of Med1 and FOG-1 as GATA-1 coregulators, we knocked-down FOG-1 in G1E-ER-GATA-1 cells using the identical timeline depicted in Figure 5A and quantitated GATA-1 target gene expression. In contrast to the modest inhibitory effects resulting from the Med1 knockdown, knocking-down FOG-1 (Figure 6A) strongly impaired both activation and repression (Figure 6B). The regulation of all FOG-1-dependent activated genes analyzed was severely impaired, including *Slc4a1* (Figure 6B), which was unaffected by the Med1 knockdown (Figure 5C). *Epb4.9*, which was shown previously to be less dependent upon FOG-1 based on studies in *Fog1*<sup>-/-</sup> cells and with a GATA-1 mutant defective in FOG-1 binding (19,22), exhibited a less severe defect (Figure 6B). The FOG-1 knockdown also strongly impaired GATA-1-mediated repression. *Gata2*, a FOG-1-dependent GATA-1 repressed gene was repressed  $<2$ -fold, versus 20-fold with the control siRNA. Thus, most targets are highly sensitive to the FOG-1 knockdown, whereas only select targets are sensitive to the Med1 knockdown, and the extent of target gene dysregulation is considerably greater with the FOG-1 knockdown.

To further compare the impact of knocking down FOG-1 and Med1, we tested whether the knockdowns altered G1E-ER-GATA-1 cell maturation. The knockdowns were conducted as described above, and cells were analyzed by Wright-Giemsa staining (Figure 7A). Control siRNA-transfected cells matured normally in response to  $\beta$ -estradiol treatment, while FOG-1 siRNA-transfected cells exhibited a maturation blockade. Med1 siRNA-transfected cells matured normally. To quantitatively analyze maturation, we measured the nuclear diameter, and calculated the nuclear area for each condition (Figure 7B). Nuclei from control siRNA-transfected, uninduced cells and FOG-1 siRNA-transfected, induced cells were similarly large. In contrast, nuclei from control siRNA-transfected, induced cells and Med1 siRNA-transfected, induced cells were



**Figure 3.** GATA-1 recruits Med1 and FOG-1 with similar kinetics. **(A)** Quantitative ChIP analysis of ER-GATA-1, Med1 and FOG-1 chromatin occupancy at various times post-induction with 1 μM β-estradiol (mean ± standard error, three independent experiments). **(B)** ChIP controls. Quantitative ChIP analysis of GATA-1, FOG-1 and Med1 occupancy at active (*RPII215*), and repressed (*necdin*) genes that are not regulated by GATA-1 (mean ± standard error, three independent experiments).



**Figure 4.** GATA-1-mediated Med1 recruitment is FOG-1-independent. (A) Potential FOG-1-dependent and -independent modes of Med1 recruitment. (B) Quantitative ChIP analysis of GATA-1 and Med1 occupancy at select sites in *Fog1*<sup>-/-</sup> cells (mean  $\pm$  standard error, three independent experiments). (C) Quantitative ChIP analysis of GATA-1 and Med1 occupancy in *Fog1*<sup>-/-</sup> cells stably expressing ER-GATA-1, treated with 1  $\mu$ M  $\beta$ -estradiol for 48 h (mean  $\pm$  standard error, three independent experiments).

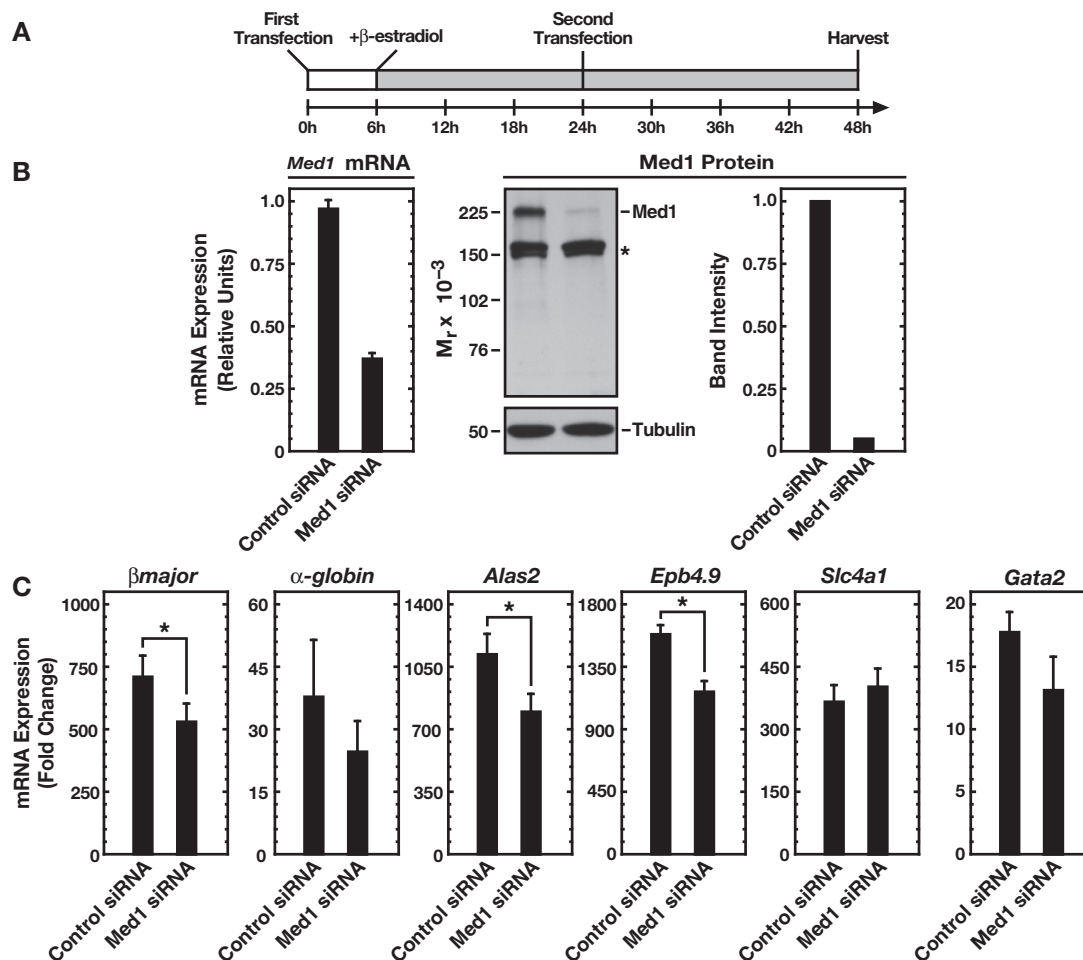
significantly smaller ( $P < 0.0001$ ). Thus, contrasting with the critical role of FOG-1 for conferring G1E-ER-GATA-1 maturation, the modest changes in GATA-1 target gene expression resulting from Med1 knockdown are insufficient to impair maturation.

### Mechanistic insights

The phenotype of *Med1* knockout mice indicates that Med1 has critical nonredundant functions *in vivo* (31–34). Though molecular and biochemical analyses have demonstrated that Med1 can bind GATA factors (32) and function as a GATA-1 coregulator (27), it is unclear how important Med1 is for GATA-1-mediated control of transcription at endogenous target genes and for erythropoiesis. Extensive genetic analysis has established FOG-1 as a crucial GATA-1 coregulator (15,16,18). Herein, we compared the capacity of GATA-1 to recruit and utilize FOG-1 and Med1. GATA-1 recruited Med1 to GATA-1-activated genes, similar to FOG-1 (Figure 3),

and FOG-1 was not required for Med1 recruitment (Figure 4). Although FOG-1 persists at loci upon GATA-1-dependent repression (20), GATA-1-instigated repression was associated with eviction of Med1 from chromatin (Figure 1). In contrast to the consequences of reducing FOG-1 levels, which dramatically compromised GATA-1-dependent activation, repression, and maturation, reducing Med1 levels had a significant, but modest, inhibitory effect at select endogenous targets, with no effect on maturation.

An important quantitative consideration is whether a small percentage of total cellular Med1 suffices to function as a GATA-1 coregulator, as if this is the case, knockdown conditions might not suffice to deplete Med1 below a critical threshold. This seems unlikely, however, as the knockdown reduced the Med1 protein level by 95%, and hypomorphic mutant mice expressing 20% of wild-type Med1 levels exhibit embryonic lethality with defective erythropoiesis and megakaryopoiesis (33).



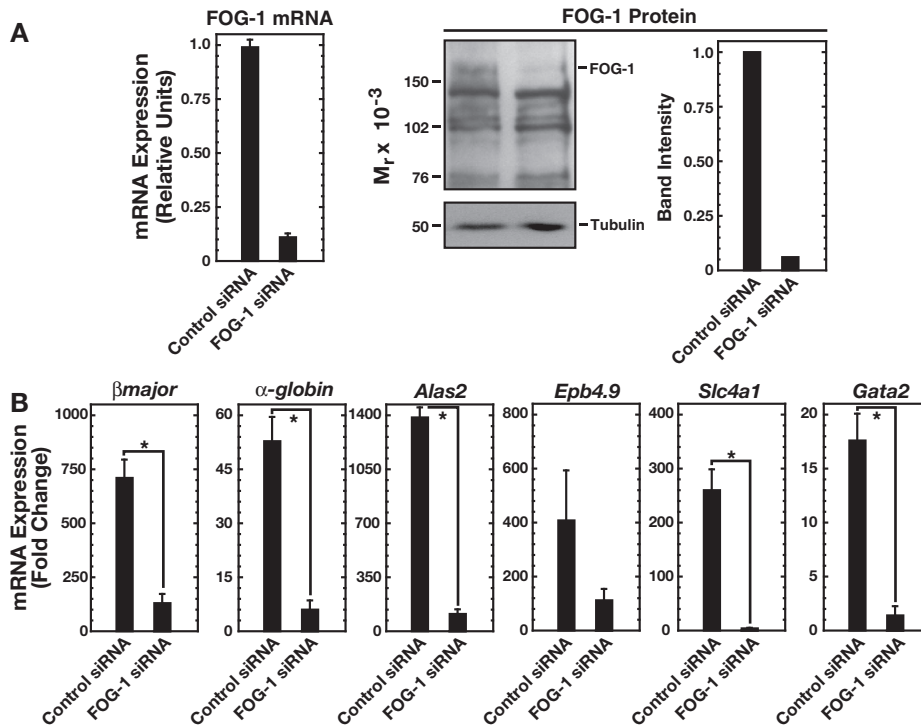
**Figure 5.** Knockdown of Med1 significantly, but modestly, alters expression of select endogenous GATA-1 target genes. (A) Diagram illustrating the knockdown strategy employed. Cells were transfected twice allowing 24 h between transfections, and treated with  $\beta$ -estradiol (shaded period) for 42 h. (B) Knockdown of *Med1* mRNA and protein by siRNA treatment, as measured by real-time RT-PCR and semi-quantitative western blotting respectively (mean  $\pm$  standard error, six independent experiments). Asterisk indicates non-specific band. Right, densitometric quantitation of protein level. (C) Real-time RT-PCR quantification of mRNA in G1E-ER-GATA-1 cells treated with either 240 pmol control siRNA or Med1 siRNA for 48 h and 1  $\mu$ M  $\beta$ -estradiol for 42 h (mean  $\pm$  standard error, six independent experiments). The fold change in expression was plotted, with GATA-1 activating  $\beta$ -major,  $\alpha$ -globin, *Alas2*, *Epb4.9* and *Slc4a1* and repressing *Gata2*; \* $P < 0.05$ .

Furthermore, the degree of knockdown of Med1 protein was comparable to that of the FOG-1 knockdown, which had devastating effects on transcription and maturation.

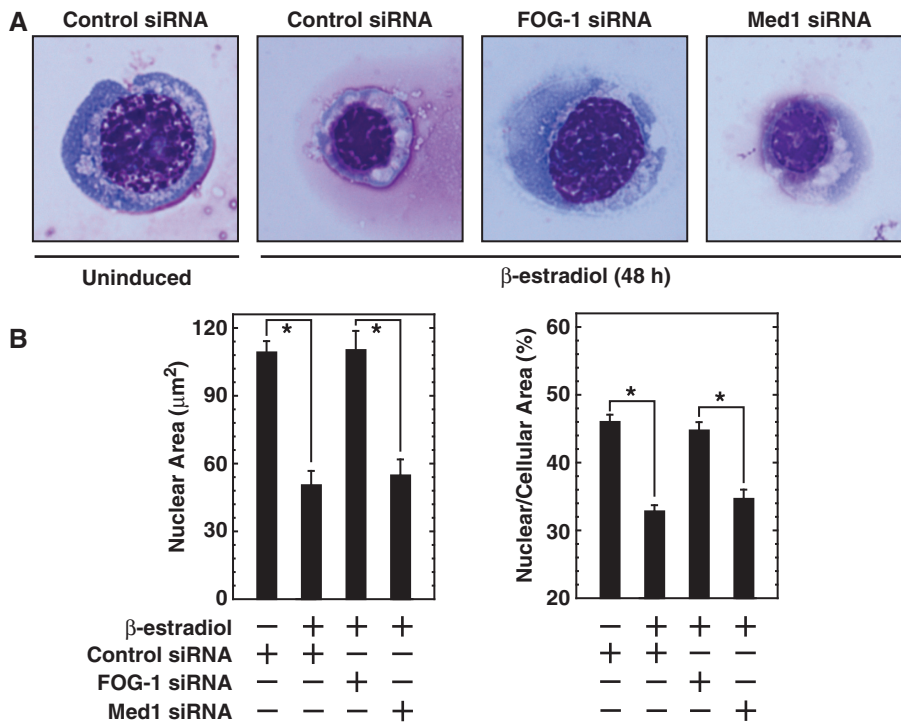
If Med1 is not broadly critical to generate GATA-1 activity at endogenous GATA-1 targets, perhaps Med1 is particularly important only at select targets. However, we analyzed multiple targets, and not a single target was critically dependent upon Med1. Med1 might also control erythropoiesis in a GATA-1 independent manner by mediating the function of other erythroid transcription factors, or through a non-cell autonomous mechanism, e.g. by regulating genes required for the function of non-hematopoietic cells within the hematopoietic niche. As Med1-null hematopoietic progenitors are defective in differentiating into CFU-E and BFU-E colonies *in vitro* (27), Med1 appears to have cell autonomous functions in hematopoietic cells, but such a mechanism has not been established *in vivo*.

Based on results described herein, in conjunction with loss-of-function studies (32,33), it is attractive to propose that Med1 confers maximal GATA-1 activity at crucial GATA-1 target genes to ensure normal erythropoiesis. This modulatory function differs from FOG-1, which is essential for GATA-1 activity at most target genes (Figure 6). Alternatively, Med1 might exert important GATA-1-independent functions to control erythropoiesis. Analogous to this fine-tuning of GATA-1 activity, the GATA-1 N-terminus, which is lacking in a leukemogenic mutant (44), also confers maximal GATA-1 activity in certain biological contexts (45–47). This truncation is linked to specific hematopoietic disorders in humans, specifically transient myeloproliferative disorder and Down's syndrome-associated acute megakaryoblastic leukemia (11). Though the truncation mutant sustains megakaryopoiesis and erythropoiesis (45,46), and therefore apparently controls the requisite target genes, in





**Figure 6.** FOG-1 knockdown severely dysregulates endogenous GATA-1 target gene expression. (A) siRNA-mediated knockdown of *Fog1* mRNA and protein as measured by real-time RT-PCR and semi-quantitative western blotting, respectively (mean ± standard error, six independent experiments). Densitometric quantitation of protein level in whole boiled cell samples. (B) Real-time RT-PCR quantification of mRNA in G1E-ER-GATA-1 cells treated with either 240 pmol control or FOG-1 siRNA for 48 h, and 1 μM β-estradiol for 42 h (mean ± standard error, six independent experiments). The fold change in expression was plotted, with GATA-1 activating *β-major*, *α-globin*, *Alas2*, *Epb4.9* and *Slc4a1* and repressing *Gata2*; \**P* < 0.05.



**Figure 7.** FOG-1 knockdown, but not Med1 knockdown, blocks G1E-ER-GATA-1 erythroid maturation. (A) Representative photos of Wright-Giemsa stained G1E-ER-GATA-1 cells treated with either 240 pmol control siRNA and no β-estradiol; or 240 pmol control siRNA, Med1 siRNA or FOG-1 siRNA and 1 μM β-estradiol for 42 h. (B) Quantitation of nuclear area and nuclear area as a percentage of total cellular area (mean ± standard error, 50 cells measured for each condition). \**P* < 0.0001.

certain contexts, the mutation dysregulates proliferation and induces leukemogenesis. As this relatively subtle perturbation of GATA-1 function underlies human hematologic malignancy, presumably the Med1 requirement for maximal GATA-1 activity also has considerable biological importance.

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